**Intramural Activities**

October 1, 1989–September 31, 1990

Division Of

# Cancer Biology, Diagnosis, and Centers

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

NATIONAL CANCER INSTITUTE

annual report

Division Of

# Cancer Biology, Diagnosis, and Centers

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ANNUAL REPORT

October 1, 1989 through September 30, 1990

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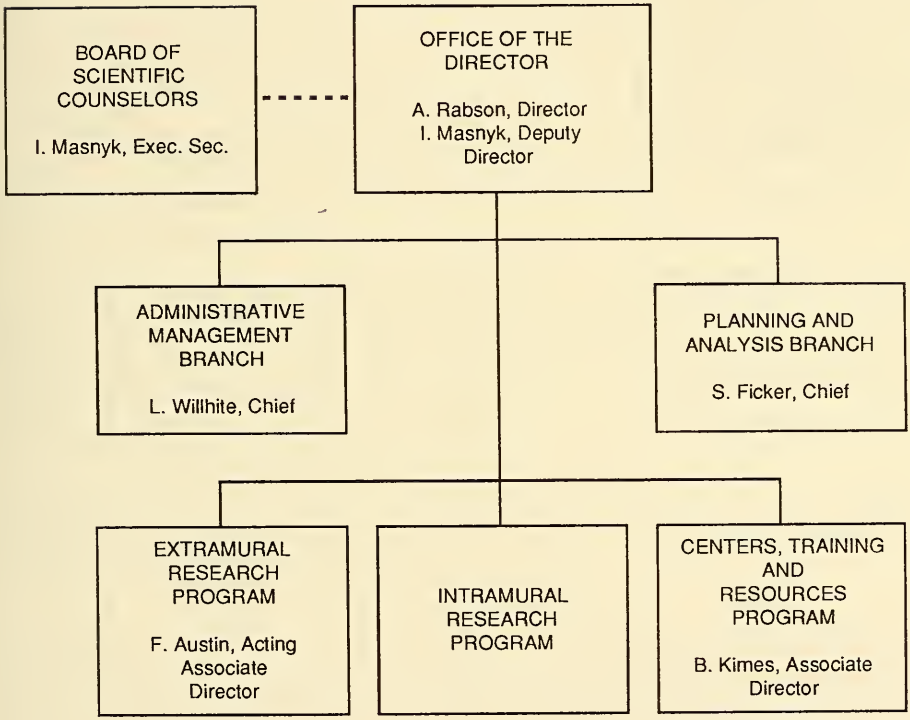
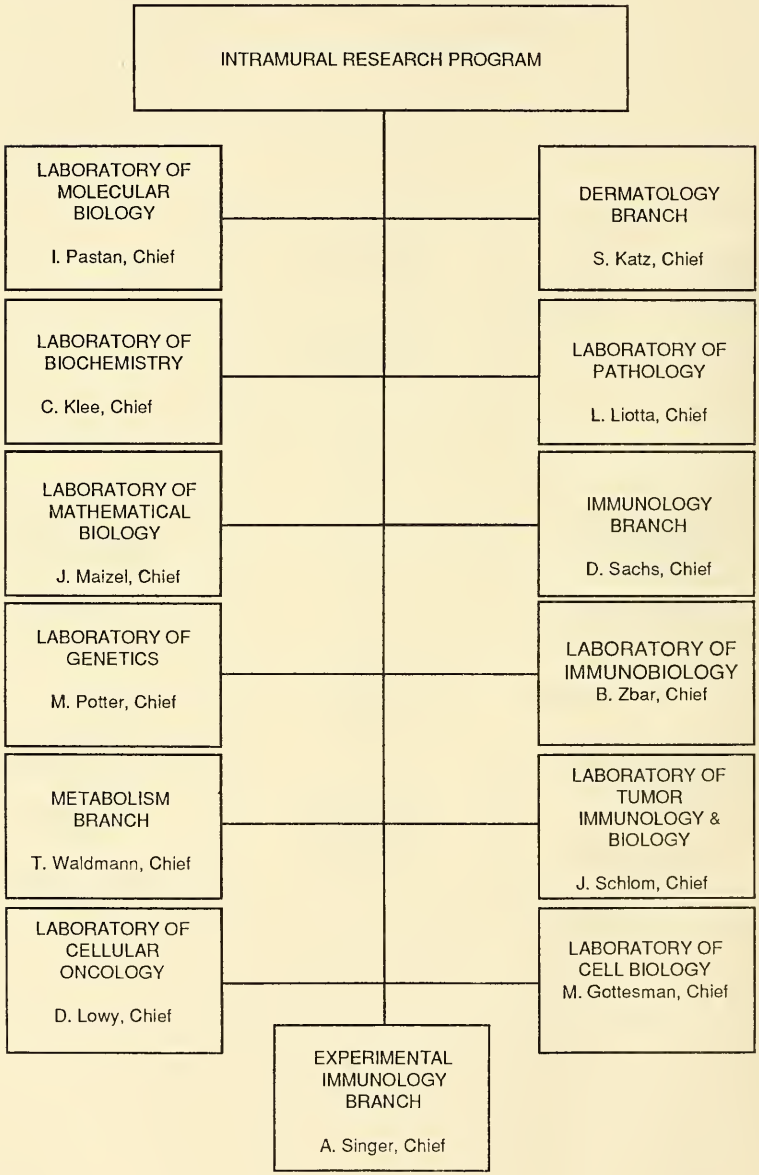


# DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS INTRAMURAL RESEARCH PROGRAM ORGANIZATIONAL CHART



DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS

NATIONAL CANCER INSTITUTE

INTRAMURAL RESEARCH PROGRAM

SUMMARY REPORT OF THE DIRECTOR

October 1, 1989 through September 30, 1990

## INTRODUCTION

The Division of Cancer Biology, Diagnosis, and Centers supports laboratory and clinical investigations in cancer biology, immunology, and diagnosis. A common theme of many of these studies is to understand the molecular mechanisms involved in the regulation of normal cell growth and differentiation, with the ultimate goal of elucidating the genetic alterations responsible for neoplastic transformation. A broad based program in immunology supports studies which relate to the regulation of the immune response and potential of the immune system to prevent or control cancer. The ultimate goal of many of these studies is to determine how to augment the immune response in a way that will lead to tumor regression.

The scope of the responsibilities of the division has been expanded to include four programs which are of paramount importance to the success of the National Cancer Program. They are the Cancer Centers Program, the Research Training Program, the Construction Program, and the Organ Systems Program. Administratively, they have been placed under the direction of an Associate Director for Centers, Training and Resources in DCBDC. The name of the division has been officially changed to reflect the importance of the Centers program to the division and to the National Cancer Institute.

In addition to the newly established Centers, Training, and Resources Program, the division is comprised of an extramural and an intramural research program. The Extramural Research Program consists of three branches: cancer biology, cancer immunology, and cancer diagnosis. Collectively they administer a portfolio of over 1250 active grants and contracts.

The Intramural Research Program of the Division of Cancer Biology, Diagnosis, and Centers consists of thirteen laboratories and branches on the NIH Bethesda campus and at the Frederick Cancer Research and Development Center. They are the Laboratory of Genetics, the Laboratory of Molecular Biology, the Laboratory of Biochemistry, the Laboratory of Cellular Oncology, the Laboratory of Pathology, the Dermatology Branch, the Metabolism Branch, the Immunology Branch, the Experimental Immunology Branch, the Laboratory of Cell Biology, the Laboratory of Tumor Immunology and Biology, the Laboratory of Immunobiology, and the Laboratory of Mathematical Biology. During the past year, Dr. Lloyd Law, chief of the Laboratory of Cell Biology, became a Scientist Emeritus in the Laboratory of Genetics. Dr. Michael Gottesman was appointed chief of the Laboratory of Cell Biology and will direct a research program on the genetic analysis of acquired and intrinsic multidrug resistance in tumor cells.

Research conducted in the intramural laboratories covers a broad range of investigations from gene regulation in prokaryotes to clinical studies of human cancer. Particularly exciting advances have been made in our understanding of the genetic basis of metastasis. The discovery of gene products involved in the development of an invasive cancer cell have led to the development of new strategies for blocking tumor cell invasion and preventing the subsequent development of metastasis.

New cytotoxic agents have been developed for the diagnosis and treatment of cancer and other human diseases. Molecular genetic techniques have been used to fuse cell targeting genes, such as growth factors or monoclonal antibodies, to genetically modified bacterial toxin genes to produce molecules that are effective in killing certain types of cancer cells and HIV-infected lymphocytes and macrophages. A different approach, conjugating monoclonal antibodies to radioisotopes, has resulted in the development of agents that are already being used for the localization and treatment of certain types of cancer.

The identification of the molecular genetic changes associated with some cancers has greatly improved our ability to determine tumor type and predict prognosis. Tumors that were indistinguishable by histologic or immunologic techniques can now be accurately identified, leading to earlier and more appropriate therapeutic intervention.

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

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

LABORATORY OF GENETICS

The primary focus of the Laboratory of Genetics, directed by Dr. Michael Potter, is on understanding the pathogenic mechanisms involved in the development of mineral oil (pristane) induced plasmacytomas. BALB/cAn mice are highly susceptible to developing these tumors, while most other inbred strains of mice are resistant. Over 95% of these tumors have chromosomal translocations [rcpt (12;15), rcpt (6;15)] involving directly or indirectly the c-myc locus on chromosome 15.

Genetics of Susceptibility to Plasmacytoma Induction

Dr. Potter's group has continued its efforts to identify and characterize the gene(s) responsible for determining susceptibility or resistance to plasmacytomagenesis. Previous studies led to the identification of two genes involved in resistance to plasmacytoma (PCT) induction. Recent efforts have focused on finding the exact location of one of these (Pctr-1) on chromosome 4. Current data suggest that Pctr-1 has both susceptibility and resistance alleles. It appears to have a weak effect and may be only one of many genes that influence the development of plasmacytomas. Dr. Beverly Mock has also isolated a new $D_2$/BALB/c allelomorphic marker 1B-4 located in chr 4 that has been valuable in mapping genes on distal 4. She has also shown that genes on chr 17 play a role in the susceptibility of mice to the highly plasmacytomagenetic RIM (carrying Eμmyc and v-Ha-ras) virus. Although the precise physiologic function of Pctr-1 is not known, there is some evidence to suggest that it may be involved with DNA repair.

A specific pathogen-free BALB/cAnPt mouse has been derived and induction studies currently at between 250 and 300 days post-pristane injection have shown that the incidence of plasmacytomas is below 10%, while over 40% PCTs have occurred in the control BALB/cAn mice in the conventional colony. This suggests that the presence of microbial flora and infectious viruses in a conventional colony must be essential for stimulating cells in the immune system to undergo changes that eventually result in the development of plasmacytomas. Preneoplastic lesions have been found in susceptible mice injected with pristane months before a tumor is manifest. During the past year it was shown that these early lesions originate in the mesenteries of the small intestine and appear to progress slowly by forming new foci.

Dr. Fred Mushinski's group is continuing to search for genes that cooperate with c-myc in plasmacytomagenesis. The v-abl oncogene has been found to cooperate with over-expressed c-myc in a retrovirus (ABL-MYC) which induces plasmacytomas more rapidly than previously reported and, for the first time, in 100% of adult mice, even in the absence of pristane priming. The rapidity of this induction has permitted the production of antigen-targeted myeloma proteins by ABL-MYC induction of plasmacytomas in immunized mice. An important family of protein kinases, Protein Kinase C (PKC), is being studied for its possible role in tumorigenesis. Dr. Mushinski and his colleagues have shown that the members of this gene family are differentially expressed in tumors of hemopoietic cells and that there is at least one undescribed form of PKC expressed in myeloid tumors.

Dr. Konrad Huppi has cloned a cDNA for PVT-1. PVT-1 is a chromosomal breaksite located 100-300 kb 3' of c-myc that is involved in illegitimate exchanges with Ig light chain loci. Drs. Huppi and Mushinski have shown PVT-1 is transcribed and

that unusual transcripts are generated in plasmacytomas. The mechanism whereby PVT-1 is involved with the deregulation of transcription of c-myc is not yet understood but is being vigorously pursued.

## 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 diagnosis and treatment of cancer, AIDS and other human diseases. Genetically modified bacterial toxin genes have been fused to growth factors or monoclonal antibodies to create cell specific cytotoxic agents. These chimeric toxin molecules can be expressed in E. coli and readily purified to homogeneity. Using this approach, TGFa-PE40, IL2-PE40, IL4-PE40, IL6-PE40, CD4-PE40 and several single-chain antibody toxin fusion proteins have been made. More recently, chimeric toxins have been made by linking two antibody variable domains to Pseudomonas exotoxin. One such chimeric molecule is anti-Tac(Fv)-PE40 which binds to the p55 subunit of the human and monkey IL2 receptor. This chimeric toxin is extremely active in killing human cell lines displaying the IL2 receptor, human and primate activated T cells, and many leukemia cells from patients with adult T cell leukemia. Anti-Tac(Fv)-PE40 is being developed for the treatment of allograft rejection and leukemias. To simplify the construction of single-chain antibody toxin fusion proteins, new methods have been developed to directly clone the antibody variable region DNAs from RNA using the polymerase chain reaction technique (PCR) and specific DNA primers.

The activities of TGFa-PE40 and IL2-PE40 have been shown to be effective in eliminating tumors in animal models. Furthermore, an IL6-PE fusion protein has been found to inhibit the growth of certain hepatomas, multiple myeloma and some human prostate cancer cell lines. A mutant form of Pseudomonas exotoxin, Lys-PE40, has been made and coupled to an antibody against the human transferrin receptor. This agent is very effective against several types of human tumors. All these studies show that chimeric toxins directed at antigens present on tumor cells can be effective against solid tumors. The current goal is to increase the activity and specificity of these molecules. To this end, Dr. Pastan's group has carried out several types of genetic and biochemical experiments to further understand how Pseudomonas exotoxin works. Mutagenesis studies indicated that Arg276 and Arg279 in domain II were important for toxin action. Another region required for toxin activity lies at the carboxyl end of PE in the last five amino acids (REDLK), a sequence similar to the endoplasmic reticulum sequence. This indicates that the toxin probably utilizes for its toxic action the same cellular machinery that retains proteins in the endoplasmic reticulum.

In collaboration with Drs. E. Berger and B. Moss, a chimeric toxin, CD4-PE40, has been made which kills HIV infected cells expressing gp120 on HIV infected cells. CD4-PE40 is active against many different isolates of HIV and SIV. It suppresses the spread of HIV, and in combination with AZT it eliminates HIV from infected cultures. CD4-PE40 is under preclinical development by the Upjohn Company for the possible treatment of AIDS.

### Multidrug Resistance

A collaborative program in the genetic analysis of the multidrug resistance in tumor cells has been developed with Dr. Michael Gottesman, Chief of the Laboratory of Cell Biology, DCBDC. Several lines of investigation concerning the

multidrug transporter have been pursued. Dr. Glenn Merlino has constructed transgenic mice in which the human MDR1 mRNA is expressed in the bone marrow at levels comparable to those found in human tumors. This level of MDR1 expression is sufficient to confer resistance to leukopenia induced by MDR drugs.

Regulation of the Gal Operon in Escherichia coli

Dr. Shankar Adhya has continued his studies of the regulation of the gal operon of Escherichia coli. The gal operon of E. coli is negatively controlled by two regulatory proteins, the Gal repressor, and a newly discovered factor termed UIF (ultrainducibility factor). Both repressor and UIF act by binding to the same DNA sequences, OE and OI, providing an example of cellular regulation by different factors acting through common DNA elements. While repressor inhibits gal transcription under normal conditions, UIF down regulates gal only in cells devoid of Gal repressor. Both proteins are inactivated by galactose. Inactivation of UIF, in the absence of repressor, causes extreme derepression which is termed ultrainduction.

Repression involves formation of a DNA-multiprotein complex of higher order structure, comprised of DNA, one molecule of RNA polymerase, one molecule of cyclic AMP receptor protein and two molecules of repressor. In this complex, two repressor molecules, bound to two spatially separated sites, interact with each other looping out the intervening DNA segment to which the other proteins are bound. Adhya and colleagues have proposed that repression of transcription is achieved by a novel mechanism in which repressor freezes the bound RNA polymerase by a direct contact with the latter. The formation of a DNA loop with a higher order structure facilitates such contact(s). Dr. Adhya is currently investigating the various protein-protein contact sites and the state of transcription initiation at which RNA polymerase is frozen.

Genetic Regulatory Mechanisms in Escherichia coli and Its Bacteriophage

In E. coli, protein degradation plays an important role in regulating cell growth control, particularly proteases which carry out ATP-dependent proteolysis. Dr. Susan Gottesman has continued her studies of the Lon protease that degrades RcsA, a positive regulator of capsular polysaccharide synthesis, and SulA, a cell division inhibitor. RcsA interacts in the cell with another positive regulator, RcsB to promote efficient capsule synthesis. The Clp protease, a second major ATP-dependent protease, contains a large subunit, ClpA, with ATPase activity. This subunit has been well conserved in other prokaryotic cells and in eukaryotic cells. The smaller subunit, ClpP, contains the active site of the protease, and undergoes a self-processing reaction. The small subunit is also conserved in many cells. The structure and general organization of the Clp protease suggests that it has many similarities to the eukaryotic energy-dependent proteases which degrade ubiquitin-conjugated proteins. Alp, a third energy-dependent protease activity, which can substitute for Lon when overproduced, consists of a set of closely linked genes and a positive regulator. In a new approach to the study of bacterial cell growth control, mutants which may be defective in partition of the bacterial chromosome have been selected, mapped and characterized.

LABORATORY OF BIOCHEMISTRY

The Laboratory of Biochemistry, directed by Dr. Claude Klee, is composed of a group of protein chemists and molecular biologists whose research interests are

focused on understanding the complex network of factors controlling normal cell growth and differentiation.

Regulation of Gene Expression

The regulation of gene expression remains one of the major topics of interest in the Laboratory of Biochemistry. Dr. Carl Wu and his colleagues have continued to study the structure, regulation and mode of action of two classes of sequence-specific DNA-binding proteins in Drosophila. A major achievement this year was the characterization of the transcriptional activator of the heat shock genes (Heat Shock Factor, HSF). HSF was cloned and expressed in E. coli. Functional domains of the HSF protein responsible for DNA binding, oligomerization, and transcription activation were identified. The recombinant HSF which is constitutively active, lacking the negative regulation to which HSF is subjected in vivo, can now be used to isolate the inhibitory factor responsible for the inactivation of HSF. Studies of the interaction between HSF and the inhibitory factor should allow this group to elucidate the mechanism by which the inactive form of HSF acts as a sensor of cellular stress. A second project deals with the regulation of temporal and spatial expression of the segmentation gene fushi tarazu during early embryogenesis. The transcriptional activator of this gene, FTZ-F1, was cloned and found to encode a new member of the steroid receptor family. A transcriptional repressor of fushi tarazu, FTZ-F2, was also cloned and found to encode a new "zinc finger" protein. Thus, the complex expression pattern of a developmental gene is mediated by a combination of positive and negative transcription factors.

Major progress has also been made by Dr. Dean Hamer's group in the elucidation of the basic mechanism of metallothionein gene regulation in yeast. Dr. Hamer and his colleagues showed that metal ions directly interact with a regulatory protein, ACE1, altering its conformation so that it can bind to the metallothionein gene control sequences and thereby activate transcription. Remarkably, the predicted structure of the regulatory protein is similar to that of metallothionein itself. The isolation of a metallothionein regulatory factor from mouse cells is in progress.

Another aspect of the regulation of gene expression is studied by Dr. Bruce Paterson and his colleagues, who are focusing their attention on the regulation of a group of genes involved in determining the muscle phenotype in both vertebrates and invertebrates. They have isolated two different myogenic genes from chicken (CMD1 and Cmgn1) and a potential homolog from Drosophila. The avian genes not only activate a cotransfected muscle specific promoter/reporter gene but are also capable of the phenotypic conversion of mouse 10T1/2 fibroblasts whereas the Drosophila gene can only activate the muscle specific reporter gene. The stimulation of the mouse genes by their avian homologs suggests a complex regulatory network. All the proteins encoded by these genes share a common structural motif which consists of a basic domain adjacent to a putative "helix-loop-helix" element.

The role of DNA methylation in the modulation of gene expression is a 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). The genetic basis for selective demethylation and expression of IAP provirus is being investigated in normal mouse lymphocytes while relevant

transcription factors are being studied in nuclear extracts of various transformed rodent and primate cells.

Proteins and Control of Cellular Processes

The structure of biologically important proteins and their role in the regulation of cell function is the focus of interest of others in the laboratory. Dr. Shelby Berger and her colleagues are studying the newly discovered role of prothymosin α in cell division. This highly acidic protein contains a nuclear targeting signal which is both necessary and sufficient for its transport to the nucleus, and its synthesis is regulated in a cell cycle-dependent manner. Cell division requires sufficient quantities of prothymosin α in order to take place. Dr. Berger's group has also conducted comparative studies of the mechanism of action of the ribonuclease H activity of reverse transcriptase from avian myeloblastosis virus and human immunodeficiency virus. Using enzyme kinetics, they have shown that only one binding site for the template:primer suffices for both polymerase and RNase H catalysis. This information may be important for the future design of enzyme inhibitors for the treatment of HIV infection.

There are few biological systems which are not regulated by calcium ($Ca^{2+}$); in all eukaryotic cells $Ca^{2+}$ regulation is at least partially mediated by calmodulin. Dr. Klee's group has demonstrated that $Ca^{2+}$ destabilizes and $Mg^{2+}$ stabilizes the central helix of calmodulin. The cloning this year of the two subunits of Drosophila calmodulin-regulated protein phosphatase, calcineurin, has revealed the extreme conservation of the catalytic and regulatory domains of this protein during evolution. The calmodulin-binding and autoinhibitory domains have been characterized and the structural organization of the functional domains determined at the gene level.

Organization of the Human Genome

Dr. Maxine Singer and her colleagues continue to carry out experiments designed to elucidate the mechanism of LINE-1 transposition in the human genome. A second human teratocarcinoma cell line (2102) has been found to promote LINE-1 transcription at a high rate, confirming earlier conclusions. Previous work on the internal LINE-1 promoter, which measured expression of a reporter gene, was confirmed at the RNA level by analyzing transcripts directly. Dr. Singer's group also demonstrated that in addition to the presence of an essential DNA sequence motif in the first 100 bp at the 5' end of human LINE-1s, several other significant regulatory sequences occur between base pairs 100 and 600 from the 5' end. Antibody to the ORF1 protein allowed the detection of a protein of the approximate expected size in the cytoplasmic fraction of human teratocarcinoma cell lines - NTera2D1 and 2102-EP. This is the first demonstration of a LINE-1 encoded protein in human or other mammalian cells.

Dr. O. Wesley McBride has continued his work on human chromosome mapping as a tool to identify genes involved in genetic diseases and human neoplasia. Among the highlights of this year's achievements is the mapping of the hydroxyindole o-methyltransferase gene (isolated by Drs. Klein and Donohue) to the pseudoautosomal region of the X (and Y) chromosome. This is only the third gene assigned to this region. In addition, an alphoid satellite probe from a clone isolated by Dr. H. Ozer has been found to identify chromosomes 1, 5, and 19. This probe may have a clinical human cytogenetic application. A linkage map of four probes spanning a 15 cM region of chromosome 22q11.2, including a region

involved in the DiGeorge Syndrome, has been completed and additional genes have been added to a cluster previously mapped in the laboratory at chromosome 22q12.

DNA Replication

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 preliminary evidence to suggest that the precise control of β-polymerase expression is important for normal cell growth. They have isolated 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 and have demonstrated that A1 hnRNP binding protein has annealing activity toward both RNA and ssDNA. This suggest that the protein may play a role in mRNA splicing and DNA recombination.

Dr. Wilson's group has continued to make significant progress in the studies of the structure-function relationship of HIV reverse transcriptase. The domain responsible for the interaction between the two subunits of the dimeric enzyme has been identified and a peptide inhibiting the dimerization process is being tested as a specific inhibitor of the enzyme and as an antiviral agent.

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.

Studies of the ras Oncogene

Dr. Lowy's group has continued to study the function of the ras oncogene by examining proteins that influence the activity of ras protein and by performing structure-function analysis with mutants and chimeric genes. They have made a series of chimeras between ras and the Krev-1 gene, which encodes a ras-like protein that can suppress ras-transformed cells. The results indicate that the ras-specific and Krev-1-specific amino acids immediately surrounding residues 32-44, which are identical between the two proteins, determine whether the protein induced cellular transformation or suppressed ras transformation. This suggests that Krev-1 may suppress ras-induced transformation by interfering with the interaction between ras protein and its effector. The influence of GAP (GTPase activating protein) on cell transformation by ras and other oncogenes was also examined. Overexpression of GAP in NIH 3T3 cells inhibited and suppressed transformation by c-ras, c-src and an activated c-src mutant, but not by v-ras or v-mos. Dr. Lowy concluded that GAP as a negative regulator is limiting in cells and overexpression of GAP can inhibit transformation by ras-dependent oncogenes. These results make it unlikely that GAP by itself is the ras target, but are compatible with the possibility that GAP may be part of a complex that forms the ras target.

Analysis of Papillomaviruses

A second focus in the laboratory is the study of transformation by human and animal papillomaviruses. Certain human papillomaviruses (HPV16 and 18) are frequently detected in genital cancers, while others (HPV6 and HPV11) are associated with benign disease but rarely with carcinomas. Since the viral E6 and E7 genes are selectively retained and expressed in carcinomas, Dr. Lowy's group has undertaken a comparative analysis of these genes from the presumed low

risk and high risk HPV types. They have determined that the in vitro transforming activities of E6 and E7 from HPV6 are lower than those of E6 and E7 from HPV16 and 18; these results have also been correlated with lower efficiency of Rb binding and casein kinase II phosphorylation by the E7 encoded protein of HPV6, compared with E7 protein of HPV16 and 18. The data provide strong experimental support for the hypothesis that the E6 and E7 genes in HPV 16 and 18 play an important role in the genesis of human genital cancer and offer insight into the possible mechanisms of E6 and E7 induced carcinogenesis.

The E1 protein of bovine papillomavirus type-1, although only 44 amino acids in length, can independently transform rodent fibroblasts. Studies conducted during the past year have shown that E1, which is a transmembrane protein, can potentiate the activity of co-transfected receptors in a ligand independent manner. Analysis of co-transfected EGF receptors suggested that E5 may transform by interfering with receptor processing.

LABORATORY OF PATHOLOGY

The Laboratory of Pathology, directed by Dr. Lance Liotta, is responsible for all the diagnostic services in anatomic, surgical and ultrastructural 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.

In the Pulmonary and Postmortem Pathology Section, Dr. William Travis is conducting a detailed review of interstitial fibrotic lung disease. A comprehensive study of the pulmonary pathology of the Acquired Immune Deficiency Syndrome (AIDS) is currently being conducted based on surgical and autopsy lung pathology material from AIDS patients seen at the NIH. The Cytopathology Section, led by Dr. Diane Solomon, provides diagnostic services to the NIH in both exfoliative cytology and fine needle aspiration. During the past year there has been a 200% increase in outside consult cases submitted to this section; fine needle aspirations have increased 100% Dr. Solomon has been a leader in developing procedures to improve the quality control of cervical/vaginal examinations and in the development of "The Bethesda system" for cervical/vaginal cytopathology diagnoses.

Tumor Invasion and Metastases

A major focus of experimental research in the Laboratory of Pathology centers on the molecular genetics of tumor cell invasion. The results of these experimental studies have led to the development of new therapeutic approaches designed to inhibit the development of cancer metastasis. Dr. Liotta's group has identified a set of gene products which may regulate tumor invasion and metastasis: a) laminin receptor; b) autocrine factor; c) NM23 metastasis suppressor protein, d) type IV collagenase, and e) TIMP-2 metalloproteinase inhibitor. These metastasis-associated gene products may be different from those which regulate tumor growth, supporting the concept that benign tumors may grow in an unrestrained fashion, but fail to invade.

The laminin receptor is a cell surface protein which binds laminin, a glycoprotein of basement membranes, and is augmented in certain aggressive human cancers compared to non-malignant counterpart cells. Dr. Mark Sobel has cloned

and sequenced the full-length cDNA for the laminin receptor and is now studying its structure and function.

The autocrine motility factor (AMF) is a cytokine secreted by tumor cells that profoundly stimulates their locomotion. Previously, Dr. Elliott Schiffmann's group identified the biochemical pathway required for the action of AMF. Screening of inhibitors blocking this pathway led to the identification of a compound, carboxyamide-imidazaole (CaI), that is a potent inhibitor of AMF. Preclinical toxicology studies and the design of clinical protocols are now complete for this compound, and a Phase I clinical trial is scheduled to begin in 1991.

Dr. Liotta's group has continued its study of NM23, a human gene that appears to encode a putative metastases suppressor protein. The gene has been cloned and sequenced, and the NM23 protein was shown to be virtually identical to a Drosophila gene (awd) product which plays a critical role in morphogenesis and tissue pattern formation. The negative correlation of NM23 expression with metastases was confirmed in four different animal model systems; a strong negative correlation was found between human breast carcinoma aggressiveness and NM23 mRNA expression. Immunogenicity does not appear to play a role in the mechanism of action of NM23. During the past year it was shown that the NM23 gene is located on chromosome 17, and is associated with allelic loss in a variety of carcinomas. Additional data have demonstrated a strong suppression of experimental metastases in a series of tumor cell clones transfected with NM23 compared to a series of control transfections. NM23 may provide the basis for a number of significant prognostic and therapeutic strategies.

The structural backbone of the basement membrane is a genetically distinct type of collagen, type IV collagen. Type IV collagenase is a unique member of the metalloproteinase family which specifically cleaves type IV collagen. The enzyme is secreted in a latent 70 kDa form which fails to degrade type IV collagen. When the enzyme is activated, it becomes reduced to a molecular size of 62 kDa and can specifically cleave type IV collagen. This enzyme is secreted in large amounts by tumor cells and its rate of elaboration is correlated with the metastatic phenotype in several systems. Type IV collagenase production shows a strong correlation with invasive grade in human colorectal and gastric cancer, and may be an important correlate of tumor progression in human breast cancer. A very strong correlation has been observed between the levels of type IV collagenase and AMF in the urine and bladder carcinoma grade, stage of invasion, and recurrence. A large study is in progress to determine the value of these tests as a potential adjunct in the early detection of bladder cancer recurrence.

Synthetic, natural, or recombinant inhibitors of type IV collagenases may have significant therapeutic potential as agents which block angiogenesis and tumor invasion, with minimal toxicity, since basement membrane turnover is rare in normal tissues. Analysis of the full-length cDNA clone encoding the human latent type IV collagenase has revealed three separate functional domains, suggesting innovative strategies for enzyme inhibition.

In previous studies, Dr. William Stetler-Stevenson discovered a novel proteinase inhibitor which binds to type IV collagenase. The inhibitor has been purified, the complete primary structure determined, and the full-length human gene has been cloned. The novel inhibitor, termed TIMP-2, is the first new member of the TIMP (tissue inhibitor metallo- proteinases) family with conservation of all 12

cysteine positions. TIMP-2 can be considered in the class of tumor suppressor gene products.

Hematopathology

The Hematopathology Section, led by Dr. Elaine Jaffe, conducts a major program in diagnostic and experimental hematopathology. She has continued her research on the immunological characterization of malignant lymphomas; in particular, on the study of the relationship of malignant lymphomas to the normal immune system. The results of her studies continue to contribute to the improved classifications of disease, and to distinguish new clinicopathologic entities.

Dr. Maryalice Stetler-Stevenson has continued her studies of the expression of bcl-2 in follicular lymphoma and Hodgkin's disease. She has developed a procedure for isolation of RNA followed by reverse transcription and PCR amplification and has used this procedure to measure the levels of expression of bcl-2 in biopsy specimens. In a small series of follicular lymphoma cases, she found that the levels of expression had a strong correlation with prognosis. Her studies indicate that bcl-2 may confer a growth advantage to cells but that involvement of other oncogenes, especially myc, is necessary to attain full malignant potential. Previously she demonstrated the t (14;18) translocation is present in 32% of Hodgkin's lymphomas. However, Hodgkin's disease and follicular lymphoma are two distinct clinical-pathological entities. Future studies will examine the expression of the bcl-2/JH transcript and of the bcl-2 protein product in Hodgkin's disease patients and again correlate this information with the clinical course. This data will provide further information concerning the role of bcl-2 in hematopoietic malignancies.

Gene Regulation

Dr. David Levens has developed a novel method for identifying sequence-specific DNA binding proteins. The gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity resides 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.

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

Dr. Kathleen Kelly is 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. She has isolated over 60 novel cDNA clones that represent mRNA species induced by mitogens in human peripheral blood T cells. A subset of inducible genes (including two putative transcription factors) are constitutively expressed at high levels in T cells infected with HTLV I, and also following transfection of T cells with HTLV I-derived DNA encoding the overlapping reading frames of $p42^{tax}$ and $p27^{rex}$. Such abnormally expressed genes may play a role in the deregulated growth of adult T cell leukemia, a cancer that shows a 100% infection rate with HTLV I. The mechanism of $p42^{tax}$ and $p27^{rex}$ action that leads to over-expression is being examined.

**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 NIH Clinical Center.

Immunopathologic Mechanisms Involved in Inflammatory and Neoplastic Skin Diseases

Dr. Katz's group has continued to study the immunological functions of cells of the epidermis. In collaborative studies with investigators in the Experimental Immunology Branch, they have found that Langerhans cells express markedly diminished amounts of H-2K, whereas they express normal, if not increased, amounts of H-2D compared to keratinocytes. The potential significance of this finding may relate to the ability of Langerhans cells to function as targets and/or stimulators of cytotoxic lymphocyte responses. In another series of experiments, they have found that, although there appears to be a strain specific difference in the ability of Langerhans cells to process antigen, freshly prepared cells from all strains can process antigen and present the relevant peptides after culture. Cultured human Langerhans cells exhibit increased class II MHC molecules and are potent alloantigen presenting cells. Current studies are in progress to assess their ability to present protein antigens and then to attempt to generate primary *in vitro* responses to viral-or tumor-associated antigens. Dr. Katz and his colleagues are also assessing the role of class II-bearing human keratinocytes in the activation or suppression of specific T cell responses.

Regulation of Cutaneous Accessory Cell Activity in Health and Disease

Dr. Mark Udey, who recently joined the laboratory, is conducting studies to determine how ultraviolet B (UV-B) radiation modulates the epidermal immune system. He has found that low doses of UV-B radiation completely inhibit the ability of freshly isolated Langerhans cells (fLC) to support the mitogenic response of T cells to anti-CD3 antibodies. LC that have been cultured for 24-72 hours (cLC) become progressively more resistant to UV-B radiation, which correlates with the progressive increase in the expression of the intercellular adhesion molecule ICAM-1 by cLC. Subsequent studies have confirmed the importance of the ICAm-1/LFA-1 interaction in this system.

Molecular Basis of Autoimmune Skin Diseases

Dr. John Stanley's laboratory studies 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) antigen is known to be a component of the hemidesmosome, a basal cell-substrate adhesion junction. Dr. Stanley's group has determined, using immunochemical techniques, that the BP antigen is a 230 kD protein with a pI of 8. During the last year they have cloned overlapping cDNAs encoding almost the entire BP antigen. 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.

Molecular and Cell Biology of Immunoreactive Molecules in the Skin

ICAM-1, or intercellular adhesion molecule -1, is a cell surface glycoprotein that serves as the major ligand for LFA-1, an anchoring receptor on all T cells whose interaction with ligand is critical for proper T-cell function. The regulation of ICAM-1 expression is critically important in both normal inflammatory and pathologic processes. Dr. Wright Caughman's group has established the kinetics and molecular regulatory level for cytokine-induced de novo ICAM-1 expression by human keratinocytes (HK) in culture and has cloned and characterized the transcriptional regulatory region of the human ICAM-1 gene.

METABOLISM BRANCH

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

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

A major focus of the Metabolism Branch continues to be the identification, purification, and genetic analysis of the multi-chain interleukin-2 receptor on normal and malignant lymphocytes, and the development of different forms of IL-2 directed therapy for cancer and other human diseases. Previously, Dr. Waldmann's group identified two peptides that bind IL-2, the 55 kD protein reactive with the anti-Tac monoclonal antibody and a novel 70/75 kD (p75) protein reactive with a monoclonal antibody termed Mikβ1. Dr. Waldmann proposed a multichain model for the high affinity receptor in which both p55 α and the p75 β IL-2-binding proteins are associated in a receptor complex. His data suggest that IL-2 must initially interact with the p55 α protein with subsequent communication of the IL-2 signal to the p75 α chain. In earlier studies, Dr. Waldmann demonstrated that a 95 kD protein identified by two monoclonal antibodies interacts physically with the 55 kD/IL-2Rα protein of the high-affinity IL-2 receptor. Over the past year, in conjunction with Dr. Jack Burton, he purified the p95 protein and defined its amino acid sequence and thus showed that this protein is ICAM-1. This association of ICAM-1 with the IL-2 receptor may facilitate the paracrine IL-2-mediated stimulation of T cells expressing IL-2 receptors.

A state of T cell activation reflected by a marked degree of spontaneous proliferation in vitro exists among patients with HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP). Using a modification of the polymerase chain reaction, Dr. Waldmann and his colleagues have undertaken studies to define the mechanism by which the circulating cells from HAM/TSP are driven. Their studies suggest that immune activation in HAM/TSP is virally driven by the transactivation and coordinate expression of IL-2 and the IL-2Rα protein. This deregulated autocrine process may contribute to the development of inflammatory nervous system damage in HAM/TSP.

T cells in patients with certain lymphoid malignancies and select autoimmune disorders, as well as individuals rejecting allografts, express the IL-2 receptors identified by the anti-Tac monoclonal antibody, whereas normal resting cells and their precursors do not. This observation has led to the development of a new therapeutic approach for a broad range of human diseases. Unmodified anti-Tac monoclonal antibody has already shown promising results in preliminary clinical studies of patients with adult T cell leukemia. In a randomized prospective kidnet transplantation trial, anti-Tac was added to the treatment of one of two patient groups who received otherwise identical immunosuppression. There was no increased toxicity, but there was a significant reduction of early rejection episodes following renal transplantation. In conjunction with Dr. Otto Gansow, Dr. Waldmann has shown using in vitro assays and in vivo cardiac allograft and xenograft models that $^{212}Bi$, an alpha-emitting or $^{90}Y$, a beta-emitting radionuclide conjugated to anti-Tac by use of bifunctional chelates were effective and specific immunocytotoxic agents for the elimination of Tac expressing cells. In conjunction with Dr. Cary Queen, Dr. Waldmann and his coworkers have constructed a "humanized" anti-Tac antibody by placing the complementarity determining regions of the murine anti-Tac antibody into the human variable region framework and IgG1 constant regions in an attempt to circumvent the host immune response to the murine antibody. Preliminary studies show that 'humanized' anti-Tac retains high binding affinity, is very effective in inhibiting IL-2 mediated events, and furthermore, that it adds the new functional activity of antibody dependent cellular cytoxicity (absent in the parent mouse anti-Tac). This could substantially enhance its efficacy in therapy of T cell leukemia/lymphoma. "Humanized" hyperchimeric anti-Tac significantly prolonged cardiac allograft survival in cynomolgus monkeys when compared to the group treated with murine anti-Tac.

Dr. David Nelson has identified a soluble form of the p55 component (Tac protein) in the sera of patients with human lymphoproliferative disorders, including adult T-cell leukemia, hairy cell leukemia, non-Hodgkins lymphoma, and AIDS. In some cases, an elevated serum level of Tac protein was indicative of tumor burden or disease recurrence. Elevations of Tac protein in the serum were also indicative of allograft rejection episodes in patients with liver and heart-lung transplants.

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

As an approach to understanding development in the B lymphocyte lineage, Dr. Louis Staudt has characterized trans-acting factors that mediate lymphoid-specific gene transcription of immunoglobulin (Ig) genes. Oct-2 is a transcription factor expressed in B lymphocytes which is critical for the lymphoid-specific expression of immunoglobulin genes. During the past year he

has demonstrated that Oct-2 is also expressed in some T lymphocytes, myeloid cells and in the fetal spinal cord, suggesting that Oct-2 must therefore cooperate with other B cell restricted transcription factors to fully account for lymphoid-specific gene expression.

The molecular cloning of Oct-2 helped to identify a new class of transcription factors that share two structural domains, a homeobox and POUbox. Dr. Staudt has molecularly cloned and characterized a new member of this POU-homeobox family, termed Oct-3. Oct-3 binds to the same DNA motif as Oct-2, but 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 cells of the germ line. This is in marked contrast with previously studied homeobox genes which are not expressed in pluripotent stem cells or in the germ line but rather are expressed in cells that have differentiated along one of the somatic lineages.

Somatic Gene Therapy for Human Genetic Disease

Dr. Michael Blaese has continued his research to develop gene transfer for the therapy of human disease. He and his collaborators, W. French Anderson and Steven A. Rosenberg, have now performed the first authorized clinical experiment in which a foreign gene has been introduced into man. The bacterial gene encoding Neomycin resistance (neoR) was used to label tumor infiltrating lymphocytes (TIL) which were reinfused into patients with malignant melanoma. The next phase of these studies will involve the insertion of a gene encoding tumor necrosis factor into TIL in an attempt to improve their tumoricidal activity for a variety of tumors. Dr. Blaese's laboratory has prepared vector constructs with the genes for various cytokines including IL-1β, TNF and IL-6. Major studies of the biological effects of cells constitutively secreting these molecules are also underway, as are studies of the potential utility of using lymphocytes as cellular vehicles for gene therapy of non-malignant diseases such as adenosine deaminase (ADA) deficiency, severe combined immunodeficiency (SCID) and hemophilia. A clinical protocol for the treatment of ADA(-)SCID with gene-treated cultured T cells has now been approved by the Human Gene Therapy Subcommittee of the Recombinant DNA Advisory Committee. With the expected early approval by two additional Federal regulatory agencies, the first use of gene transfer for the therapy of human disease could begin as early as this autumn.

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

Dr. Jay Berzofsky has studied the mechanisms by which T cells recognize antigens on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, and the application of these principles to the design of synthetic vaccines for AIDS and malaria. In his studies of HIV, Dr. Berzofsky found that the same peptide can also bind to both class I and class II MHC molecules for recognition by CD8 cytotoxic T lymphocytes (CTL) and CD4 helper T cells, respectively. Thus, this peptide can provide help for induction of CTL to itself. This peptide is also recognized by CTL from HIV-infected and uninfected immunized human volunteers, in association with two common human HLA types, A2 and A3, making it broadly useful in a vaccine. Dr. Berzofsky also identified a conserved CTL epitope in the HIV-1 reverse transcriptase recognized by CD8 cells from both mice and 5 of 12 HIV-infected humans. He has identified

multideterminant helper T-cell regions of the HIV envelope that are broadly recognized by mice and humans. These may be useful in the diagnosis of exposed individuals who are still seronegative, as well as in vaccine development. Dr. Berzofsky has shown that some of the helper peptides can elicit help for an enhanced antibody response to HIV envelope in monkeys the first time the monkeys are exposed to a suboptimal dose of whole envelope protein, and that humans immunized with a recombinant gp160 vaccine make much higher levels of IL-2 in response to these peptides than do HIV-infected individuals. Thus, vaccine induced immunity can exceed that produced by natural infection. Dr. Berzofsky has developed a way to get purified nonliving subunit vaccines, which do not normally induce CD8 CTL, to do so reproducibly by incorporating them into immunostimulatory complexes (ISCOMS). This may make it possible for the first time for subunit vaccines to induce all 3 types of specific immunity, CTL as well as helper T cells and antibodies.

## IMMUNOLOGY BRANCH

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

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

Dr. Sachs and his colleagues have developed a method of inducing intentional mixed chimerism across MHC barriers as a means of producing specific transplant tolerance. 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 may be useful in the treatment of leukemia, since the procedure does not appear to eliminate the graft-vs-leukemia (GVL) effect. Treatment with monoclonal anti-T-cell subset antibodies plus selective thymic irradiation results in long-term mixed chimerism and specific tolerance across an MHC barrier. This regimen has also been successfully applied to rat-mouse xenogeneic chimeras.

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 following bone marrow transplantation. The effect is additive to that of T-cell depleted syngeneic marrow previously described. It avoids both acute and chronic GVHD while preserving a graft-versus-leukemia effect.

### Miniature Swine Model

Partially inbred strains of miniature swine have been developed as a large animal pre-clinical model for transplantation studies. This model has made it possible to determine the relative importance of class I versus class II antigens for allograft survival. Transplantation protocols based on the results of the mouse studies have been developed for use in miniature swine.

A molecular approach to the analysis of class II genes in the miniature swine has been initiated. 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. 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. 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.

## EXPERIMENTAL IMMUNOLOGY BRANCH

The Experimental Immunology Branch, directed by Dr. Alfred Singer, carries out laboratory investigations in basic immunobiology with particular emphasis on lymphocyte differentiation and regulation; cell biology of immune responses; signal transduction; structure, regulation and function of genes involved in immune responses; transplantation biology; and tumor immunology. A flow cytometry laboratory is maintained that supports over 80 individual research projects each year.

### Lymphocyte Differentiation and Regulation

Dr. Alfred Singer's laboratory continues to focus on the role of CD4 and CD8 accessory molecules in T cell differentiation and function. Dr. Singer found that antibody induced engagement of CD4 on immature $CD4^+8^+$ (double positive) murine thymocytes caused the immature double positive thymocytes to markedly increase their expression of T cell receptor (TCR). Since increased TCR expression is a necessary step in the differentiation of immature double positive thymocytes into mature single positive thymocytes, these studies indicate a critical role in T cell differentiation for CD4 engagement on developing thymocytes. Dr. Singer's studies to elucidate the molecular basis for the increased expression of T cell receptor on immature thymocytes in response to CD4 engagement revealed that the great majority of receptor proteins are retained and degraded within the endoplasmic reticulum of immature thymocytes, and that the amount of TCR retained and degraded in the endoplasmic reticulum is regulated by CD4-mediated signals. Dr. Singer's laboratory has also examined the role of T cell receptor expression in thymocyte development, and the mechanisms by which T cell tolerance is induced in the thymus.

Identifying and subsequently characterizing T cell subsets has been essential to our current understanding of cellular function and interactions in the immune system. Studies from the laboratory of Dr. Stephen Shaw continue to elucidate the phenotypic heterogeneity of normal human T cells by defining two further subpopulations within human memory T cells. Data on further diversity of human CD4+ T cells is being systematically accumulated to enable a much more complete classification of T cell subsets. Limited preliminary studies have been undertaken to characterize CD4 T cell subsets in HIV-infected individuals.

Dr. Gene Shearer's laboratory has investigated a series of murine models of autoimmunity, including those with thyroiditis-like and with lupus-like characteristics. These studies may provide a better understanding of immune regulation and dysregulation in autoimmune disease. Studies of T lymphocyte responses in $HIV^+$, asymptomatic individuals by Dr. Shearer's laboratory indicate that approximately 50% of these patients exhibit selective $CD4^+$ T helper cell (Th) functional defects without any AIDS symptoms and without loss of $CD4^+$ cell numbers. Dr. Shearer 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 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. The results of this study 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 and implicate an antigen-presenting cell (APC) defect in AIDS patients but not early in disease progression.

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. His findings indicate that maintenance of tolerance to a variety of self determinants results in substantial deletions in available TCR Vß repertoire. The self determinants that function as ligands for Vß-specific T cell deletions were shown generally to represent the products of non-MHC-encoded genes in association with MHC gene products. Ligands responsible for deletion of Vß11- and Vß12-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.

Structure, Regulation and Function of Genes Involved in Immune Responses

Studies carried out in Dr. Dinah Singer's laboratory have demonstrated that the swine class I MHC multigene family contains only seven members. However, within this family three sub-groups of genes can be discerned based on their sequence homologies; these sub-groups also display discrete patterns of expression. In order to investigate the molecular basis for the differential patterns of expression, Dr. Dinah Singer's laboratory has begun to generate and analyze transgenic mice containing isolated swine class I genes. For one gene encoding a classical transplantation antigen, PD1, it has been demonstrated that the pattern of expression of the swine gene in the transgenic parallels that observed in situ in the pig. Lines of transgenic mice containing various constructs of another class I gene, PD7 have been generated. Analysis of PD7 expression in these mice, as a function of the various regulatory elements present, reveals the presence of a lymphoid tissue specific silencer which contributes to the regulation of PD7 in vivo. Dr. Dinah Singer's group has also initiated studies to characterize DNA sequence elements and cognate trans-acting factors associated with class I MHC genes. Their analysis has 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 a DNA regulatory complex.

Transplantation Biology

Dr. Alfred Singer's laboratory has continued to investigate the basic cellular mechanisms responsible for initiating and mediating transplantation responses in vivo. They have found that in vivo skin allograft rejection responses require the initial activation of lymphokine secreting Th cells and the subsequent activation of lymphokine responsive Tk cells. Monoclonal antibodies with specificity for cell surface antigens expressed by human T cells have been used in Dr. Ronald Gress' laboratory to deplete normal T cells from allogeneic marrow and to remove malignant T cells from autologous marrow in an attempt to permit

allogeneic bone marrow transplantation and avoid GVHD. A clinical protocol has been developed to assess the feasibility of utilizing these marrows in the treatment of aggressive T cell malignancies in combination with marrow ablative chemotherapy and radiotherapy. The initial stage of a phase one study has been completed with definition of a new chemotherapy regimen, the efficacy of which is under evaluation.

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL), may play a significant role in mediating allogeneic marrow graft rejection. In a murine model system developed in Dr. Gress' laboratory it was shown that IL-2 enhances suppressor activity of T-cell-depleted donor marrow, and enhances marrow engraftment in sublethally irradiated, MHC-disparate hosts. The administration of monoclonal antibodies with specificity for CTL surface antigens into sublethally irradiated hosts results in T cell activation within hours of administration. The injection into sublethally irradiated hosts of anti-CD5 monoclonal antibody with donor marrow results in allogeneic chimerism. If the antibody is given seven days before the marrow, such facilitation of engraftment is not observed, although host T cell responses are suppressed.

Studies in murine marrow chimeras have suggested that MHC mismatched marrow transplantation is associated with immunoincompetence of the T cell arm of the immune response. Preliminary data from studies in a Rhesus monkey model suggest that the generation of T cells in marrow grafted primates may not be the same as in the mouse. This has potentially very important implications for the transplantation of HLA-mismatched marrow in man. Studies of T cell regeneration in patients receiving T cell depleted autologous marrow demonstrate the regeneration of $CD4^+$ and $CD8^+/CD28^+$ subsets of T cells which appear, by preliminary evaluation, to be oligoclonal rather than polyclonal in nature.

## LABORATORY OF CELL BIOLOGY

The Laboratory of Cell Biology was reorganized this year under the direction of Dr. Michael M. Gottesman. The primary focus of research activity centers on the genetic analysis of multidrug resistance in tumor cells.

### Resistance of Cancer Cells to Chemotherapy

Resistance of cancer to chemotherapy is the major obstacle to successful treatment. Dr. Michael Gottesman, working in collaboration with Ira Pastan, Chief, Laboratory of Molecular Biology, has shown that one of the major mechanisms responsible for the development of multidrug resistance to natural product drugs is the expression of an energy-dependent drug efflux pump. This pump, called P-glycoprotein, or the multidrug transporter, is the product of the MDR1 gene in the human. Over 100 kb of this gene, consisting of 28 introns and 29 exons, has been cloned, as has a 4.5 kb cDNA. Expression of the MDR1 cDNA in a variety of eukaryotic expression vectors, a retroviral system, and a transgenic mouse system where expression occurs in bone marrow, has been used to prove that the MDR1 gene encodes a multidrug transporter which is functional in vitro and in vivo. Vectors have been developed which use the MDR1 cDNA as a dominant selectable, amplifiable marker for introduction of non-selectable genes into cultured cells and bone marrow.

Structure-function studies on the multidrug transporter have been undertaken using molecular genetic and biochemical approaches and have demonstrated specific drug interaction sites in both halves of the P-glycoprotein molecule. Energy transfer studies using doxorubicin and iodonaphthalene azide indicate that the pump is capable of removing hydrophobic drugs from the membranes of resistant cells.

Clinical studies strongly support a role for P-glycoprotein in both intrinsic and acquired drug resistance of human cancer. The multidrug transporter is found at physiologically significant levels in a wide variety of intrinsically resistant solid tumors. In acute non-lymphocytic leukemias, its expression at the time of presentation predicts poor response to chemotherapy. In other cancers, levels of MDR1 RNA increase in tumors which have acquired resistance to chemotherapy. These results point to a need for clinical trials to determine the ability of agents which inhibit the multidrug transporter to improve efficacy of existing chemotherapy for tumors which express P-glycoprotein.

Mechanisms of Growth Regulation by cAMP

Dr. Gottesman has isolated Chinese hamster ovary (CHO) cell mutants which are resistant to the growth inhibitory effects of cAMP or its analogs. These mutants have alterations in the regulatory (R1) and catalytic (C) subunits of cAMP-dependent protein kinase (PK). Some of these substrates are likely to be transcription factors. A yeast phosphodiesterase gene which breaks down cAMP as it is formed within cells has been introduced into transgenic mice under control of tissue-specific promoters to study the role of cAMP in development and function of specific organs.

ATP-dependent proteases determine the levels of important regulatory proteins in a variety of diverse cellular processes. Dr. Michael Maurizi has been studying two ATP-dependent proteases in Escherichia coli: the Lon protease, and the two-component Clp protease. These proteases have been purified to homogeneity and their substrate specificity has been determined.

LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY

The Laboratory of Tumor Immunology and Biology, directed by Dr. Jeffrey Schlom, carries out a range of investigations in tumor immunology, tumor cell biology, and molecular biology with particular emphasis on the characterization and utilization of monoclonal antibodies directed against tumor-associated antigens and their potential diagnostic and therapeutic applications.

Monoclonal Antibodies Define Carcinoma-Associated and Differentiation Antigens

Monoclonal antibodies (MAbs) directed against antigens associated with human carcinoma are being developed to better understand the cell biology and pathogenesis of several human carcinomas and to provide reagents that may be useful in the management of human cancer. The MAbs generated in this laboratory can be classified into three groups. Antigens differentially expressed in human carcinoma *versus* normal adult tissues, such as the tumor-associated glycoprotein (TAG)-72 which is detected by MAb B72.3, and carcinoembryonic antigen (CEA) which is detected by MAbs COL-1 through COL-15; tissue-associated antigens, such as the breast-associated antigen detected by MAb DF3, and the colon-associated antigens

detected by MAbs D612 and CAA; and oncogene- or retroviral-related gene products, such as *int*-2.

A series of "second generation" MAbs to the TAG-72 antigen have been developed, some of which (such as CC83 and CC49) have a higher affinity constant for TAG-72 than B72.3, and may be better suited for some clinical applications. Studies have also been conducted in the analysis of the COL MAb series and MAb D612 to define which MAbs are best suited for *in vivo* clinical applications. The usefulness of mouse MAbs in therapy 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. Recombinant/chimeric (rec/chi) MAbs to TAG-72 and anti-CEA MAb B72-3 have been produced and appear to have retained specificity and binding properties of the parental MAb. These rec/chi constructs and modified forms may be better suited for diagnostic and therapeutic applications.

Augmentation of Tumor Antigen Expression

Experimental data have shown that recombinant human interferons can increase the level of expression of human tumor-associated antigens. Using a large number of colorectal carcinoma cell lines, both Type I (i.e., IFN-α and IFN-β) and Type II (i.e., IFN-γ) can enhance the cell surface expression level of CEA and related antigens as measured by monoclonal antibody binding. The level of expression of TAG-72 was also found to be regulated by interferon. A phase I clinical trial was undertaken to determine whether IFN-gamma could augment tumor antigen expression in patients, and the results suggest that recombinant human interferon may serve as an adjunct by enhancing cell surface tumor antigen expression, thereby augmenting the localization of a monoclonal antibody.

Isolation and Characterization of Genes Coding for Carcinoma-Associated Antigens

Recent studies have demonstrated that CEA belongs to a multigene family related to the immunoglobulin supergene family, and this family of genes exists in a state of hypermethylation in normal cells. In contrast, the CEA gene(s) are relatively hypomethylated in the tumor cell lines, suggesting a correlation between the state of methylation and degree of expression of the CEA gene(s). Studies are also in progress to identify and characterize the gene(s) encoding the TAG-72 mucin antigen, and mucin genes representing other tissue types. Nucleic acid sequence analysis has shown them to be identical, with the difference residing in the sugar epitopes detected by the MAb.

Anti-Carcinoma Monoclonal Antibodies in Clinical Trials

To date, over 1,000 patients have been administered radiolabeled B72.3 in tumor-targeting studies carried out in several institutions, with similar findings of approximately 70-80% tumor targeting observed. 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 also in progress. The use of recombinant/chimeric MAbs has begun and other MAb-isotope conjugates are planned.

Additional studies are in place to determine the optimal route of inoculation of antibody as well as the antibody form. Major emphasis will be placed on the

pharmacokinetics of plasma clearance of recombinant/chimeric monoclonal antibodies and their ability to target tumors.

Identification and Characterization of Genes Associated with Breast Cancer

Dr. Robert Callahan's laboratory has continued to focus on the identification and characterization of genes associated with the development of breast cancer. In the mouse, mouse mammary tumor virus (MMTV) appears to induce mammary tumors by acting as an insertional mutagen that leads to the activation of previously silent cellular genes (*int* genes). Previous studies demonstrated that the inbreeding program used to develop mouse strains with a high incidence of MMTV-induced mammary tumors may also have fixed other cellular mutations that affect the frequency with which *int*-1 and *int*-2 are activated. The *int*-1 and *int*-2 genes are much less frequently activated in feral mouse mammary tumors, suggesting that other *int* loci are active in these tumors. Dr. Callahan's group has described one such locus, designated *int*-3, in CZECHII $V^+$ feral mice. The organization of *int*-3 differs from that of *int*-1 or *int*-2. MMTV insertions occur within a 500-bp region of the cellular genome on chromosome 17. Viral insertion at this locus leads to the activation of the expression of two cellular RNA species that correspond to host flanking sequences located 5' and 3' of the viral insertion site, respectively. Dr. Callahan's group is currently determining the nucleotide sequence of recombinant cDNA clones corresponding to each of these RNA species.

Dr. Callahan's group is also pursuing studies aimed at determining on a molecular level those genetic alterations in primary human breast tumor DNA that have a statistically significant association with patient history, tumor characteristics, and patient postsurgical prognosis. The *int*-2 gene (frequently activated by MMTV in mouse mammary tumors) is amplified 2- to 15-fold in 14% of primary human breast tumor DNAs and has a significant association with a poor prognosis for the patient. Dr. Callahan has now defined the amplification unit on chromosome 11q13 and found that 17 of 18 tumors amplified for *int*-2 were also amplified for *hst* and *bcl*-1. The *hst* and *int*-2 genes are both related to basic fibroblast growth factor. The *bcl*-1 locus defines the translocation t(11;14) breakpoint in B-cell chronic lymphocytic leukemias. In a lymph node metastasis of a breast cancer patient, *int*-2 but not *hst* was expressed. In 10% of the primary breast tumor DNAs, the c-*erb*B-2 gene was amplified. However, in contrast to published reports, Dr. Callahan has found no significant association with patient postsurgical prognosis or other aspects of patient history and tumor characteristics. In other studies, Dr. Callahan has detected the expression of MMTV-related human RNA sequences in breast tumor-derived cell lines and has obtained recombinant cDNA clones of these sequences for further analysis.

LABORATORY OF IMMUNOBIOLOGY

The genetic basis of human renal cell carcinoma has been a major focus of the research effort of the Laboratory of Immunobiology directed by Dr. Berton Zbar. His group has continued to study hereditary and sporadic renal cell carcinomas and to isolate and characterize new probes from human chromosome 3. Von Hippel-Lindau disease (VHL) is an autosomal dominant trait characterized by a predisposition to develop retinal angiomas, cerebellar and spinal hemangioblastomas, renal cell carcinomas, pheochromocytomas and benign tumors of the epididymis and pancreas. Earlier studies supported the concept that the VHL gene is a recessive oncogene. Renal cell carcinomas obtained from patients with von

Hippel-Lindau disease showed 3p allele loss from the chromosome 3 bearing the wild-type allele of the VHL gene. This suggested that the unaltered (normal) form of the VHL gene is essential for normal growth of several cell types. Loss of function of both copies of this gene is a critical early step in the development of certain benign and malignant tumors.

Most recent studies have enabled a more precise determination of the location of VHL on chromosome 3. The results of multipoint linkage analysis suggest that VHL is located between RAF1 and D3S18. The D3S18 marker was inherited with the disease gene in about 95% of affected individuals, and thus may be useful in identifying asymptomatic gene carriers in VHL families. Asymptomatic individuals, predicted to be gene carriers by genetic analysis, were shown to have one or more of the manifestations of the illness when screened at the National Institutes of Health.

The results suggesting the VHL is located between RAF1 and D3S18 have been guiding efforts to isolate the VHL gene. A small region of human chromosome 3 (from RAF1 to D3S18) has been found to be conserved and located on mouse chromosome 6. This conserved region of human-mouse homology will help identify human DNA fragments that are close to the VHL gene. A large number of new polymorphic probes are being tested in VHL families to find probes that do not recombine with the disease gene. As yet no probes have been identified between D3S18 and RAF1. A grant to construct a high resolution genetic map of human chromosome 3 was awarded to the Laboratory of Immunobiology by the National Center for Human Genome Research.

Cohen et al. (New England Journal of Medicine, 1979) identified a family with a 3;8 translocation associated with the development of renal cell carcinoma. Ninety percent of family members with the translocation developed renal cell carcinoma by age 60. Dr. Zbar's group has used cytogenetic and molecular techniques to test for the presence and source of 3p loss in renal cell carcinomas associated with the 3;8 translocation. They found that the derivative chromosome 8 (bearing the distal portion of 3p) was lost in the renal cell carcinomas of two patients with this form of hereditary renal cell carcinoma. This suggests that there are at least three events in the origin of renal cell carcinoma associated with the 3;8 translocation: (1) inheritance of the translocation chromosome; (2) a somatic mutation of the renal cell carcinoma gene located on the "normal" chromosome 3; and (3) loss of the derivative 8 chromosome.

## 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.

Sequence Analyses in Virology, Cell and Molecular Biology

New analytical tools continue to be developed for studies of proteins and nucleic acids; numerical methods that aid in the prediction of secondary structures, splice sites, promoters, and recombination sites in nucleic acids; and graphic representations which reveal homology, and reverse complementarity. These programs were developed and have been installed on a variety of computer systems at the Advanced Scientific Computing Laboratory. Structures up to 2000 bases in size have been predicted.

RNA secondary structure methods have been refined to include alternate energy parameters, extended Monte Carlo simulations, and comparative studies to establish firmly the uncommon structural features in subregions of a number of sequences of HIV and other retroviruses. These predicted structural features have been correlated with biological features, leading to deeper understanding of the replication and expression processes in this group of viruses. In HIV-1 and related viruses, predicted stable features have been correlated with sites of <tat>-regulation elements, <rev> responsive elements and sites of translational frame-shift. Conserved secondary structure is predicted to be absent in regions of hypervariability in the envelope gene m-RNA's. Use of the supercomputer has allowed development of an improved 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.

Molecular Structure

Dr. Robert Jernigan's group has developed a novel approach to the problem of protein folding that examines the complete range of folded topologies accessible in the compact state of globular proteins. The procedure is to generate all conformations, with volume exclusion, upon a lattice in a space restricted to the individual protein's known compact conformational space. This affords an enormous reduction in the number of conformations that must be considered for the complete problem. Five proteins have been treated using this method: avian pancreatic polypeptide (36 residues), crambin (46 residues), rubredoxin (52 residues), pancreatic trypsin inhibitor (58 residues), and neurotoxin (62 residues). Additional methods are being developed to further narrow the choice of the best among the good conformers. One approach superimposes all atoms onto a lattice conformation, and refines this structure with conventional molecular dynamics and energy minimization.

Work on nucleic acid structure has continued in a variety of collaborations and directions. Dr. Bruce Shapiro has expanded his analysis of RNA structure to include more functionality for analyzing RNA conformations from various perspectives. This has involved the development of new algorithms to explore secondary and tertiary structural motifs.

Simulation, Analysis and Modeling of Physiological Systems

A long-standing research interest of the laboratory is the development of the simulation, analysis, and modeling (SAAM/CONSAM) computer programs. The development of a version of SAAM30 to run under the DOS operating system on personal computers that makes use of the Intel 80386 and 80486 central processor continues. The SAAM group participated in an international committee to

contribute advice regarding the direction of kinetic analysis. A special workshop was again organized to discuss the kinetics of lipids and lipoproteins, the investigation of which centers around the use of SAAM and CONSAM.

Theoretical Immunology

The long-term aim of Dr. John Weinstein's laboratory 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. Recent work has centered on quantitative modeling of the pharmacology of monoclonal antibodies. Previously, Dr. Weinstein's group found that dipyridamole (DPM) potentiates the activity of AZT and other dideoxynucleosides against HIV in monocytes and stimulated T-lymphocytes. Further, in a T-lymphoblastoid cell line, DPM simultaneously potentiates the antiviral potency of AZT while it significantly decreases AZT's toxic effect on bone marrow stem cells. The AZT-DPM combination has been approved for study within the AIDS Clinical Trials Group. Initial clinical trials will begin in the summer of 1990 in collaboration with groups at two other institutions.

Membrane Structure and Function

Dr. Robert Blumenthal's group has continued its study of the mechanisms of membrane fusion mediated by viral spike glycoproteins. The mode of action of the envelope protein of HIV, the G protein of Vesicular Stomatitis Virus (VSV), the HN and F proteins of Sendai Virus, and the HA protein of influenza virus are studied.

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. Dr. Blumenthal has developed biophysical techniques to study the initial steps of viral envelope protein mediated membrane fusion and has applied these techniques to the study of the initial steps of HIV envelope protein-mediated membrane 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 antiviral antibodies. 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.

SUMMARY REPORT

LABORATORY OF GENETICS, DCBDC, NCI

October 1, 1989 through September 30, 1990

The Laboratory of Genetics is in its 8th year. Many projects in the lab are based on understanding the pathogenesis of plasma cell tumor development in mice; these include biological, biochemical and molecular genetic studies. Linda Wolff continues to investigate the pathogenesis of myeloid tumors in mice, Hayden Coon is studying the neurons of the olfactory epithelium, and Dr. Smith-Gill is investigating the chemistry and biology of antibodies to hen egg white lysozyme.

Stuart Rudikoff. This has been a year of transition in which Dr. Rudikoff has begun investigating pristane induced plasmacytomagenesis. He has established a good breeding colony of SCID mice and is carrying out reconstitution experiments by transplanting BALB/cAn or DBA/2 bone marrow into SCID recipients and then injecting them with pristane. The resulting plasmacytomas will be tested for their strain of origin. In the course of these studies he has discovered that Peyers Patch cells can dramatically reconstitute immunoglobulin production in SCID recipients. In collaborative studies with Alberto DeGrassi and David Hilbert, adhesion dependent primary plasmacytomas have been established in culture. These cells maintain their dependence on the pristane conditioned environment for growth.

Wendy Davidson continues to develop as an independent investigator in her detailed work on the differentiation switch of B-cell precursors into myeloid cells. A critical gene in this process is CD45 (Ly5) which Dr. Davidson has shown to express differentiation specific isoforms by splicing out exons of the CD45 gene. She has continued her work on the characterization of unusual T-cell subsets in lymphoproliferative diseases associated with lpr and gld (autoimmune) genes. She has demonstrated some cell types that inappropriately express differentiation antigens.

Konrad Huppi has cloned a cDNA for PVT-1. PVT-1 is a chromosomal breaksite located 100-300 kb 3′ of c-myc that is involved in illegitimate exchanges with Ig light chain loci. Drs. Huppi and Mushinski have shown PVT-1 is transcribed and that unusual transcripts are generated in plasmacytomas. The mechanism whereby PVT-1 is involved with the deregulation of transcription of c-myc is not yet understood but is being vigorously pursued.

Linda Wolff continues her studies on the induction of acute pre-monocytic leukemias by Moloney virus in pristane conditioned mice. Evidence that prostaglandin ($PGE_2$) may play a critical role in the pathogenesis of these tumors has been demonstrated by showing that $PGE_2$ can reverse the effects of indomethacin (which inhibits the development of these leukemias by Moloney virus in pristane-conditioned mice). Splenectomy dramatically decreases the incidence of these acute myeloid tumors.

Sandra Smith-Gill continues her structural analysis on the interaction of monoclonal antibodies to hen egg white lysozyme (HEL). Crystals of Fab-HEL complexes have been refined, and these have been used to locate epitopes on HEL to define the structural basis of interaction of antibody molecule and protein antigen and to carry out experiments on site-specific mutagenesis. Dr. Smith-Gill has given out these α-HEL mAbs to other workers, and her system is widely used as a model beyond NIH for studying antiproteins Igs. Dr. Smith-Gill has characterized over 500 α-HEL mAb from mice immunized in different ways to HEL. Surprisingly, thus far she has not been able to gain evidence to support the concept of affinity maturation; rather it appears even high affinity antibodies can be isolated from primary responses. This study has intriguing implications for explaining mechanisms of antibody diversity. The α-HEL system has many potentially valuable biological applications.

Drs. Mushinski, Mischak, Weissinger and Smith-Gill are working with Rex Risser's helper free ABL/myc transforming retrovirus. This virus induces plasmacytomas in non-pristane conditioned mice and virtually 100% plasmacytomas in pristane conditioned mice with short latent periods. When ABL/myc is injected into immunized mice, transformation of the antibody producing cells occurs. High titers of antibody can be found in the mice as well as with the transplants. It is not clear whether this will be an alternative to the Kohler-Milstein method for generating monoclonal antibodies. Current work is directed to finding means for propagating these tumors in vivo and in vitro.

Grace Shen-Ong has begun working on an unusual mutation which arose in our colony. One hundred per cent of mice carrying this recessive mutation develop between 6 and 12 weeks of age a severe, crippling destruction of the foot bones and tail vertebrae. Dr. Shen-Ong has found the mutant gene is located on chr 18. Slides of the lesions have been given to several pathologists, and it appears the earliest lesions are found in the bone marrow cavities as inflammatory cells associated with the endosteum and the periosteum. As this disease occurs in a specific period of development, it interferes with the remodelling of foot and tail bones. The exact human counterpart is not clear at this time. Dr. Shen-Ong continues her work on the myb structure and expression of the myb gene in mice.

Hayden Coon has successfully cultured odorant responsive olfactory receptor neuroblasts (ONR) from the neonate rat and is currently applying his method to culture comparable cells from humans. Biochemical markers and responses to odorants all confirm that these cells are neurons. These cells represent one of the first mammalian neuronal systems that have been successfully cultured.

Plasmacytomagenesis model

General. At 220 days none of the 56 SPF BALB/cAnPt mice have developed plasmacytomas as compared with 32% in controls. This experiment carried out by Linda Byrd (graduate student) confirms previous results from our laboratory that genetically susceptible BALB/c mice raised in specific pathogen free conditions are resistant to plasmacytoma induction by pristane. Ann McDonald has shown that the peritoneal exudates and oil granulomas of SPF BALB/cAnPt are highly depressed in $CD4^+$ T-cells. Morphological studies of the oil

granuloma during the latent period of plasma cell tumor development (days 25-120) has shown that foci of (?proliferating) plasma cells appear to originate in the mesenteries of the small intestine.

Genetic studies. Beverly Mock has studied 320 BALB/c x (BALB/c x DBA/2)$F_1$ progeny in which she has given pristane to induce plasmacytomas; of 41 (13%) plasmacytomas, 30 have been typed for DBA/2 and BALB/c genetic markers, and it has been found that susceptibility is associated with genes in a 12 cM switch on chromosome 4. This finding suggests that Pctr-1 (chr 4) gene has both susceptibility and resistance alleles. Dr. Mock has also isolated a new $D_2$/BALB/c allelomorphic marker 1B-4 located in chr 4 that has been valuable in mapping genes on distal 4. Dr. Mock has now shown that genes on chr 17 play a role in the susceptibility of mice to the highly plasmacytomagenetic RIM (carrying E$\mu$myc and v-Ha-ras) virus. The physiologic function of the Pctr-1 gene is not known. Wilhelm Bohr, Jeff Beecham and Emily Shacter have, however, found a new phenotypic difference between BALB/c and DBA/2 relating to DNA repair that is linked to chr 4, i.e., C.D2 Fv-1 N19 mice express the DBA/2 phenotype.

Emily Shacter is continuing to search for a biochemical marker that is relevant to plasmacytomagenesis. The non-steroidal anti-inflammatory agent, indomethacin, profoundly inhibits plasmacytomagenesis and the major effect of this drug is as an inhibitor of cyclooxygenase, a key enzyme in prostaglandin biosynthesis. Dr. Shacter has shown that peritoneal macrophages stimulated by pristane produce relatively large amounts of (indomethacin inhibitable) $PGE_2$. Her work is now directed to finding how this exogenous agent affects B-cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 05552-21 LGN

PERIOD COVERED
October 1, 1989 to September 30, 1990

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 |
| | A. Mancini | Guest Researcher | LGN, NCI |
| | S. Minucci | Fogarty Int. Fellow | LGN, NCI |
| | B. Wolozin | Staff Fellow | NIMH, LCS |
| | S. Freeman | Staff Fellow | NHLB, MH |

COOPERATING UNITS (if any)
Prof. P. Graziadei and Dr. A. Monti-Graziadei, Florida State University, Tallahasse, FL

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 1.0 | 1.0 | 0.0 |

CHECK APPROPRIATE BOX(ES)

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

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:

Since the publication in March 1989 of our successful culture of neuroblasts of the rat olfactory epithelium and the demonstration of the sensitive and selective response to chemical odorants by the resulting cell lines, a revolution has started that has been silent until very recently. I am pleased to report that we have made some progress in the past year.

I have succeeded in repeating the isolation of cloned strains of odorant responsive olfactory receptor neuroblasts (ONR) from new born rats. We have found conditions that make more sensitive and less noisy the cAMP assays that are used in characterizing the SNIF cell odorant response. We need, however, to extend these observations to individual cell (image analysis) using our two year old discovery of an odorant dependent $Ca^{++}$ response using fura-2 and further to analyze electrical activity using the voltage sensitive dyes.

I have succeeded in developing strains of sensory cells from the rat vomeronasal organ (VNO) and have shown that these cells are NOT responsive (cAMP accumulation) to the same chemical odorants that stimulate the ONR SNIF cells but rather, they are actively stimulated by high molecular weight fractions in male rat urine. At this writing it is not known whether the response is to a macromolecule or to smaller molecule tightly bound to a carrier molecule. One of the prime motives for investigating VNO cells is the hope of finding an olfactory cell that responds to a macromolecular ligand (the isolation and cloning of the much sought after olfactory receptor would be much simplified with such a ligand). We are hoping that RUP, the rat urinary protein or some similar molecule will prove to be such a ligand. This study represents the first time that a cell from the VNO has been isolated and shown to be responsive to physiological stimulants. Cell strains of this type should prove especially useful is assaying pheromones and in dissecting a wide range of mammalian behavioral responses.

One of the important medical benefits of culturing mammalian neuroblasts would be the possibility that such cells could be used as strategic impants in damaged or deficient regions. In culture many of our SNIF cells suggest an incompletely differentiated neuron. Accordingly, in collaboration with Prof. P. Graziadei and Dr. Monti-Graziadei (Florida State University, Tallahassee) we have initiated studies seeking to test whether more perfect olfactory diferentiation of the cultured SNIF cells can be achieved by reimplantation into various sites in the CNS, or whether (as we both suspect) the SNIF neuroblast series represent cells that are a kind of "generic" neuron that may differentiate consistently with the requirements of the local environment in which they mature. In order to be able to identify these cells in grafts, we have collaborated with Dr. Scott Freeman (NHLB,MH) and transfected the SNIF cells with a defective retroviral construct that confers both G418 resistance and the bacterial $\beta$-galactosidase gene to cells that stably integrate the viral construct. Preliminary experiments have established the conditions for preparing pseudotissues from the cultures, the grafting operations (olfactory bulb, frontal cortex, cerebellum, anterior eye chamber) into new born rat pups and the subsequent differential staining of the grafted cells. In the exploratory experiments that have established the feasibility of the protocol, the SNIF cells proved to be highly malignant and

killed all the host animals within 10 days. The first transfections had to be done with a cell strain, SNIF-11, that had been in culture for over 500 cell generations and had apparently become "spontaneously transformed". Alternatively, the transfection/integration itself might have caused the tumorigenesis.

In collaboration with Dr. Ben Wolozin (NIMH,LCS) and with the kind support of the NIMH, we have succeeded in adapting the methods (worked out in my laboratory over the past 5 years) to the culture of human olfactory epithelium! Seven cloned strains have been isolated so far from cadaver material of both normal and Alzheimer persons. A biopsy protocol has been approved. Because of its accessibility, the olfactory epithelium can be biopsied easily and could supply cells of the central nervous system for in vitro diagnosis and physiological as well as pharmacological study. A U.S. patent application has been filed. These cell strains are especially promising because they apparently have more highly differentiated phenotypes in culture than do the corresponding rat cells (SNIF). I hope to use them in our attempts to produce a cDNA library that could help us to isolate and clone the receptors for the individual odorants (for which we have demonstrated a vigorous cAMP response) using the FRTL transfection, TSH replacement technique described by Richard Axel last year.

In spite of my spectacularly successful, innovative and exciting basic work in developing the first in vitro systems for study of the regulation of growth and differentiation in the only portion of the vertebrate central nervous system that is renewed throughout life from a stem cell population, I have been frustrated at every turn in my efforts to follow it up as vigorously as it deserves. During this year I requested a position for a sabbatical visitor who is considered the acknowledged leader in the biochemistry of our field: this was summarily denied. My timely request to replace our technician who left to go on to study neurobiology at Yale was ignored. My repeated pleas for equipment with which to make crucial measurements on our cells were denied. The position of a staff fellow who was contributing needed support in biochemistry was withdrawn.

Recently the announcement of culture of human brain cells from a two year old child has captured national attention and been described by high officials of the NIH as "astounding" and "very important breakthroughs". The national press reports have preceded the publication, but as far as one can make out, this achievement pales into insignificance compared to the importance of Ben Wolozin's cell strains that can be obtained by biopsy from any person. I find it sad that a mean spirited lack of vision is depriving the National Cancer Institute of an opportunity to be celebrated at the forefront of this revolution in our understanding of the blastematic cells of the central nervous system.

We hope to find support for these and other worthy projects during the coming year.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 05553-21 LGN

PERIOD COVERED
October 1, 1989 to September 30, 1990

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 |
| W. Davidson | Expert | LGN, NCI |
| J. Hyde | Staff Fellow | LGN, NCI |
| D. Hilbert | Biotechnology Fellow | LGN, NCI |

COOPERATING UNITS *(if any)*
Dr. J. Pierce, NCI; Dr. K. Holmes, Dr. A. Hubin, Dr. C. Calkins, NIAID, NIH

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Laboratory of Genetics

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NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 6 | 4 | 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.)*

(1) A SCID mouse colony has been established to examine in vivo aspects of both neoplastic and normal development. In the first instance, this system is being used to analyze the basic genetic requirements for plasmacytoma induction. SCID mice have been reconstituted with bone marrow from either susceptible or resistant strains and have been subjected to tumor induction protocols to determine the relative contributions of genetic background versus potential B-cell defects in the induction process. The SCID model is also being used to assess the genetic potential of various normal lymphocyte populations through appropriate reconstitution experiments. (2) The initial development of plasmacytomas has been difficult to study due to the inability to grow early stage tumors in culture or prevent them from progressing to later stage phenotypes. This problem has now been significantly lessened by the demonstrated ability to grow primary plasmacytomas in vitro on feeder layers consisting of autologous adherent cells derived from the same tumor. Characterization of these feeders and their interactions with plasmacytoma cells are currently being evaluated. (3) Two longstanding studies of genes that regulate the differentiation of haematopoietic cells have continued. In the first system it was shown that a number of events including production of GM-CSF and IL-6, down regulation of myb and up regulation of fos and fms mRNA transcripts and changes in CD45 isoform expression may contribute to the switch to myeloid differentiation by bipotential B lymphocyte progenitors. A detailed study of CD45 isoform expression during devlopment of B lymphocytes and myeloid cells was initiated. Further studies of mice homozygous for lpr or gld, two genes that interfere with normal T cell differentiation and function, showed that following cross linking of the TcR, the expanded DN T cells were unable to produce a spectrum of lymphokines, whereas the diluted $CD4^+$ T cells overproduced lymphokines involved in inflammatory responses and thus may contribute to the development of autoimmune disease.

Major Findings:

Dr. S. Rudikoff

1. The study of plasmacytomagenesis has been one of the major interests among this group for more than 30 years. One aspect of this program has involved the establishment and use of in vitro plasmacytoma cell lines to approach various aspects of the problem including karyotypic alterations, oncogene expression, and factor requirements. Although useful, these lines have been derived from established plasmacytomas which differ from newly arising plasmacytomas in that they are transplantable in vivo in the absence of pristane priming. In order to study earlier stages of plasmacytomagenesis reflected by the retention of priming dependence, we have attempted to culture plasmacytoma cell lines from the primary site of oil induced tumor induction, the granuloma. To date, 4 newly arising plasmacytomas, each of which is dependent on an autologous, granuloma derived adherent cell layer consisting of mesothelial, endothelial, fibroblastic and macrophage-like cells have been successfully cultured. It has been determined that the subsequent in vivo growth of at least one of the resultant plasmacytoma lines is still dependent on pristane priming indicating that growth on these feeders has maintained the tumor in an 'early' stage of tumor progression. The growth requirements of the other lines are currently being tested. The adherent feeder cell lines have been cloned and are currently being characterized in terms of phenotype as well as their individual ability to support plasmacytoma growth and the long term growth and differentiation of normal B-cells. Initial experiments suggest that normal B-cells, when cultured on the appropriate adherent cell line, survive for 4-6 weeks during which time they are induced to secrete immunoglobulin predominantly of the IgM isotype.

2. A second set of experiments relating to the plasmacytoma question has recently been initiated to attempt to address one of the fundamental issues concerning the genetic basis of induction. It has long been appreciated that the BALB/cAn strain of mice is susceptible to tumor induction whereas DBA/2 is resistant. However, the relationship between the requirements for the BALB/c background versus the BALB/c B-cell is largely unknown. We are approaching this question through the use of our recently developed SCID mouse colony. The SCID mouse is an immune defective animal which, essentially, has no B- or T-cells. This animal thus serves as an ideal recipient for the introduction of donor cells to be tested for biological responses in an in vivo situation.

We have taken advantage of the SCID model to reconstitute these animals with bone marrow from either susceptible (BALB/c) or resistant (DBA) strains. Such animals appear fully reconstituted by 8 weeks in terms of serum immunoglobulin and have now been introduced into a conventional colony where tumor induction protocols are being initiated. It is believed that these experiments will differentiate the contribution of genetic background versus the role of the BALB/c B-cell in the tumor induction process.

3. The use of SCID mice to examine the genetic potential of specific cell populations has been extended in that we have also reconstituted SCIDs with Peyer's patch cells. Peyer's patches are of particular interest in that these

organs form what is believed to be the initial immunologic response to antigens encountered through the gut. These organs are unusual in that Peyer's patch B-lymphocytes express either IgM or IgA, but none of the other subclasses. Surprisingly, eight weeks post reconstitution SCID mice display serum immunoglobulin patterns indistinguishable from normal mice with the predominant isotype being IgG. These animals are currently being examined for the extent of reconstitution by cell sorting analysis and the determination of B-cell Ig gene repertoire. Similar studies are also being carried out with peritoneal cells as the reconstituting population.

4. A novel set of spontaneously arising hematopoietic cell lineage tumors have been identified which arise in 18-24 month old BALB.H-$2^b$ (BALB.B) but not in the parental BALB/cAn or H-$2^k$ congenic BALB.K mice. These tumors occur in virtually all aged BALB.B mice, and characteristically involve severe splenomegally. As the disease progresses, gross enlargement of the mesenteric lymph nodes, thymus, and peripheral lymph nodes is sequentially observed. FACS analyses of cells from within affected tissues suggests they are myeloid in origin, however, histologically the cells are lymphoblastic in nature. To date, we have been unable to establish in vitro lines nor have we been able to adoptively transfer the disease to syngeneic hosts, nude mice, or SCID mice. It is, however, of note that adoptive transfer of several tumors to either H-2 compatible nude mice or SCID mice results in host death within two weeks of tumor injection. No tumor mass has been observed in these animals. Consequently the cause of death remains unclear, although we are pursuing the possibility that an infectious agent, most likely a virus, is involved.

5. A project unrelated to the above entails the mapping and characterization of murine genes involved in eukaryotic cell division. To date, the structural gene for proliferating cell nuclear antigen has been mapped and similar studies are in progress with the 70 KDa family of heat shock proteins. An additional family under investigation consists of the cyclin genes which are believed to effect cell cycle regulation. These genes are conserved in evolution having been identified in invertebrates, yeast and humans although sequence conservation is limited. Using available sequence data, degenerate probes have been constructed and used in the polymerase chain reaction to produce DNA probes which might encode murine cyclin genes. cDNA clones hybridizing to these probes have been isolated and their characterization is in progress.

Dr. W. Davidson

In preceding annual reports two areas of research of longstanding interest were outlined. The first was a study of genes controlling the differentiation of haematopoietic progenitor cells and their commitment to a particular cell lineage. 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. Regulation of differentiation

1. In earlier published studies, a large panel of cell lines with the properties of $CD5^+$ B lymphocyte progenitors were characterized extensively and evaluated for their capacity to differentiate in vitro. Three of these cell lines, HAFTL-1, HAFTL-3 and BAMC1, derived by transformation of fetal liver or bone marrow cells with v-Ha-ras or v-bas, spontaneously differentiated at low frequency into $CD5^+$ monocytes and macrophages. Following the switch to myeloid differentiation, the cells lost markers associated with the B cell lineage (the B cell-restricted isoform of CD45, Lyb-2, Lyb-8 and CD2 antigens and sterile IgM, λ5 and V pre-B mRNA transcripts) and acquired macrophage surface markers (Mac-1, Mac-2, Ia) and functions (phagocytosis, enzyme production, secretion of IL-6, TNF and GM-CSF) that were consistent with normal macrophage differentiation. To better understand the mechanisms promoting this differentiation process, progenitors and their differentiated progeny were examined for differences in levels of mRNA transcripts of oncogenes and lymphokines and for lymphokine secretion. The progenitors also were treated with various differentiation-inducing agents in an effort to increase the efficiency of differentiation into macrophages.

These studies revealed that in contrast to $CD5^+$ pre-B cell lines transformed with oncogenes other than ras, HAFTL-1, HAFTL-3 and BAMC1 uniquely secreted low levels of GM-CSF. Following stimulation with LPS, GM-CSF production was increased 5-10 fold and low levels of IL-6 were detected. For HAFTL-1, LPS-induced $Ia^+$ cells that in some instances spontaneously differentiated into macrophages, produced the greatest quantities of GM-CSF and may represent a cell population intermediate between the B lymphocyte progenitor and a committed monocyte or macrophage. To determine whether secreted IL-6 plays a role in the switch from B lineage to macrophage differentiation, the three cell lines were treated with a neutralizing rabbit anti-IL-6 antibody. This treatment had no measurable effect on spontaneous or induced differentiation, cell growth or GM-CSF production. Parallel studies for GM-CSF are proposed with a neutralizing antibody to GM-CSF that recently became available. Studies of oncogene expression revealed that when pre-B cells differentiated into macrophages, mRNA transcription of fms and bcl-2 was initiated, fos transcription was upregulated and myb transcription ceased. These observations are consistent with published studies of oncogene expression during macrophage differentiation from conventional myeloid precursors. Because studies of changes in gene expression relating to differentiation are facilitated greatly by having a high proportion of precursors differentiating into more mature cells, we attempted to accelerate differentiation of the three cell lines with various differentiation inducing agents. Combinations of recombinant M-CSF, GM-CSF, IL-6 and IL-3 had no accelerating effect but PMA induced Mac-1 expression on 45% of BAMC1 cells and caused a moderate increase in the proportion of $Ia^+$ cells in HAFTL-3 cultures. The proportions of macrophage-like cells also were increased in the PMA-treated cultures. Treatment with PMA therefore may facilitate the study of genes regulating early events in the switch to myeloid differentiation by B cell precursors.

From the foregoing studies we conclude that the switch to macrophage differentiation by ras-transformed B lymphocyte precursors probably requires a combination of factors possibly involving GM-CSF production, down regulation of myb and up regulation of fos, fms and bcl-2. It is not clear yet what role ras plays in these processes. Although we have not established whether a similar related $CD5^+$ B cell/macrophage differentiation pathway exists in normal haematopoiesis, this model may have important implications for clinical situations where lineage switching in leukaemia patients has been reported and often leads to more serious disease.

2. One observation reported in the foregoing study was that the macrophages derived from HAFTL-1 and HAFTL-3 had a smaller-sized (4.5 kb) mRNA transcript for the cell surface antigen CD45 (Ly-5) than the precursors (5 kb) and were no longer recognized by the mcAb6B2 that recognizes the B cell isoform of CD45, although they were still recognized by a pan reactive antibody to CD45. Because of our interest in genes that are expressed in a lineage-restricted fashion during differentiation, we have further examined CD45 expression during B cell and macrophage development. Recent reports in the literature showed that differences in the sizes of CD45 proteins precipitated from the surface of T cells and B cells resulted both from differential glycosylation and also from differential splicing of exons 5,6 and 7 at the N-terminal end of the gene. In other studies CD45 was reported to be the predominant PTPase in the cell membrane of haematopoietic cells and in T lymphocytes to associate with a number of surface molecules involved in signal transduction. We initiated this project by cloning CD45 from a cDNA library prepared from the HAFTL-1-derived macrophage cell line, 3G4. Full length clones were sequenced and the change in the size of the mRNA from 5 kb to 4.5 kb was shown to result from the splicing out of exons 5, 6 and 7. Examination of mRNAs from a large panel of macrophages, pre-B cells, B cells and plasmacytomas showed that macrophages universally express the 4.5 kb mRNA and pre-B cells and B cells the 5 kb mRNA. Interestingly, plasma cells express an mRNA of intermediate size (4.8 kb). Low levels of smaller sized CD45 mRNA transcripts were also detected in all cell types. These minor transcripts as well as the 4.8 kb transcript in plasmacytomas and transcripts in freshly isolated normal lymphoid cells and macrophages are being examined by PCR technology with exon specific primers. Novel PCR fragments will be cloned and sequenced. Proposed future studies include: 1) the examination of isoform changes in normal B cells following stimulation; 2) the identification of molecules that associate with CD45 in the membranes of resting and activated B cells and macrophages and how this association influences cell activation and; and 3) comparisons of glycosylation patterns in resting and activated cells. This project will be a major focus of future research.

B. T cell abnormalities associated with lpr and gld

The mutant autosomal recessive genes lpr and gld when introduced into C3H mice cause remarkably similar abnormalities. In published studies we showed that C3H mice homozygous for either gene develop severe lymphoproliferative disease characterized by the expansion of an unusual T cell subset of unknown origin that expresses Thy-1, CD3 and the TCR $\alpha/\beta$ heterodimer but not CD2, CD4 or CD8 antigens. These double negative (DN) T cells also express high levels of the B cell restricted isoform of CD45 [Ly-5(B220)], Ly-24, Ly-22, PC-1 and Ly-6C

and in addition, exhibit abnormalities in the phosphorylation of the zeta component of CD3. Using a variety of stimuli the DN cells were shown to be functionally inert. Most recently, our interest has focused on the source of these cells, their capacity to produce factors that may contribute to their accumulation and the role of other T cell subsets in their genesis. Purified DN cells and unfractionated LN cells from diseased mice were evaluated for spontaneous and induced lymphokine production. The cells were stimulated by cross-linking of the TCR complex with mcAb specific for CD3 and TCR $\alpha/\beta$ in the presence of PMA. The results showed that DN cells did not produce lymphokines spontaneously or after stimulation nor did they proliferate or express CD4 or CD8. By comparison, stimulated unfractionated LN cells produced low levels of IL-3, IL-4, IL-6 and GM-CSF and high levels of TNF-$\alpha$ and IFN-$\gamma$. The levels of all lymphokines were higher than expected when the number of potentially reactive $CD4^+$ T cells (~ 5% of the total LN population) was considered, suggesting that some $CD4^+$ T cells may be hyperactive. In other experiments, we were unable to induce CD4 or CD8 expression on the DN cells using protocols known to induce the differentiation of immature thymocytes. During the course of these experiments, we also identified a subpopulation of $CD4^+$ T cells that expressed Ly-5(B220) and was functionally inert. On the basis of these observations we propose that the DN cells may arise from hyperactivated $CD4^+$ T cells which acquire Ly-5(B220), become refractory to further stimulation but do not die and later lose CD4. Inappropriate expression of Ly-5(B220) may contribute to the inability to respond to stimuli by interfering with phosphorylation/dephosphorylation of the TCR complex. Experiments are planned to test this hypothesis. Future studies also include more detailed analyses of isolated $CD4^+,B220^-$; $CD4^+,B220^+$; and $CD8^+$ T cell subsets for surface antigen expression, immunologic function and TCR complex phosphorylation.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 05596-21 LGN

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (90 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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☐ (a) Human 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 two genes that determine resistance to plasmacytoma induction and are concentrating on finding an exact location of one of the Pctr-1 on chr 4. We have found three oncogenes (v-abl, v-Ha-ras, and v-raf-1) that can cooperate with myc in transforming plasmacytomas and we are testing new viruses containing new combinations of oncogenes. Plasmacytomas originate in the mesenteries of the small intestine and appear to progress slowly by forming new satellite foci.

A. Genetics of Susceptibility to Plasmacytoma Induction by Pristane - Dr. Michael Potter

BALB/cAn mice are uniquely susceptible to developing plasmacytomas following the intraperitoneal injection of paraffin oils such as pristane while most other inbred strains are resistant, e.g., DBA/2. We have developed a number of BALB/cAn.DBA/2(C.Ds) congenic strains of mice to determine the location of genes of DBA/2 origin that reduce the incidence of plasmacytomas. We are currently focusing on the identification of a resistance gene (Pctr-1) located on the distal end of chromosome 4. The characterization of this gene has proved to be a difficult problem.

First, the congenic mouse in which the resistance effect was found was C.D2-Fv-1 and this mouse carried a large (~30 cM) segment of the distal end of chromosome 4. We have found two other congenics in our collection, C.D2TolFam3 and BALB/cDAG, which have DBA/2 chromosome 4 genes and which are also partially resistant to plasmacytoma induction by i.p. pristane. C.D2TolFam3 carries MMTV-13 but not the DBA/2 Fv-1b allele. This suggests Pctr-1 may be somewhere in the 30 cM stretch between these two markers; unfortunately, there are few allelic markers that distinguish BALB/c and DBA/2 chromatin in this segment. We have turned to M. spretus as a potential new source of the Pctr-1r allele and are attempting to rapidly develop a BALB/c.Spretus chr 4 congenic by selecting at each generation mice that carry spretus chr 4 genes but lack other spretus genes. M. Spretus carries multiple useful markers in the distal end of chr 4.

Second, the assay for detecting Pctr-1 is by utilization of an induction study and we have encountered considerable variation in the incidence and rate of development of plasmacytomas in the C.D2Fv-1 congenic mice. We recently tested the N19F1 stock and obtained an incidence of approximately 20% plasmacytomas; a repeat study done on the N19F3 mice was 40%. At the same time we have experienced fluctuations in BALB/cAn controls; however, in neither case were matched BALB/c controls run with these experiments. In retrospect, we have realized that the testing of the effects of these genes in induction experiments must be rigidly controlled each time with a BALB/cAnPt sample. We suspect seasonal factors may influence the incidence and rate of plasmacytoma induction. Despite these variations in incidence Pctr-1 appears to consistently product a measurable decrease in susceptibility.

Third, we do not know the function of Pctr-1. In previous studies with K. Sanford we found a difference in the efficiency of repairing X-ray induced damage in chromatin in BALB/cAn and DBA/2 mice and further that C.D2Fv-1 mice at N19 had the DBA/2 phenotype. In unpublished studies it was found that the C.D2TolFam3 mice had the BALB/c inefficient repair phenotype. As this mouse is relatively resistant to plasmacytoma induction, this result suggested the Pctr-1 is not the same as Rep-2. We have sought to find other phenotypes related to DNA repair. In collaborative work with V. Bohr and J. Beecham, new repair phenotypic difference has been identified that distinguishes BALB/c and DBA/2. In this assay LPS blasts from the respective strains are UV radiated and the removal thymidine dimers is assayed. Certain regions of DNA such as the region 5' of c-myc are repaired at different rates. This region in DBA/2 and C.D2Fv-1

congenics is repaired efficiently while that from BALB/cAn is not. Thus this is the second repair difference that has been found suggesting the possible function for Pctr-1. However, this is hypothetical at this point and many other possibilities can be intertained, e.g., the distal end of chromosome 4 contains several oncogenes. Pctr-1, however, behaves like a quantitative (polygene) gene in its weak effects and also that may be only one of many genes that influence tumor development. Pursuit of identifying this gene may be a formidable task but could potentially be rewarding if the gene was found to elucidate a biological function or biochemical pathway.

1. Plasmacytoma Induction in nu/nu Mice: Role of Environmental Conditions (with Linda Byrd, Graduate Student)

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

A specific pathogen free BALB/cAnPt mouse has been derived and induction studies currently at between 250 and 300 days post pristane have shown the incidence of plasmacytomas is below 10%, while over 40% PCTs have occurred in the control BALB/cAn mice in the conventional colony.

2. Rapid Induction of Plasmacytomas: Oncogenes that Cooperate with c-myc

We are continuing to explore this system to find other oncogenes that cooperate with c-myc in collaborative experiments with U. Rapp and J. Troppmair at Frederick and K. Marcu from Stony Brook. In addition, we are preparing new constructs in our laboratory. Joy Williams has made two new viruses; the first contains myc and Pkc and the second, myc and cdc-2. These are currently being tested.

We have received viruses that contain combinations of Il-6, p53, Ha-ras, myc and E1A, and these are being tested for their ability to induce plasmacytomas.

The protocol now being used for testing the plasmacytomagenicity of these viruses utilized 3 week old BALB/c mice and a 0.2 ml dose of pristane that thus far is non-plasmacytomagenic. Pristane thus appears to act in a different manner than with the induction by pristane alone where a dose of 0.5 ml is given.

We have found that 13% MSV 3611 given to mice primed with 1.0 ml pristane develop plasmacytomas before 100 days (rapid induction). In some of these tumors the mechanism of myc activation is by enhancer insertion of Moloney LTRs as shown by J. Shaughnessy.

3. Biological Effects of Pristane: Pre-neoplastic Lesions in Pristane Plasmacytomagenesis

We have previously described proliferations of plasma cells during the pre-neoplastic period, i.e., 25-120 days in BALB/cAn mice. This work has been continued for several reasons. First, can the pre-neoplastic foci of plasma cells be localized in the peritoneal cavity? We have examined mice at days 40 and 90 by taking tissues from various regions of the peritoneum. Approximately six 2-3 cm wedge sections of the ileum and jejunum intestine containing gut, mesentery and mesenteric node were removed. After fixation the mesentery folded near the gut, and when the distal surface of the gut was trimmed away, sections of the tissue provided a semi-quantitative sample of the intestinal mesentery. In addition, sections were taken from duodenal mesentery, omentum, pelvic fat·and mesenteries, and retroperitoneal fat in adjacent sections suggesting regional-local spread. The omentum, pancreatic connective tissues and pelvic fat contained rare lesions indicating the mesentery of the small intestines are the preferential site of origin of the earliest plasma cell lesions. Histologically, the plasma cells are frequently found in the connective tissues between the organized oil granuloma (OG) and the underlying mesenteric tissues. Also foci tend to invade into the distal OG tissue. Occasionally mega-foci can be found that contain thousands of plasma cells. These are interpreted to be composed of cells that have not yet acquired the ability to spread. Mega-foci arise in the same regions as the smaller spreading type of lesion. These data now allow us to predict the optimal location of the developing plasmacytoma; this will be useful in attempts to culture these early plasma cells.

What type of cell is the precursor of the plasma cell that proliferates in foci? The predominant focus produced IgA-kappa. Rare lesions that contained lambda L chains or IgG subclass heavy chains could be used to assess the morphology of spreading lesions. In BALB/cAn mice the predominant plasma cell in foci and plasmacytomas secretes IgA-kappa. From previous studies we know that the predominant non-proliferating plasma cell in the peritoneal OG is also an IgA producing cell. The reason why these cells migrate into the OG is not known. The finding suggests that cells differentiating to make IgA are at increased risk of becoming plasmacytomas in pristane treated BALB/cAn mice. Possibly, too, it is during the switching process that some of the earliest neoplastic mutations occur, e.g., the myc activating rcpt(12;15) chromosomal translocations in which switch alpha regions are joined to the c-myc gene.

Can the number of foci per mouse be used to determine the susceptibility/resistance phenotype of the mouse? The cells in foci have a tendencey to spread and form new foci; thus, more foci per mouse are found with increasing time, but also more mice with foci also accumulate. These features make it possible to study the progression of the disease during the pre-neoplastic period. Further plasma cell foci are seen rarely if at all in plasmacytoma resistant strains. Thus the number of foci can be used to determine plasmacytoma susceptibility and resistance. We are currently carrying out studies in which the number of foci is being determined in groups of mice that are being followed for plasmacytoma incidence. If these studies are predictive, we can eliminate many of the long term incidence studies and determine the susceptibility/resistance phenotype by 5 months after the injection of pristane.

Can the rate of development of foci be used to study early and late acting steps in plasmacytomagenesis? One of the crucial unanswered questions in pristane induced plasmacytomagenesis in BALB/cAn mice is when does the myc activating translocation occur? Is it an initiating step or a late event? It has been difficult thus far to detect the presence of the rearranged myc genes in tissues. PCR reactions are complicated by the fact that switch alpha region cannot be used in amplification. In situ labelling is also indirect. Hopefully we can obtain earlier lesions by culturing foci or cloning DNA from putative foci (i.e., regions enriched with foci). It is strongly suspected that myc activating chromosomal translocations are not the only oncogenic lesions in plasmacytomagenesis and that progression depends upon the development of other oncogenic events. In previous studies we have shown that the development of foci is inhibited by indomethacin. We are extending these studies to gather more quantitative data on the period between 60 and 120 days. Indomethacin treatment initiated 60 days post pristane inhibited plasmacytomagenesis. We would now like to know if it actively inhibited the progression of early multiple foci. Experiments on this question are in progress.

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

1. Pristane-Induced (PCTs)

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

So far, 500 (CxD)F2 progeny have been typed for susceptibility to pristane-induced PCTs. Only 6 progeny (equivalent to 12 meiotic events) were susceptible, indicating an incidence of PCT formation consistent with a 3 gene model of plasmacytoma susceptibility. DNAs have been isolated from the susceptible F2s and southern analysis has been performed for ca. 37 markers distributed over 17 different chromosomes to examine the extent of cosegregation between the susceptible phenotype and BALB/c alleles for the markers. To date, the lowest recombination frequencies have occurred with Ifa on chr 4 (0.16) and N-myc3 on chr 5 (0.25). In addition, relatively low recombination frequencies have been observed for the following loci: myb (0.33), IL-9 (0.33), and Tcra (0.33)

The significance of these linkage analyses have been hampered by the low number of susceptible F2 progeny. As a result, we have generated an additional 320 backcross progeny and are currently in the process of typing these mice for PCT susceptibility. To date 41 of 321 mice have developed plasmacytomas. This incidence (13%) of PCTs among backcross mice is consistent with the 3 gene model of PCT susceptibility observed among the F2 progeny. The first 30 mice have had their DNAs extracted and subjected to RFLP analysis. So far only 12 markers distributed across 7 chromosomes have been examined for the degree of cosegregation between the susceptible phenotype and BALB/c alleles for the markers. Since the data from the CDF2 study showed the best correlation between

susceptibility and BALB/c genotypes for Ifa on chromosome 4, we focused the majority of our efforts in analyzing 6 markers spanning a 60 cM segment of chr 4 from Mtv-14 (10 cM distal to the centromere) to Anf (66 cM distal to the centromere). Significant deviations from 0.50 were observed for 4 of the 6 markers indicating linkage of a gene controlling susceptibility to PCT formation near the middle of mouse chromosome 4. Preliminary analyses of the recombinants within this region are suggestive of a location between D4Lgm1 (an anonymous DNA fragment) and Mtv-13 (a mammary tumor integration site); thus localizing one of the susceptibility genes (Pct-1) to a 12 cM stretch of chromosome 4. This induction study should be completed by August 1990, at which time additional mice can be included in a more extensive linkage analysis of susceptibility phenotypes with BALB/c genotypes in search of other genes influencing PCT formation.

The linkage analyses indicating the presence of a gene involved in PCT susceptibility on chromosome 4 lead us to generate a chromosome 4 specific library from a somatic cell hybrid known to carry mouse chromosome 4. We purified 70 random clones from this library and are in the process of mapping them in an effort to obtain markers more closely linked to the disease locus. To date, 3 out of 13 clones have shown BALB-DBA RFLPs and at least one of them (D4Lgm1) has been mapped within 13 cM $\pm$ 6 cM of Pct-1.

In conjunction with these studies, we have collected and analyzed a large panel of probes for restriction fragment length polymorphisms (RFLPs) upon hybridization with BALB/cAn and DBA/2 DNAs. Our goal is to obtain a minimum of 3 equally spaced markers/chromosome to insure a high probability of linkage when used in segregation analyses. To date, we have enough markers to cover roughly 50% of the mouse genome. The rate at which new genes are being cloned makes this approach extremely feasible.

Previous studies (by M. Potter) involving C.D2 bilineal congenic strains indicated the existence of three plasmacytoma resistance genes in the BALB/c-DBA/2 model system, as revealed by studies involving C.D2 congenic mice. These genes were identified as follows: Pctr-1 (linked to Fv-1 on chr 4), Pctr-2 (linked to Ly-6 (DAG) on chr 15) and Pctr-3 (linked to Qa-2 on chr 17). Last year molecular analyses of C.D2-Dag mice showed that they not only carry the DBA/2 allele of Ly-6 on chr 15, but that they also carry the DBA/2 allele for MMTV-13 on chr 4. These results raised the possibility that what had previously been referred to as Pctr-2 may be the same gene as Pctr-1. Subsequent studies by M. Potter have revealed that C.D2-Ly-6 mice are susceptible to PCT formation. Further molecular analyses of the C.D2-Dag mice have revealed that they also carry the DBA/2 allele for Qa-2 on chromosome 17. This may account for their increased resistance to PCT formation over and above that observed in the congenics selected solely for Fv-1 (C.D2-Fv-1$^n$) or Qa-2 (C.D2-Qa-2$^+$).

Numerous attempts to find RFLPs between BALB/c and DBA/2 from hybridization with chromosome 17 markers have resulted in identifying only one marker (Qa-2). Similar to the approach outlined for chr 4 above, we have generated a chromosome 17 specific library from a somatic cell hybrid line. To date 25 clones have

been purified; four of these have been analyzed. Three clones have been mapped to chromosome 17, but unfortunately, none of them have revealed BALB/c-DBA/2 RFLPs. Future studies will involve analyzing more of these random clones in addition to screening for RFLPs with genes which are constantly being cloned from this region of the genome.

2. Pristane and RIM-Induced PCTs

We utilized the phenotypic differences between BALB/c and DBA/2 mice to tumor formation in examining the genetics of susceptibility to RIM-induced plasmacytomagenesis. RIM is a retroviral construct containing an immunoglobulin enhancer and promoter driven c-myc gene and a Moloney MuLV-LTR driven V-Ha-ras gene. Over the course of 6 separate induction studies, by day 120, 92% (n=83) of the BALB/c and 10% (n=75) of the DBA/2 mice developed PCTs. In addition, 85% (n=85) of the (CxD)F1 mice developed tumors, indicating that susceptibility is dominant with this induction scheme. Interestingly, 48% (n=244) of the backcross progeny between BALB/c and DBA/2 mice were susceptible to PCT formation, indicating that a single gene is likely to control the development of RIM-induced PCTs in BALB/cAn mice ($X^2$=0.51, p=0.5, i.e., no significant deviation from 50% susceptible:50% resistant expected for a single gene model). Linkage analysis involving 100 of these backcross progeny for RFLPs associated with $PCT^s$ and $PCT^r$ mice, have not revealed significant linkage to ca. 30 markers distributed across 15 chromosomes. In vivo infections of several inbred and congenic strains of mice have suggested that a gene on chromosome 17 controls the development of RIM-induced PCTs in BALB/c mice. Future efforts will be focused on fine-mapping the location of this gene through extensive in vivo analysis of various recombinant congenic strains of mice selected for MHC variation. In addition, our search for chromosome 17 markers outlined in the previous section will be useful in analyzing the cosegregation of R/S phenotypes and BALB/c/DBA/2 genotypes and fine-mapping the location of the gene controlling RIM-induced plasmacytomagenesis among the 200 backcross progeny which have already been tested for susceptibility to PCT formation.

C. Mechanisms of the Action of Pristane (2,6,10,14-tetramethylpentadecane) as a Plasmacytomagenic Agent in BALB/cAn Mice - Dr. Siegfried Janz

Description: The overall goal of this research is to study the effects of pristane (2,6,10,14-tetramethylpentadecane) at the cellular level in vitro as well as in the in vivo system of the BALB/cAn mouse in order to better understand the plasmacytomagenic activity of this compound in mice. We developed a new method to solubilize the extremely hydrophobic compound pristane by forming monomolecular inclusion complexes with beta-cyclodextrin as a precondition to test pristane more effectively in the aqueous phase of various in vitro assay systems. In these tests, we determined the cytotoxicity of pristane to various B lymphocyte cell lines and murine ex vivo cells of the immune system. This approach also led to the recognition of a mitogenic effect of pristane on B cells and a putative tumor promoting activity in an experimental system of murine skin carcinogenesis (S. Yuspa). Other experiments were aimed at elucidating the effects of pristane on biological membranes. NMR (nuclear magnetic resonance) and DSC (differential scanning calorimetry) studies

demonstrated the potential of pristane to trigger the phase transition from lamellar to hexagonal in model membranes (A. Parsegian). Experiments on the interaction between viruses and mammalian cell membranes uncovered a putative fusogenic activity of pristane (R. Blumenthal). Studies on the interaction of pristane with reactive oxygen species raised the interesting possibility of an indirect genotoxic effect caused by pristane oxidation products (O. Brede).

Future directions: Further experiments will be concentrated on verifying potential DNA damaging effects or DNA damage modulating effects, respectively, caused by pristane and on in vivo effects of pristane (distribution, metabolism?). For the latter, we hope to make available 14C-pristane soon.

Collaborators: M. Potter, E. Shacter; R. Blumenthal, A. Herrmann; A. Parsegian, K. Gawrisch; O. Brede, J. Mueller; S. Yuspa

D. The Role of T Cells in Pristane-Induced Plasmacytomas - Dr. Ann McDonald

A systematic study of the role of T cells in pristane-induced plasmacytomas has been undertaken. Initial work has focused on determining the time of appearance and cell-surface characteristics of lymphocytes that enter the peritoneal cavity and oil granulomas of both plasmacytoma-susceptible and -resistant mice after intraperitoneal injection of pristane. Immunofluorescent staining and FACS analysis of peritoneal exudate and oil granuloma cells revealed 103 fold greater numbers of Thy-1.2$^+$, CD4$^+$ lymphocytes in BALB/cAnPt mice than in resistant mice, i.e., DBA/2N, (BALB/cAnPt x DBA/2N)F1 (CDF1), BALB/cDAG, BALB/CAnPt-nu/nu and BALB/cAnPt mice housed under specific pathogen free (SPF) conditions. Except for BALB/cAnPt-nu/nu and BALB/cAnPt-SPF mice which had no significant increases in either CD4$^+$ or CD8$^+$ cells, resistant mice had greater numbers of CD8$^+$ cells. Congenics of intermediate susceptibility to pristane-induced plasmacytomas (PCT), i.e. C.D2 Ly-6 and C.D2 Fv-1$^{n/n}$, had both low numbers of CD4$^+$ and CD8$^+$ cells. These differences result in dramatically higher CD4/CD8 ratios in mice susceptible to PCT induction (manuscript in preparation). The above results also suggest a correlation between Qa-2 expression, increased CD8$^+$ cells and resistance to pristane-induced PCTs, since only the resistant mice DBA/2N, CDF1 and BALB/cDAG express Qa-2. This is of particular interest since Qa-2 is a major histocompatibility complex class I-like antigen located on chromosome 17 and CD8$^+$ cells are primarily restricted by class I antigens. Therefore, the congenics C.D2 TOL-1 FAM 3 (chromosome 4, Qa-2), C.D2 Fv-1$^{n/n}$Qa-2$^+$ (chromosome 4, 17) and C.D2 Qa-2$^+$ (chromosome 17) have been challenged with pristane to further analyze Qa's contribution. In addition, attempts are being made to clone T cells from the peritoneal exudate and oil granulomas of BALB/cAnPt and DBA/2N mice. Cloned T lymphocyte populations will be characterized by both FACS analysis and cytokine secretion, e.g., IFN-$\gamma$, IL-2, IL-4 and IL-6. In the future, the cloned T cells will be used to reconstitute BALB/c-nu/nu mice in vivo to directly assess the role of T cells in the induction phase of pristane-induced PCTs.

E. Biochemistry of Induction of Plasmacytomagenesis - Dr. Emily Shacter

The work is directed at identifying the carcinogenic agents that cause pristane-induced plasmacytomagenesis in BALB/cAn mice and at determining the genetic basis for susceptibility to this disease by comparing specific biochemical pathways in different inbred strains of mice. In particular, we are investigating the apparent association between the chronic inflammatory condition induced by the intraperitoneal injection of pristane and the strict localization of tumor development within this environment. The main focus of our work over the past year has been to:

(1) Continue to try to elucidate the biochemical mechanism whereby indomethacin inhibits plasmacytomagenesis. To this end, we have begun studies to determine the level of production of prostaglandins in the oil granuloma and in the free peritoneal space following injection of pristane. The best characterized and most potent biochemical action known for indomethacin is the inhibition of prostaglandin synthesis, and the oil granuloma contains high levels of macrophages which may be expected to produce prostaglandins in situ.

(2) Determine whether inefficient DNA repair in B-lymphocytes from BALB/cAn mice may be responsible for their unique susceptibility to sustaining tumor-associated chromosomal translocations involving the c-myc oncogene. Over the past year, we have employed the alkaline elution assay to compare the capacity of LPS-blasts from different strains of mice to repair damage induced by pristane-elicited neutrophils. In addition, we have employed molecular genetic techniques to assay repair of damage within the c-myc oncogene following uv irradiation.

Major Findings:

1. Pristane-elicited macrophages produce prostaglandins constitutively, without exogenous stimulation, during short term culture following their isolation from the peritoneal cavity. HPLC analysis of eicosanoids found in cell culture supernatants reveals that the predominant prostaglandin is $PGE_2$ and that production is completely inhibited by addition of 1 $\mu$M indomethacin. Radioimmunoassays are currently being performed to determine the actual levels of $PGE_2$ being secreted so that they may be compared both to non-inflammatory macrophages and to cells elicited with other inflammatory stimuli. Thus far, experiments to determine the levels of prostaglandins generated in vivo have been unsuccessful. Nonetheless, the data obtained in vitro are consistent with the hypothesis that prostaglandins are generated in the oil granuloma. Further studies will be required to ascertain their possible effects on promoting B cell proliferation and possibly tumorigenesis. Experiments are also being set up to compare prostaglandin production in macrophages from plasmacytoma-resistant mice (experiments carried out in collaboration with Dr. Larry Wahl, Cellular Immunology Section, Laboratory of Immunology, NIDR).

2. Myeloperoxidase is a neutrophil-derived enzyme which catalyzes the formation of HOCl from $H_2O_2$ and $Cl^-$. We previously found that indomethacin is a strong inhibitor of HOCl formation by neutrophils. By carrying out

kinetic analysis of the mechanism of inhibition, we have established that the drug is competitive with respect to chloride and uncompetitive with respect to $H_2O_2$. Additional experiments have shown that inhibition of HOCl formation is a common but non-uniform property of aspirin-like non-steroidal anti-inflammatory drugs (NSAIDs). The results offer an explanation for why indomethacin inhibits only the chlorinating activity of myeloperoxidase and support the concept that NSAIDs may modulate several different pathways involved in inflammation, including but not restricted to inhibition of prostaglandin synthesis (manuscript submitted).

3. B-lymphocytes (LPS-blasts) isolated from BALB/cAn mice are inefficient in repairing chromosomal breaks induced by γ-irradiation. It has been established that pristane-elicited neutrophils generate DNA-damaging reactive oxygen intermediates in vitro. Taken together, these observations support the hypothesis that inadequate repair of the DNA damage induced in vivo by this mechanism may account for the unique mutagenicity of the oil granuloma in BALB/cAn mice. Alkaline elution assays were carried out to test whether cells from C.D2 congenic strains of mice differ in their capacity to repair neutrophil-induced DNA damage. No differences were found in the rate of repair of single-strand breaks in B-lymphocytes isolated from BALB/cAn, Fv-$1^{n/n}$ (chromosome 4 marker) and Pep-3 (chromosome 1 marker) mice; the latter two strains are resistant to plasmacytomagenesis. In contrast, in molecular genetic experiments carried out in collaboration with Dr. Will Bohr (Laboratory of Molecular Pharmacology, NCI) a specific deficiency was found in repair of uv-induced thymidine dimers in the c-myc oncogene of B-lymphocytes from BALB/cAn mice when compared to DBA and C.D2 Fv-$1^{n/n}$. The results support the concept that analysis of overall repair of single-strand breaks in the total genome is not sufficiently sensitive to detect fine differences in repair of potentially significant genes. Further studies are required to ascertain whether inefficient repair of oxidative damage within c-myc may account for the near ubiquitous rearrangement of this gene in plasmacytomagenesis.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 08727-13

PERIOD COVERED
October 1, 1989 to September 30, 1990

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 |
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Laboratory of Genetics

SECTION
Oncogene

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 10 3/4 | 4 | 6 3/4 |

CHECK APPROPRIATE BOX(ES)

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

B

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

The overall goal of this research is to study the activation of particular genes in diseased and normal cells in order to understand which genes may play important roles in the development of malignancies, autoimmune diseases and normal differentiation. We have concentrated on the expression of "oncogenes," especially abl, bcl-2, myc, myb, Pvt-1, raf, ras and rel, as well as immunoglobulin and T-cell receptor genes in tumors that represent immortalized lines of lymphocytes or myeloid cells at different stages of differentiation. The deregulated expression of the myc oncogene has been clearly shown to be essential to the induction of plasma cell tumors, but how "variant" plasmacytomas with rcpt(6;15) translocations involving the Pvt-1 gene, which is 200-300 kb 3′ of c-myc, also deregulate c-myc expression remains unknown. We have cloned several cDNAs for mouse Pvt-1, which has, for the first time, permitted the identification of mRNA from this gene in normal mouse tissues and tumors. "Variant" plasmacytomas have elevated levels of Pvt-1 transcripts which are also truncated due to the chromosomal translocation.

The v-abl oncogene has been found to cooperate with over-expressed c-myc in a retrovirus (ABL-MYC) which induces plasmacytomas more rapidly than previously reported and, for the first time, in 100% of adult mice, even in the absence of pristane priming. The rapidity of this induction has permitted the production of antigen-targeted myeloma proteins by ABL-MYC induction of plasmacytomas in immunized mice. An important family of protein kinases, Protein Kinase C (PKC), is being studied for its possible role in tumorigenesis. We have shown that the members of this gene family are differentially expressed in tumors of hemopoietic cell and that there is at least one undescribed form of PKC expressed in myeloid tumors.

Alternatively spliced transcripts of the myb proto-oncogene have been observed in normal and tumor cells in man as well as mouse. An important difference between the species is that the alternate forms of human c-myb result in truncated protein isoforms instead of the elongated ones expected in mice.

Major Findings:

I. Gene Expression (J.F. Mushinski)

A. Expression of oncogenes in normal and neoplastic hemopoietic cells (with H. Mischak). We have previously shown that expression of nuclear oncogenes such as c-myc, c-myb and c-fos was highest in tumors of pre-B lymphocytes and that it diminished in tumors of more mature B cells. A different pattern is found for expression of the serine-threonine kinase c-raf and the G protein c-Ha ras, which increases with B-cell maturation. Both of these latter oncogenes (when structurally mutated) complement over-expressed c-myc in transforming embryo fibroblasts and in rapidly inducing mouse plasmacytomas in pristane-primed BALB/c mice. We have extended these studies to two other similar proteins, the G protein/oncogene bcl-2 and the family of serine-threonine kinases called protein kinase C (PKC). bcl-2 is expressed only in maturing B lymphocytes, not in Pro-B cells nor in plasmacytomas. The different members of the PKC family are expressed variably in different hemopoietic tumors. Most B lymphocytes express substantial amounts of PKC$\alpha$ and $\beta$, but plasmacytomas express unusually large amounts of a 2.5 kb PKC$\alpha$ message and virtually no PKC$\beta$. Myeloid cells, on the other hand, show virtually no mRNA for PKC$\alpha$, $\beta$ or $\gamma$!. We have cloned several PKC cDNAs from a myeloid tumor. One appears to be PKC$\delta$, but another may be a previously undescribed, myeloid cell-specific form of PKC.

B. Complimentation of over-expressed c-myc by various oncogenes in rapid plasmacytomagenesis (with H. Mischak, E. Weissinger, R. Risser). We have shown that over-expressed c-myc or v-myc can dramatically speed up tumorigenesis in pristane-primed BALB/c mice, but the cell type targeted is usually myeloid. myc-containing retroviruses that also express mutated forms of v-raf change their target cell to B lymphocytes. We have found that v-abl also complements over-expressed c-myc and can rapidly induce plasmacytomas. This combination (in the form of the retrovirus ABL-MYC) is the most effective one reported, inasmuch as tumors develop in 100% of adult BALB/c mice (vs 30-60% with other retrovirus constructs), in 30-40 days (vs 45-80 days with other viruses), and in C57BL mice which are rather resistant to this form of tumorigenesis. What is more, this virus will induce intraperitoneal plasmacytomas in the absence of helper virus and when administered intravenously in the absence of pristane priming. If the mice have been immunized with hen egg white lysozyme or sheep red blood cells before infection with ABL-MYC, the resulting transplantable plasmacytomas will, in large measure, produce antibodies that specifically react with the immunogen. We are also attempting to induce hemopoietic tumors with three other dual-expressing viruses that coexpress c-myc and 1) PKC$\alpha$ or a mutated form of this PKC molecule; 2) v-rel or mutated c-rel and 3) bcl-2.

C. Control of c-myc expression (with J. Shaughnessy, K. Huppi). Deregulation of myc expression appears to be one of the key elements in plasmacytomagenesis, but just how this deregulation is achieved in different tumor forms is not yet clear. A substantial breakthrough appears to be at hand for the plasmacytomas with rcpt(6;15) translocations very far 3′ from c-myc with the isolation of cDNA clones for Pvt-1, the locus where these translocations occur (see next section). Another accomplishment in this area was achieved when

genomic clones of c-myc exon 1 and some of its 5' flanking sequence were cloned from normal BALB/c, from a BALB/c plasmacytoma (ABPC22) with high c-myc mRNA but no evidence of chromosomal abnormality and from normal DBA/2 (a mouse strain that is resistant to oil-induced plasmacytomagenesis). The c-myc locus was found to be identical in all three clones, indicating that the unique susceptibility of BALB/c to plasmacytomas associated with translocation involving the c-myc chromosome is probably not due to a unique DNA structure in the BALB/c. The ABPC22 plasmacytoma was found to harbor a Moloney helper virus about 1060 bp upstream of the c-myc. This is the first report of such a promoter/enhancer insertion in mouse B lymphomas, and the effects on c-myc expression are being studied.

II. The Mouse Pvt-1 Gene (K. Huppi)

A. Gene structure and organization. Approximately 10-20% of mouse plasmacytomas contain a "variant" translocation, rcpt(6;15), which breaks chromosome 15 100-200 kb downstream of c-myc. This cluster of breakpoints is known as Pvt-1 (plasmacytoma variant translocation-1) and is consistently found at the same location in rat, mouse and man. Moreover, this region is also the site of retroviral insertions in rat thymomas and mouse T lymphomas. Although it is possible that translocations, even as distant as 100-300 kb from c-myc, may be close enough to perturb the regulation of c-myc, an appealing alternative is that Pvt-1, itself, encodes a protein which directly or indirectly affects c-myc expression. Published data indicated that Pvt-1 was not expressed in mouse tissues. In order to reexamine this alternative, we screened a mouse splenic cDNA library with a series of genomic DNA probes spanning more than 20 kb of the mouse Pvt-1 locus. We identified a 1.4 kb clone, Pvt-1-1, which maps specifically to the region of the major breakpoint cluster of mouse Pvt-1. Since Pvt-1-1 contains only a small portion of the total 14 kb Pvt-1 mRNA, studies are under way to clone a more substantial part of the Pvt-1 gene. Preliminary results from these cloning experiments reveal complex patterns of alternative exon splicing. Furthermore, genomic cloning studies suggest the Pvt-1 gene may extend more than 50 kb. We are currently attempting to determine the nature of the protein product for Pvt-1 and to understand its role in lymphoid development. In addition, we are attempting to isolate the human counterpart to the mouse Pvt-1 gene (in collaboration with K. Bhatia and I. Magrath, NCI) and determine whether it plays a role in human neoplasia.

B. Expression studies (with J.F. Mushinski). Northern analyses using Pvt-1-1 as hybridization probe revealed a low level of ca. 14 kb transcripts present in mouse thymus and in early stages of B-cell development. Higher levels of Pvt-1 transcripts are found in more mature B cells, including plasmacytomas which have undergone Ig rearrangement and express Ig. Examination of RNAs from rcpt(6;15) plasmacytomas by northern hybridization with Pvt-1-1 reveals even greater levels of truncated Pvt-1 transcripts. The truncation of the transcripts presumably result from the translocation events that occur within the region comprising Pvt-1. Evidence in support of this conclusion comes from cDNA cloning studies from a rcpt(6;15) plasmacytoma, ABPC20. Several cDNAs isolated from this tumor contain chimeric transcripts, the 5' end comprised of Pvt-1 RNA and the 3' end comprised of Jk-Ck sequences of the Igκ light chain gene.

III. Study of myb proto-oncogene (G. Shen-Ong)

A. Mutation and malignant transformation. Our main goal in this study is to understand the mechanism by which the myb gene product is involved in myeloid tumorigenesis. We have found that clonal provirus disruption of the mouse myb gene resulted in the synthesis of N-terminally truncated proteins in myeloid tumors. Recently published data showed that the c-myb protein can be phosphorylated by casein kinase II (CKII), and this phosphorylation abrogates specific DNA-binding activity of myb proteins. Interestingly the site for CKII phosphorylation is lost in the N-terminally truncated tumor-specific myb proteins, resulting in the constitutive binding of these proteins to DNA even in the presence of active CKII. To determine if removal of the CKII phosphorylation site oncogenically activates the c-myb protooncogene, we are now testing the effects of point mutations (introduced by site-directed mutagenesis) in the CKII phosphorylation site on the transcriptional and biological activities of c-myb.

B. Alternative splicing (with J.F. Mushinski). We have detected the presence of a minor form of mouse c-myb protein as a result of alternative splicing that inserts an internal additional in-frame E6A exon. The same alternative splicing event is also found in the expression of the human c-myb gene. The human cDNA sequence, however, predicts a smaller alternate form of c-myb protein that truncates in the E6A exon. Another smaller alternate form of human c-myb protein is also predicted from the sequence of a different alternatively spliced c-myb transcript that contains an additional E7A exon. The region of c-myb protein affected by the various alternative splicing events had been suggested to be important in the negative regulation of the transcription activity of myb. We are now testing if the alternate and the major form of c-myb may differ in their abilities to transactivate in in vitro assays.

IV. An Animal Model for Heriditary Bone Disorder (G. Shen-Ong)

Some progeny of the F15 generation of brother-sister mating of the BALB/c.DBA/2 N6Fv-$1^{bb}$ ($Qa2^{+}$) mice was found by Dr. Michael Potter (Chief, LG) to exhibit arthritis-like phenotype. This phenotype was not observed in all litter-mates. Further breeding of this mouse by Linda Byrd (graduate student, LG) showed that the arthritis-like phenotype is inherited as an autosomal recessive trait, suggesting the presence of a spontaneous mutation of a single gene. Based on RFLP studies, we have been able to localize this mutation to mouse chromosome 18. The onset of the disease in 100% of both male and female homozygotes was apparent from approximately 4 weeks of age. The short, tubular bones of the tail (starting from the tip) are the first sites of involvement, spreading progressively to the hindlimbs (phalanges, metatarsals and tarsals), the forelimbs (phalanges, metacarpals and carpals) and the ears. Acute inflammatory episodes were occasionally observed later in the course of the disease. The disease appears to be self-limiting in the sites described. Examination of hematoxylin and eosin stained sections of samples obtained from the afflicted areas of mice of different ages revealed the presence of an appreciable number of "mesenchymal-like" cells in the bone marrow cavities and next to the periosteum in the dermis tissue. These cells appear to invade the bone cortex but spare the joint tissues. Late in the disease process, trabeculae of woven bone (bone reconstruction) were observed. Analyses of sera of these mice did not

show elevated levels of immunoglobulins, anti-DNA reactivity nor presence of rheumatoid factors. Hence, it is unlikely that these mice have rheumatoid arthritis. We are presently continuing our efforts to identify the mutated gene and to define the mechanism by which this lesion is incurred.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08950-08 LGN

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular recognition by protein effector molecules

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

| | | | |
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| P.I.: | Sandra Smith-Gill | Microbiologist | LGN, NCI |
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| | M.A. Newman | Biologist | LGN, NCI |
| | E. Weissinger | Fogarty Fellow | LGN, NCI |

COOPERATING UNITS (if any)
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LAB/BRANCH

SECTION
Laboratory of Genetics

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Protein-protein interactions underlying molecular recognition are studied using monoclonal antibodies directed against the model protein antigen, lysozyme c (HEL). The X-ray structures of two Fab-HEL complexes for 2 of these antibodies (HH5 and HH10) have been refined, and we are now studying in detail the molecular mechanisms underlying specificity and affinity in protein-protein interactions. A panel of hybridomas representing various time points in the immune response to HEL was functionally characterized by several different types PCFIA. Of over 500 primary and secondary response hybridomas analyzed, no IgM antibodies were specific for HEL, although HEL-specific IgM precursor cells are available. There is no obvious pattern of affinity "maturation" during the course of the IgG response. Most specificities are found throughout the response, and no single specificity dominates any one time point, although a few specificities are confined to very late in the immune response. All the antibodies analyzed to date fall into one of 4 complementation groups, three of which have been described previously and include the majority of the antibodies; antibodies within a complementation reciprocally cobind (complement) and competitively block one another. The patterns of interaction support the hypothesis that the entire surface of the protein is antigenic. They also suggest a fundamental relationship between protein structure and antigenicity. Analyses of interactions among three antibodies whose X-ray crystallographic structures are known suggest the apparent structural overlap of epitopes will not necessarily result in competitive exclusion, and that those antibodies showing strong competitive interference overlap significantly in their contacts with HEL. Site-directed mutations both of expressed antibody and HEL genes are being utilized to examine the specific roles of individual amino and residues to molecular specificity.

Project Description:

We have continued to place major emphasis on investigating molecular recognition, utilizing antibodies (mAbs) specific for the protein hen egg white lysozyme (HEL), 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. Until recently, our studies with HEL have relied upon the availability of a panel of evolutionary variants of avian lysozyme for epitope mapping and manipulation of the immune response, but recently site-directed mutants of HEL expressed in yeast have become available, and we are currently in the process of expressing and producing site-directed mutants of cloned antibody genes. With the X-ray structures of 3 Fab-HEL complexes available, we can now begin to experimentally investigate structure-function relationships in the interface by manipulation through site-specific mutagenesis both antigen and antibodies. These studies are beginning to define fundamental principles that are critical to such applications as antibody design and vaccine development. These results also provide a model for studies utilizing mAbs to probe structure-function relationships of other biologically important regulatory proteins whose structure and function are less well defined. In addition, we are beginning to examine the emergence of specificity from an essentially polyreactive IgM antibody repertoire during the immune response, and regulation of repertoire expression in normal and autoimmune development.

1. Functional Analysis of Antibody-Protein Interactions

The mAb HyHEL-5 is a highly specific mAb in which electrostatic interactions contribute a significant portion of the binding energy; both Arg $68_{HEL}$ and $Arg45_{HEL}$ form salt links with Glu residues from the Ab heavy chain. Site specific mutants of HEL were produced in the laboratories of A.C. Wilson and J.F. Kirsch (Department of Biochemistry, University of California, Berkeley). Binding studies with these mutant lysozymes indicated a significant quantitative difference between the energetic contribution of the 2 $Arg_{HEL}$ residues to the antibody:HEL interaction, and energy calculations by J. Novotny (Squibb Institue of Research) are in agreement with the experimental observations. The $R68_{HEL}K$ substitution lowers affinity by a factor of almost $10^4$, while the $R45_{HEL}K$ substitution lowers affinity only by a factor of 10. These results with HyHEL-5 are directly relevant to understanding viral "escape" mutants, for the R → K substitutions in 2 adjacent contact residues have dramatically different effects on antibody binding; a substitution such as $R68_{HEL}K$ which reduces antibody affinity by approximately $10^4$ might be sufficient to escape neutralization, while the smaller quantitative effect of the $R45_{HEL}K$ substitution probably would not. We are continuing detailed analysis of these interactions. (1) A series of different amino acid substitutions for Arg68 is being examined for quantitative contribution to binding energy. (2) A double mutant (at amino acids 68 and 45) has been produced and will be examined for its functional properties. (3) The cDNA clones of the rearranged H and L V-genes are being

isolated in the laboratory of R. Lerner (Scripps), which will utilize HyHEL-5 in the design of catalytic antibodies. We will express the cDNA clones and attempt to design mutants complementary to (i.e., with increased affinity for) the $R68_{HEL}$ and $R45_{HEL}$ mutants.

The serologically-defined epitopes recognized by the structurally related mAbs HyHEL-8 and HyHEL-10 are very similar, and the affinities of the 2 antibodies are comparable. However, while HyHEL-10 is very sensitive to a variety of amino acid substitutions in HEL, HyHEL-8 is relatively insensitive to the same substitutions in Antigen Inhibition PCFIA. An understanding of the structural bases of these binding properties is essential to the design of recombinant antibodies or of vaccines which would elicit natural antibodies with specificity properties that are minimally subject to the loss of neutralizing activity of the target.

We are analyzing these properties by several approaches.

(1) The rearranged genes of HyHEL-10 were cloned and expressed in SP2/0 cells by T. Lavoie. HyHEL-8 and -10 each have a somatic mutation at the J-encoded residue Ala101 of the heavy chain, to a Asp in HyHEL-10 and to a Thr in HyHEL-8. The $D101_{VH}T$ site-directed mutant of HyHEL-10 is intermediate in sensitivity to substitutions at HEL residue R21. These results demonstrate that somatic mutation can significantly affect specificity independent of affinity.

(2) Although the oligonucleotide-directed mutations demonstrated a significant effect of residue D101 on HyHEL-10 function, the X-ray structure of HyHEL-10 Fab-HEL complex (recently refined by E.A. Padlan and Dr. Davies) showed that this residue is not in contact with antigen, nor does it contact any other amino acids which either contact antigen or might be structurally influenced (stabilized) by such a contact. J. Novotny (Squibb) has done calculations on electrostatic fields of Ab combining sites and has shown that HyHEL-10 and -5 each have strong negative fields which may be important in "guiding" the Ab and Ag to complex formation. The R101 mutation would significantly alter the HyHEL-10 field.

(3) M.A. Newman has recently characterized an additional seriees of mAbs whose epitopes are very simmilar to those of HyHEL-8 and HyHEL-10 with a range of specificities and affinities. One of these, HyHEL-26, has a similar affinity to HyHEL-8 and -10, but is even more sensitive to substitutions than is HyHEL-10. We are examining its structure and function in detail.

Monoclonal antibodies recognizing a single contiguous surface region of HEL fall into 2 distinct, nonoverlapping complementation groups, one defined by the antibodies HyHEL-8 and -10, and the other defined by HyHEL-12 and -15. Antibodies within a complementation group reciprocally cobind (complement) and competitively block one another. Although the X-ray crystallographic structural epitopes of HyHEL-10 and D1.3 overlap by several residues, no functional overlap could be detected between these 2 antibodies. D1.3 clearly fell into the HyHEL-12/15 complementation group. Molecular modelling of the HyHEL-10/HEL/D1.3 ternary complex by J. Novotny and R. Bruccoleri (Squibb) showed that only 4 HEL residues make contact with both D1.3 and HyHEL-10, but that with one exception

the different Fv fragments contacted different atoms of the same residue. The calculated free energy contributed to the ternary complex by the interaction between the D1.3 and HyHEL-10 Fv fragments was essentially zero (within experimental error). These results suggest that apparent structural overlap of epitopes will not necessarily result in competitive exclusion. Understanding these results and the relationship between structural and functional overlap is important to interpretation of blocking studies to delineate ligand-receptor binding sites. The results support the hypothesis of J. Novotny that most of the energy of binding is contributed by a small fraction (3.5 out of a total of 15-17 contact residues) of contacting residues in the interface. They further imply that those antibodies showing strong competitive interference overlap significantly in their contacts with HEL, but the actual amount of structural overlap required for functional interference remains to be determined. Reactivity patterns with lysozymes containing evolutionary or site-directed mutations within the HyHEL-10 epitope of antibodies in the HyHEL-8/-10 and the HyHEL-12/-15 complementation groups indicated the effects of these antigen mutations on antibody binding are local and probably direct.

2. Structural Analyses of Antibody-Protein Interactions

As part of an effort to gain insight into processes of molecular recognition, we are attempting to determine the crystal structures of a number of antibody: antigen complexes. To date there have been three anti-lysozyme: lysozyme complexes whose structures have been determined: D1.3 Fab:lysozyme, HyHEL-5 Fab: lysozyme, and HyHEL-10 Fab:lysozyme. We are pursuing a strategy of trying to crystallize the complex of a HyHEL Fab and lysozyme and then try to crystallize the uncomplexed Fab. We have tried or are trying to crystallize the following HyHEL antibodies in collaboration with S. Sheriff (Squibb): 7,8,9,15,16 and 26.

(1) HyHEL-8, HyHEL-10 and HyHEL-26 form a set of antibodies that appear to bind to the same epitope as determined by serological mapping techniques and that have approximately equal affinity constants for chicken lysozyme, but are affected by various evolutionary variants in a ratio of approximately 1:10:100. For example, if the affinity of HyHEL-8 for a particular lysozyme is reduced by a factor of 3 compared to chicken, HyHEL-10's affinity is reduced by a factor of 32 and HyHEL-26's affinity is reduced by a factor of 630.

(2) Binding of HyHEL-15 and HyHEL-10 are affected by some of the same evolutionary variants of lysozyme, but all of the affected residues are buried in the interface between lysozyme and HyHEL-10 in the crystal structure of the HyHEL-10:lysozyme complex. A structure of the HyHEL-15:lysozyme complex will have important implications for conclusions that can be drawn from serological epitope mapping.

(3) HyHEL-9 binds in a relatively unique region on the lysozyme surface. HyHEL-9 binding to lysozyme is blocked by prior binding of D1.3. HyHEL-9 is affected by changes in residues 113 and 114, which are close to the D1.3 epitope, but are not part of it. S. Sheriff has recently collected a moderate resolution (~2.8 A) X-ray diffraction data set on the HyHEL-9 Fab-HEL complex, and crystals of the uncomplexed Fab appear suitable for diffraction studies.

(4) HyHEL-7 binding to lysozyme is blocked by prior binding of HyHEL-5, but it is unaffected by changes in residue 68 (see below). HyHEL-7 is affected by removal of the first three residues at the amino terminus of chicken lysozyme and is nonoverlapping with D1.3. HyHEL-7 is encoded by a $V_K$ and a $V_H$ that are in the same families as those encoding D1.3. A structure of HyHEL-7 would give important information on combinational mechanisms affecting specificity.

In addition, in a 3-way collaboration with D. Davies (NIDDK) and S. Sheriff (Squibb) we are attempting to crystallize uncomplexed Fab's of HyHEL-5 and HyHEL-10, antibodies for which we already have structures of the Fab-HEL complexes. To this end we have recently introduced hollowed fiber culture units into our lab in order to induce large amounts of protein in serum-free medium (eliminating the need for hundreds of mice that would be necessary for similar production in ascites fluid). We are currently harvesting 80-100 mg of HyHEL-5 protein in the culture system.

In initial epitope mapping experiments, we showed that bobwhite quail egg lysozyme (BWQEL) bound to HyHEL-5 1000-fold less strongly than did chicken lysozyme. BWQEL has four mutations relative to chicken T40S, I55V, R68K and V91T and comparison with other quail lysozymes as well as experiments with the $R68_{HEL}K$ site-directed mutant established that the mutation R68K is crucial. S. Sheriff (Squibb) has crystallized BWQEL, collected data on the crystals and is processing the data. We hope that a comparison between the structures of chicken lysozyme and BWQEL will yield an explanation for the difference in relative affinity. In addition, L. Kam-Morgan (Berkley) has produced and purified the R68K site-directed mutant in quantities (~25 mg) sufficient for crystallization studies; D. Davies (NIDDK) will attempt to crystallize and obtain a structure of the HyHEL-5 Fab-$R68_{HEL}K$ complex.

3. Development of Specificity Repertoires and Regulation of the Immune Response

In order to document the genesis of specificity from an apparently polyreactive preimmune repertoire, we have analyzed a panel of over 500 BALB/c hybridomas produced previously by C. Mallett to represent the course of the immune response to HEL. We found that HEL-specific IgM antibodies were extremely rare despite the observation that monospecific HEL-binding precursors were observed by P. Rousseau in a hybridoma panel derived from polyclonally activated spleen cells. Among 50 HEL-specific IgG mAbs, M.A. Newman found no obvious pattern of "affinity maturation," either overall or within specificity groups. Most specificities are found throughout the response, and no single fine specificity dominates any time point, although the primary response is predominated by antibodies of the Complementation Group III. A few specificities, for example recognition of $Arg68_{HEL}$, are late in the immune response.

Interactions among the 50 anti-HEL antibodies were analyzed by complementation and competitive blocking assays. All the antibodies analyzed to date fall into one of 4 complementation groups, three of which we have described previously and include the majority of the antibodies; antibodies within a complementation reciprocally cobind (complement) and competitively block one another. The patterns of interaction support the hypothesis that the entire surface of the

protein is antigenic. They also support the hypothesis that HEL contains discrete antigenic domains which can be related to the tertiary structure of the protein. The apparent absence of any antibodies overlapping these domains is unexpected and may suggest a fundamental relationship between antigenic structure and tertiary structure. This phenomenon and its possible relationship to immune regulatory processes is being investigated.

4. Plasmacytoma Induction by a Retrovirus Expressing v-abl and c-myc

E. Weissinger has been collaborating with H. Mischak and J.F. Mushinski in tumor induction studies (described in more detail in J.F. Mushinski's report) with an Abl-myc virus produced in the laboratory of R. Risser. They have shown that the virus is capable of inducing plasmacytomas in BALB/c mice with 100% incidence. In contrast to other viruses studied, MoMuLV helper virus is not necessary for tumor induction, the latency period is considerably shorter, and tumor incidence is higher. Spleen or bone marrow cells infected in vitro produce tumors when transplanted in vivo. Mice immunized with HEL prior to viral treatment produced a high frequency of plasmacytomas involving HEL-specific antibodies; the frequency of HEL-specific clones was much higher than found in either LPS-blasts, natural antibody hybridomas, RIM-induced plasmacytomas, or pristane-induced plasmacytomas. Recently, Dr. Weissinger has been able to culture HEL-specific clones from spleens of mice immunized and inoculated with Abl-myc virus in vivo. We are continuing studies on plasmacytoma induction and B-cell differentiation utilizing this virus.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 08952-04 LGN

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Retrovirus-induced acute myeloid leukemias 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 |
| K. Nason-Burchenal | Graduate Student | LGN, NCI |
| R. Mukhopadhyaya | Visiting Fellow | LGN, NCI |
| R. Koller | Biologist | LGN, NCI |

COOPERATING UNITS *(if any)*
J.F. Mushinski, LGN, NCI; R. Feldman, LCO, NCI; L. Wahl, LMI, D; M. Sitbon, Hopital Cochin, Institut National de La Sante et de la Recherche Medicale U152 Centre National de laRecherche Scientifique UA628, Paris

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 3.8 | 3.8 | 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.)*

Our laboratory has previously demonstrated that mice with a severe and chronic inflammatory granuloma, precipitated by pristane treatment, provide an excellent model for studies on the development of acute myeloid leukemia. Injection of such mice with retroviruses can result in myeloid leukemias that resemble AML in man. Studies on the preleukemic events leading up to the development of one of these types of leukemias, called M-ML or MF-ML, has been a recent focus. These promonocytic leukemias are caused by injection of Moloney murine leukemia virus (M-MuLV) or MF-MuLV in adult pristane-treated BALB/c mice and have been shown to be transformed at least in part by insertional mutagenesis of the c-myb gene. Evidence now indicates that, although transformed cells appear in the peritoneal cavity during the acute stage of disease, leukemia induction can involve the spleen in early stages. In addition, Pristane may facilitate preleukemic events in hematopoietic organs, since, in response to pristane, the spleens and livers show increased levels of progenitor cells committed to the granulocyte/macrophage lineage and the bone marrows have an increased rate of multipotential stem cell turnover. Results also suggest that prostaglandin $E_2$ ($PGE_2$) produced during the developmental stages of the leukemia may facilitate the induction of leukemia by virus. $PGE_2$ is produced by the leukemic cells and $PGE_2$ can abrogate the inhibitory effects of indomethacin on leukemia development. Other models of acute myeloid leukemia are being developed in the laboratory and a recent murine protocol involves inoculation of amphotropic MuLV (4070) into pristanetreated DBA/2 mice. Data suggest that novel genetic mechanisms involving the c-myb gene may be implicated in the transformation of some of these tumors, since these leukemias produce small aberrant c-myb proteins, but their c-myb genetic locus shows no overt signs of DNA rearrangement or viral insertion.

Major Findings:

I. The preleukemic phase of Moloney-MuLV-induced acute promonocytic leukemias.

Promonocytic tumors resulting from intravenous inoculation of M-MuLV into pristane-treated mice are apparent in the peritoneal cavity after a latency of 2-3 months. Our laboratory has been interested in the preleukemic events that might occur in hematopoietic organs such as the bone marrow, spleen and liver prior to the final outgrowth of 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. Studies were performed to evaluate the effect of pristane treatment on the hematopoietic makeup of the mouse. The data show that the number of progenitor cells committed to the granulocytic/macrophage lineage are significantly increased in the spleens and livers of treated mice. Differences in overall numbers of pluripotenial stems cells [GEM(M), for granulocyte, erythrocyte, macrophage and megakaryocyte] as measured in colony-forming assays was less pronounced, however it was found, using a [$^3$H]thymidine suicide technique, that there is a higher percentage of cycling bone marrow stem cells in pristane-treated mice compared to untreated mice. Experiments were carried out to determine if removal of the spleen has inhibitory effects on development of acute promonocytic leukemia in BALB/c mice inoculated with Moloney MuLV. When splenectomies were performed 3 days pre and 2,4,6 and 8 weeks post virus inoculation there was in all cases a decreased incidence of disease. The greatest inhibition, at 71% (relative to sham splenectomized mice), was observed when splenectomies were carried out at 4 weeks post virus inoculation. Our data suggests that the spleen is a natural and stimulatory environment for developing monocytic tumor cells. The organ may provide a source of cells that are targets for the primary transforming event (for example, insertional mutagenesis of c-myb by M-MuLV) or the spleen may provide an attractive intermediate environment for cells that are initially transformed in the bone marrow and will be passing into the peritoneal cavity for further development.

II. Leukemia inhibitory effects of indomethacin and its reversal by prostaglandin $E_2$ ($PGE_2$).

Indomethacin, administered in the drinking water inhibits leukemias induced by M-MuLV or MF-MuLV inoculation in pristane-treated mice. We found that $PGE_2$ is produced by tumor cells and its production is inhibited by indomethacin. Furthermore, preliminary evidence has suggested that $PGE_2$, when administered intraperitoneally twice daily during the early stages of disease, can abrogate this inhibitory effect. In our recent studies 42% of the mice receiving $PGE_2$ and indomethacin in combination with the typical leukemia induction protocol developed leukemia whereas those receiving indomethacin but no $PGE_2$ did not develop leukemia. It was also found that mice receiving less $PGE_2$, for example, once a day for 1 month, in combination with continuous treatment with indomethacin developed leukemias with an incidence of 20 percent. The leukemias induced by this regimen are still dependent on pristane treatment indicating

that $PGE_2$ treatment cannot substitute for pristane treatment. The leukemic cells were found to be clonal with multiple integrations of M-MuLV and to have c-myb rearrangements, confirming that the leukemic cells in this study were derived by the same genetic changes as promonocytic tumors cells generated in earlier studies and not by a new mechanism related to $PGE_2$ itself.

III. Retroviral genes specifically required for promonocytic leukemia induction.

Our laboratory reported evidence that there are at least two genetic elements of the Moloney MuLV that are crucial to induction of promonocytic leukemias in pristane treated mice, but are not required for viral replication overall in hematopoietic tissues such as spleen and bone marrow or for induction of lymphoid disease (J. Virol. 64:155,1990). Analysis of Moloney/Friend MuLV reciprocal recombinants in which the LTR and structural gene region [GAG-POL-ENV] had been exchanged showed that one of these genetic elements that is uniquely required for this myeloid cell disease, is within the structural gene region of Moloney, and is not present in Friend MuLV. Presently, in collaboration with M. Sitbon, the laboratory is testing other reciprocal recombinants between Friend and Moloney MuLV in mice in order to determine important differences between the two viruses which may account for their different abilities to induce promonocytic leukemia.

Evidence suggested that their is another genetic element within the U3 region of the LTR of M-MuLV that is uniquely specific to promonocytic leukemia induction and not other viral functions including induction of other leukemia types, since interruption of sequences just upstream of the 75-base-pair enhancer region by insertion of nonretroviral sequences resulted in loss of leukemogenicity for cells in the monocytic lineage. Using a linker scanning approach, mutants throughout 200 bp region of the LTR are presently being prepared and will be tested for their ability to replicate and induce leukemia.

IV. Additional models of acute myeloid leukemia in pristane-treated mice.

Previously we had developed two different types of acute myeloid leukemia in pristane-treated mice. One type is a monocyte/macrophage leukemia induced by intraperitoneal inoculation of a c-myc containing retrovirus and the other is a promonocytic leukemia resulting from intravenous inoculation of Moloney MuLV and subsequent insertional mutagenous of the c-myb gene. Both of these tumors develop only in conjunction with pristane treatment. Surprisingly it was also found that an amphotropic MuLV (4070) which had a reputation for being non-leukemogenic in newborn mice was extremely leukemogenic when inoculated intravenously in adult DBA/2 mice that have been treated with pristane. The incidence of promonocytic leukemia induced by this virus is 50% in pristane-treated mice but 0% in mice not treated with pristane.

Molecular analysis of leukemias induced in DBA/2 mice with amphotropic virus indicates that the leukemias fall into at least two classes: those that have c-myb gene rearrangements resulting from insertion of amphotropic MuLV into this locus and those that show no gross rearrangement of the c-myb locus. When

leukemic cells were shown to have c-myb rearrangements it was also possible to demonstrate altered transciption of the c-myb gene, (with 5.3kb and 3.9 kb transcripts instead of a single normal transcript of 3.9kb). However, when leukemic cells were demonstrated not to have c-myb gene rearrangements they were found to have only a normal size transcript. Interestingly, when two cell lines derived from the latter leukemias were examined for c-myb protein expression, it was observed that these cells do not produce the normal size 75,000 and 85,000 c-myb proteins, but instead produce two small c-myb proteins of approximately 25,000 and 30,000 daltons and the larger of the two was shown to be more highly phosphorylated than the smaller species. It is reasonable to speculate that these proteins, which appear to contain the DNA binding domain of the c-myb protein, are involved in promoting the transformed phenotype of these cells.

Publications:

Wolff L, Nason-Burchenal K. Retrovirus-induced tumors whose development is facilitated by a chronic immune response: a comparison of two tumors committed to the monocytic lineage. Curr Top Microbiol Immunol 1989;149:79-87.

Chung S-W, Wolff L, Ruscetti, SK. Transmembrane domain of the envelope gene of a polycythemia-inducing retrovirus determine erythropoietin-independent growth. Proc Natl Acad Sci 1989;86:7957-60.

Wolff L, Koller R. Regions of the Moloney murine leukemia virus genome specifically related to induction of promonocytic tumors. J Virol 1990;64:155-60.

SUMMARY STATEMENT

LABORATORY OF MOLECULAR BIOLOGY

DCBDC, NCI

OCTOBER 1, 1989 to SEPTEMBER 30, 1990

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 diagnosis and treatment of cancer, AIDS and other human diseases.

Immunotoxin and Oncotoxin Therapy of Cancer

I. Pastan, D. FitzGerald, M. C. Willingham, and V. K. Chaudhary

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 toxin genes. This is done by fusing DNAs encoding growth factor or other cell recognition molecules to a DNA encoding a modified form of *Pseudomonas* exotoxin that has no cell killing activity by itself. The chimeric toxin molecules are expressed in *E. coli* and readily purified to homogeneity. Using this approach, TGFα-PE40, IL2-PE40, IL4-PE40, IL6-PE40, CD4-PE40 and several single-chain antibody toxin fusion proteins have been made. Previously, antibody toxin fusion proteins were made by chemically attaching antibodies and toxins. In this new approach, chimeric toxins are made by linking two antibody variable domains to *Pseudomonas* exotoxin. One such chimeric molecule is anti-Tac(Fv)-PE40 which binds to the p55 subunit of the human and monkey IL2 receptor. This chimeric toxin is extremely active in killing human cell lines displaying the IL2 receptor, human and primate activated T cells, and many leukemia cells from patients with adult T cell leukemia. Anti-Tac(Fv)-PE40 is being developed for the treatment of allograft rejection and leukemias. To simplify the construction of single-chain antibody toxin fusion proteins, new methods have been developed to directly clone the antibody variable region DNAs from RNA using PCR and specific DNA primers.

The activities of TGFα-PE40 and IL2-PE40 have been examined in tumor models. TGFα-PE40 inhibits the growth of A431 tumors in nude mice. IL2-PE40 inhibits the growth of T cell lymphomas in mice. Furthermore, an IL6-PE fusion protein has been found to inhibit the growth of certain hepatomas. A non-toxic mutant form of *Pseudomonas* exotoxin, Lys-PE40, was made and coupled to an antibody against the human transferrin receptor. This agent is very effective against human tumors. All these studies show that chimeric toxins directed at antigens present on tumor cells can be effective against solid tumors. The current goal is to increase the activity and specificity of these molecules.

To understand how *Pseudomonas* toxin works, several types of genetic and biochemical experiments have been carried out. Mutagenesis studies indicated that $Arg^{276}$ and $Arg^{279}$ in domain II were important for toxin action. Chemical studies by M. Ogata and D. FitzGerald have shown that a wild type toxin is cleaved near $Arg^{279}$ in target cells, but nontoxic mutant toxins are not cleaved. This cleavage of PE generates a 37 kd fragment containing domain III and part of domain II which is translocated to the cytosol. Another region required for toxin activity lies at the carboxyl end of PE in the last five amino acids (REDLK). These amino acids comprise a recognition sequence which when deleted leads to a nontoxic molecule. The REDLK sequence can be replaced by KDEL (the endoplasmic reticulum sequence) indicating the toxin probably utilizes for its toxic action the same cellular machinery that retains

proteins in the endoplasmic reticulum. It has also been shown that most of domain Ib, amino acids 365-380 can be deleted, but extending the deletion into domain II leads to a fall in cytotoxic activity probably by also interfering with translocation.

In collaboration with E. Berger and B. Moss, a chimeric toxin, CD4-PE40, has been made which kills HIV infected cells expressing gp120 on HIV infected cells. CD4-PE40 is active against many different isolates of HIV and SIV, it suppresses the spread of HIV, and in combination with AZT it eliminates HIV from infected cultures. CD4-PE40 is under preclinical development by the Upjohn Company for the possible treatment of AIDS.

Development of Immunotoxins for Cancer
D. J. FitzGerald

To determine how Pseudomonas exotoxin (PE) acts to kill cells, Dr. FitzGerald and colleagues prepared $^{3}$H-PE in bacteria. When incubated with cells, it was proteolytically cleaved to produce an N-terminal 28 kDa and a C-terminal 37 kDa fragment. The latter is composed of a portion of domain II and all of domain III (the ADP-ribosylating domain). Cleavage occurs near $Arg^{279}$ and is blocked if $Arg^{276}$, $Pro^{278}$, and $Arg^{279}$.are mutated. Cytosol from toxin treated cells was greatly enriched in the 37 kDa fragment which is essential for toxicity, since mutant PE molecules that do not produce this fragment or deliver it to the cytosol, fail to kill cells. Cell membranes were shown to have a calcium-dependent protease with a pH optimum of 5.5 that cleaves PE near $Arg^{279}$. In other studies, PE40 was cleaved between domains II and III by two retroviral proteases. The HIV protease hydrolyzed the Leu-Leu bond at position 389-390 while the corresponding AMV protease cleaved at the Asp-Val bond at position 406-407.

Morphologic Mechanisms of Organelle Function and Transformation in Cultured Cells
M. C. Willingham

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 are being studied. Using hybridoma technology and a new screening method, several new monoclonal antibodies to surface epitopes on human tumor cells were isolated. By immunizing mice with MCF-7 cultured human breast tumor cells, a series of related monoclonal antibodies (B1, B2, B3, K2, K3) were generated that react with mucin-like materials expressed in adenocarcinomas of breast, colon, stomach and other organs. These antibodies are under evaluation for diagnostic and therapeutic use. In addition, an antibody (K1) that reacts with ovarian cancer was generated which could prove useful for diagnostic screening and perhaps for therapy of ovarian cancer. In other studies, the intracellular distribution of the chemotherapeutic drug daunomycin is being studied by a novel electron microscopic approach.

Genetic Analysis of the Multidrug Resistance Phenotype in Tumor Cells
I. Pastan and M.M. Gottesman (Laboratory of Cell Biology)

Resistance to multiple drugs is major impediment to the successful chemotherapy of human cancers. One mechanism of multidrug-resistance is the expression of a 170,000 dalton energy-dependent drug efflux pump (P-glycoprotein or the multidrug transporter, the product of the human *MDR1* gene) which confers resistance to colchicine, adriamycin, vincristine, vinblastine, puromycin, and actinomycin D. Several lines of investigation concerning the multidrug transporter have been pursued: 1) Evidence for ATP-dependent

transport activity of P-glycoprotein in vesicles and across epithelial monolayers has been obtained; 2) Novel expression vectors which utilize a full-length *MDR*1 cDNA as a dominant selectable marker are used to introduce and amplify non-selectable genes in cultured cells and *MDR*1 retroviral vectors have been developed; 3) Many human tumors express *MDR*1 RNA, and this expression may predict drug-resistance in some cancers, and correlate with the development of drug-resistance in others; 4) Transgenic mice have been constructed in which the human *MDR*1 mRNA is expressed in the bone marrow at levels comparable to those found in human tumors. This level of *MDR*1 expression is sufficient to confer resistance to leukopenia induced by MDR drugs; 5) Increased expression of *MDR*1 RNA has been demonstrated in regenerating rat liver, in cultured rodent cells after exposure to chemotherapeutic agents, and in kidney cancer cells after heat shock; 6) The intron-exon structure of the human *MDR*1 gene has been determined and supports a model of independent evolution of the two halves of the multidrug transporter. Similarly, a deletion analysis of the *MDR*1 cDNA is consistent with important functions being contributed by both the amino and carboxy-terminal halves of the molecule as is a study demonstrating photoaffinity labeling of both halves of P-glycoprotein by the hydrophobic drug $^{3}$H-azidopine; and 7) Evidence that P-glycoprotein acts by pumping hydrophobic drugs out of the lipid bilayer has been obtained.

Functions, Structure and Regulation of Receptors for Cell Adhesion Proteins
K. M. Yamada

Receptors for key extracellular proteins such as fibronectin, collagen, and laminin are under study. These are thought to mediate adherence, migration, and invasion by normal or tumor cells. Using several subunit-specific monoclonal antibodies, the $\alpha_5\beta_1$ fibronectin receptor was found to have an important role in cell adhesion, extracellular matrix assembly, and actin cytoskeletal organization. It was also found to synergize with the T-cell receptor in lymphocyte proliferation. Other members of the $\beta_1$ integrin family were found to be involved in cell-cell as well as cell-matrix interactions. Inhibition of $\beta_1$ integrin function disrupted fibronectin matrix assembly and blocked normal gastrulation in embryonic development. Biosynthesis and localization of integrin receptors was found to differ in certain tumor cells. For example, the rate of maturation of $\beta_1$ subunits was accelerated in transformed human cells, and localization was disrupted in these cells and in squamous cell carcinomas. Another integrin receptor termed $\alpha_4\beta_1$, which binds to a cell-type specific sequence in fibronectin, was isolated from metastatic human melanoma cells. The distribution of integrin receptors reflects their function. When localized to adhesion plaques, they appear to be part of an extracellular matrix assembly system and to help to retard cell migration. In rapidly migrating embryonic cells or certain tumor cells, they can be diffusely organized and more directly involved in cell migration.

Structures and Roles of Transformation-Sensitive Cell Surface Glycoproteins
K. M. Yamada

Fibronectin and other extracellular molecules play crucial roles in cell adhesion, migration, and invasion. Polypeptide recognition sequences necessary for cell interactions with fibronectin and other proteins were characterized by synthetic peptide analyses and site-directed mutagenesis. Synthetic peptide inhibitors from fibronectin, laminin, and collagen were compared as inhibitors of the migration of human tumor cells. Peptides containing the Arg-Gly-Asp sequence and anti-integrin antibodies were effective inhibitors. Anti-$\alpha_5$ and anti-$\alpha_2$ monoclonal antibodies displayed specificity depending on the ligand used for the migration substrate. An

anti-$\beta_1$ monoclonal antibody was a general inhibitor on various substrates, and inhibited tumor cell invasion at microgram concentrations. In collaborative studies on a cell-type specific adhesion site used by melanomas and some lymphocytes, it was found that the minimal critical sequence appears to be Leu-Asp-Val, a sequence present in other adhesion proteins. A novel heat-shock protein previously shown to be decreased after malignant transformation was found to bind specifically to fetuin as well as to collagen, indicating a broader function than previously known.

Development and Uses of Eukaryotic Vectors
B. H. Howard

Studies on the control of cell proliferation in mammalian cells, with emphasis on the investigation of negative growth regulatory pathways are continuing. Several results of interest have been obtained. i) The short sequence motif GAGGCNGAGGC, which is partially responsible for growth regulatory properties of 7SL RNA and Alu elements, has been shown to bind a previously undiscovered protein containing tandem $C_2H_2$ class zinc fingers of the TFIIIA/*Krüppel* type. Characterized members of this family are transcription factors and master control genes in early embryogenesis. ii) A serious technical obstacle to the use of RNA mobility shift assays in crude nuclear extracts has been overcome. The high non-specific binding activity (which has completely masked specific RNA-protein interactions) was eliminated by high levels of the homopolymer poly(rG). This finding has opened the way for the investigation of small RNA polymerase III-transcribed RNAs as nuclear regulatory factors. iii) Recombinant circle polymerase chain reaction (RCPCR), a novel and very rapid method for site-specific mutagenesis and other molecular cloning procedures, has been developed.

Regulation of Gene Activity
I. Pastan

The human epidermal growth factor receptor (EGFR) is overexpressed in many human tumors implicating it in the pathogenesis of some cancers. The promoter region of the EGFR was isolated and shown to contain many transcription factor binding sites including four Sp1 sites and sites for at least three other factors. To isolate transcription factors that bind to the GC-rich region, a [$^{32}$P]-labeled GC-rich DNA fragment was used to screen an expression library and a cDNA that encodes a novel 91 kilodalton protein was isolated; the protein it encodes has been termed GCF. Transfection studies of GCF into CV1 cells indicate that GCF may be a transcriptional regulator that represses gene expression.

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

Transgenic mice are being used to study carcinogenisis and to develop new cancer treatments. A line of transgenic mice expressing the human gene (*MDR*1) in their bone marrow was generated and is now an established colony. As a result of expression of the human *MDR*1 gene in the normally drug-sensitive bone marrow of these transgenic animals, the marrow becomes resistant to the cytotoxic effects of a number of commonly used chemotherapeutic drugs. These animals are being used to screen for drugs that overcome this type of resistance.

To elucidate the role of transforming growth factor alpha (TGF$\alpha$) in cancer, transgenic mice were made bearing DNA fragments encoding human TGF$\alpha$. Over-expression of TGF$\alpha$ in these mice was shown to induce hepatocellular

carcinoma, mammary adenocarcinoma, and a lesion resembling chronic pancreatitis.

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

To study the molecular mechanism of thyroid hormone nuclear receptor-thyroid hormone-DNA interactions, the human $T_3$ nuclear receptor (h-TR$\beta$1) was expressed in *E. coli*, purified and used for biochemical studies. In the presence of $Zn^{+2}$, $T_3$ binding to h-TR$\beta$1 was reduced; this inhibition was rapid and fully reversible. The binding data indicate that $Zn^{+2}$ is a non-competitive inhibitor of $T_3$ binding to h-TR$\beta$1. The rapid and reversible effects of $Zn^{+2}$ suggests that $Zn^{+2}$ may play a role in modulating hormone binding. Three monoclonal antibodies (mAbs) recognizing different domains of h-TR$\beta$1 were developed. The epitopes for these are located in the DNA binding domain, D domain and hormone binding domain, respectively. These mAbs will be useful in studying the function of TR$\beta$1.

The human cytosolic thyroid hormone binding protein (p58) was shown to be a monomer of pyruvate kinase, subtype $PKM_2$, and to have intrinsic pyruvate kinase activity. Analysis of competitive binding data indicated that alanine, at a physiological concentration, is a non-competitive inhibitor of $T_3$ binding to p58. Alanine was also found to be a "mixed" inhibitor of the substrate phosphoenol pyruvate. These data suggested that alanine might play a role in the regulation of $T_3$ binding to p58 *in vivo*.

Regulation of the *gal* Operon of *Escherichia coli*
S. Adhya

The gal operon of *E. coli* is negatively controlled by two regulatory proteins: One is the Gal repressor. The other is a newly discovered factor termed UIF (ultrainducibility factor). Both repressor and UIF act by binding to the same DNA sequences, $O_E$ and $O_I$, providing an example of cellular regulation by different factors acting through common DNA elements. While repressor inhibits *gal* transcription under normal conditions, UIF down regulates *gal* only in cells devoid of Gal repressor. Both proteins are inactivated by galactose. Inactivation of UIF, in the absence of repressor, causes extreme derepression which is termed ultrainduction.

The mechanism of repression by Gal repressor has been studied in detail. The repressor does not act as previously thought by sterically hindering RNA polymerase binding to the promoter. Instead, repression involves formation of a DNA-multiprotein complex of higher order structure, comprised of DNA, one molecule of RNA polymerase, one molecule of cyclic AMP receptor protein and two molecules of repressor. In this complex, two repressor molecules, bound to two spatially separated sites, interact with each other looping out the intervening DNA segment to which the other proteins are bound. Adhya and colleagues have proposed that repression of transcription is achieved by a novel mechanism in which repressor freezes the bound RNA polymerase by a direct contact with the latter. The formation of a DNA loop with a higher order structure facilitates such contact(s). He is currently investigating the various protein-protein contact sites and the state of transcription initiation (isomerization or promoter clearance) at which RNA polymerase is frozen.

Genetic Regulatory Mechanisms in *Escherichia coli* and Its Bacteriophage
S. Garges and S. Adhya

Cyclic AMP binds to its receptor protein CRP, and induces a conformational change (allosteric transition) in the protein. The cyclic AMP•CRP complex binds at or near many promoters of *E. coli* in a sequence-specific way and can either repress or activate transcription. To determine the structural changes in CRP, induced by cAMP, that are required to make the former proficient for gene regulation and to determine the points of contact between the cAMP•CRP complex and RNA polymerase needed to modulate transcription, several classes of mutations in the *crp* gene were isolated and characterized, which identify the amino acids participating in the cyclic AMP-induced allosteric shift in the protein. Various substitutions for Asp138 located at the apex of the hinge connecting the cAMP-binding domain to the DNA-binding domain of CRP cause biochemically distinct phenotypes and show that the hinge connecting the two domains of CRP is a key area for the transmission of the cAMP-induced allosteric change. In addition, this region is directly involved in a DNA interaction. To study the mechanism by which CRP activates transcription, mutants that have lost the ability of CRP and RNA polymerase to interact with each other were selected and mutations of *E. coli* which co-transduce with the $\beta$ and $\beta'$ genes encoding two RNA polymerase subunits were identified. The mechanism by which a DNA-bound CRP communicates with RNA polymerase to activate it is also being studied by *in vitro* transcription using DNA templates with altered location or orientation of the CRP binding site with respect to the promoter or with a single stranded gap introduced between the CRP binding site and the promoter.

Bacterial Functions Involved in Cell Growth Control
S. Gottesman

In *E. coli*, protein degradation plays an important role in regulating cell growth control, particularly proteases which carry out ATP-dependent proteolysis. The Lon protease degrades RcsA, a positive regulator of capsular polysaccharide synthesis, and SulA, a cell division inhibitor. RcsA interacts in the cell with another positive regulator, RcsB to promote efficient capsule synthesis. The Clp protease, a second major ATP-dependent protease, contains a large subunit, ClpA, with ATPase activity. This subunit has been well conserved in other prokaryotic cells and in eukaryotic cells. The smaller subunit, ClpP, contains the active site of the protease, and undergoes a self-processing reaction. The small subunit is also conserved in many cells. The structure and general organization of the Clp protease suggests that it has many similarities to the eukaryotic energy-dependent proteases which degrade ubiquitin-conjugated proteins. Alp, a third energy-dependent protease activity, which can substitute for Lon when overproduced, consists of a set of closely linked genes and a positive regulator. In a new approach to the study of bacterial cell growth control, mutants which may be defective in partition of the bacterial chromosome have been selected, mapped and characterized.

DNA Replication *in vitro*
S. Wickner

Two major questions of DNA replication are being studied. First, what are the molecular mechanisms involved in the initiation of replication? Second, how is DNA replication regulated? To address these questions, an *in vitro* replication system was established that replicates plasmid DNA carrying the bacteriophage P1 origin of replication and many host functions have been identified that participate in the replication reaction, including DnaA, DnaB,

DnaC, DnaG, RNA polymerase, DNA gyrase and DNA polymerase III. Three of the *E. coli* heat shock proteins, DnaJ, DnaK (the hsp70 homolog) and GrpE are also required. DnaK and DnaJ activate the specific DNA binding function of RepA by about 100-fold; this activation is ATP-dependent and DNA-independent.

This *in vitro* system is also used to study the mechanisms involved in the regulation of replication. If both the control region and the origin region are present, plasmid DNA is not replicated. Replication is restored when seven or more of the nine RepA binding sites are deleted from the control region or when wild-type RepA protein is substituted with RepA protein isolated from a *repA* mutant that has an increased copy number *in vivo*. It is possible that DNA looping between the origin and the control locus explains this type of regulation.

Dr. Michael Gottesman has left LMB to become Chief of the Laboratory of Cell Biology, DCBDC, NCI.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08000-20 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Regulation of Gene Activity

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

| | | | |
|---|---|---|---|
| PI: | I. Pastan | Chief, Laboratory of Molecular Biology | NCI |
| Other: | G. Merlino | Expert | LMB, NCI |
| | A. Johnson | Staff Fellow | LMB, NCI |
| | L. Jefferson | Chemist | LMB, NCI |

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LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Biology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The EGF receptor (EGFR) is overexpressed in many human tumors implicating it in the pathogenesis of some cancers. To study regulation of the EGFR gene, the promoter region was isolated and shown to contain many transcription factor binding sites including four Sp1 sites and sites for at least three other factors. To isolate transcription factors that bind to the GC-rich region, a labeled GC-rich DNA fragment was used to isolate from an expression library a cDNA that encodes a novel 91 kilodalton protein. When GCF cDNA was attached to a SV40 promoter and cotransfected into CV1 cells with EGF receptor CAT constructions, GCF repressed EGF receptor promoter activity suggesting GCF may be a negative regulatory protein.





GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08010-17 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Morphologic Mechanisms of Organelle Function & 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 Sect. | NCI, NIH |
| Other: | I. Pastan | Chief, Lab. of Molecular Biology | NCI, NIH |
| | K. Chang | Visiting Associate | NCI, NIH |
| | G. Merlino | Expert | NCI, NIH |
| | M. M. Gottesman | Chief, Lab. of Cell Biology | NCI, NIH |

COOPERATING UNITS *(if any)*

| | |
|---|---|
| Molecular Biology Section, LMB | NCI, NIH |
| Laboratory of Cell Biology | NCI, NIH |

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Ultrastructural Cytochemistry Section

INSTITUTE AND LOCATION

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| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

There are many changes in the properties of cells following malignant transformation. We have recently 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 continued to isolate monoclonal antibodies to surface epitopes on human tumor cells. By immunizing mice with MCF-7 cultured human breast tumor cells, we have generated a series of related monoclonal antibodies (B1, B2, B3, K2, K3) to mucin-like materials expressed in adenocarcinomas of breast, colon, stomach and other organs. These antibodies are under evaluation for diagnostic and therapeutic use. Using OVCAR-3 human ovarian cancer cells, we have generated a new antibody, K1, that recognizes a unique epitope on an antigen similar to the CA125 antigen found in ovarian cancer. This new antibody may prove useful for diagnostic screening and perhaps for therapy of ovarian cancer. We have also evaluated an antibody to the external domain of the Neu oncogen product, which may be useful for therapy in selected tumors of breast, ovary or other organs. In other studies, we have begun examining the intracellular distribution of the chemotherapeutic drug daunomycin by a novel electron microscopic approach, and we have also localized a transgene product in mice expressing the human EGF receptor exclusively in testis at specific stages of spermatocyte differentiation.

Major Findings:

We have continued to generate monoclonal antibodies to the surface of tumor cells using morphologic screening methods that select for antibodies reactive to tumors, but not to normal tissues. In the previous year, we isolated OVM3, an IgM antibody to ovarian tumors. We have now shown that this antibody reacts to an antigen present in a distribution similar to our previously isolated IgG antibody OVB3, but OVM3 is less useful because of its reactions to more normal tissues than OVB3.

Using MCF-7 cells as the immunogen, we have more recently isolated a series of closely related monoclonal antibodies (B1, B2, B3, K2, K3) reactive to mucin-like material in ovarian, breast, colon, gastric and some prostate tumors. Normal tissue reactivity of these antibodies is limited and varies slightly between these antibodies, with the major important normal tissue reactivity found in gastric mucosa. All of these antibodies also react with monkey tissues in a pattern similar to that seen in human tissues. These antibodies are being evaluated further for their use in the generation of therapeutic and diagnostic agents.

Using OVCAR-3 human ovarian cancer cells and a method in which spleen lymphocytes were selected by panning on tumor cells prior to fusion, another antibody, K1, has been isolated. This antibody reacts with ovarian carcinomas, as well as some squamous carcinomas of cervix and esophagus. In normal tissues, this antibody reacts with mesothelium, and only a few other less vital tissue sites. This antibody also cross-reacts with monkey tissues in the same pattern. We have shown that this antibody reacts with an antigen that is very similar to the CA125 antigen, but the epitope recognized by K1 is completely separate from that recognized by monoclonal antibody OC125. In preliminary experiments, K1 has been found to be potentially useful in the construction of an immunotoxin for therapy. In addition, K1 may be useful to enhance the sensitivity and/or specificity of the diagnostic assays for the CA125 antigen, which may increase the value of this serum assay for diagnosis of primary or recurrent ovarian cancer.

In developing methods for the efficient selection of these hybridomas, we have devised a new method for selecting isotype switch variants of hybridomas using a panning technique. Two antibodies, K1 and K2, both originally isolated as IgM antibodies, were isotype switched to IgG variants using this successive panning technique using commercially available anti-gamma heavy chain antiglobulin antibodies. This method may have significant advantages in simplicity and efficiency over previous isotype switch selection methods.

We have also evaluated a number of other "tumor-specific" antibodies isolated in other laboratories using our morphological methods. Some of these included antibodies directed against prostate tumors and lung tumors. With our methods we were able to demonstrate previously unrecognized normal tissue reactivities of most of these antibodies, making them not useful as therapeutic agents.

We have evaluated an antibody to an epitope of the *Neu* oncogene product that is present on the external domain of this protein, in collaboration with investigators in Japan. This antibody localizes strongly in some tumors of

breast and ovary. In normal tissues, we have found a significant reactivity on the basal surface of cells in distal tubules in the kidney. Otherwise, the normal tissues reactive with this antibody are very limited. As a result, this antibody is continuing to be evaluated as a candidate for immunotherapy.

The methology developed for our morphologic hybridoma screening strategies has some novel elements. One of these is a device for rapid immunofluorescence screening of small volumes. After evaluation by the government, the patent rights for this device were returned to the inventors (M.Willingham and I.Pastan), and a patent has been filed for this invention, allowing the development of a commercial version of this device, now available and manufactured by Life Technologies, Inc. under the tradename *ScreenFast*.

In other studies, we have begun a high-resolution evaluation of the distribution of the chemotherapeutic drug daunomycin using electron microscopy. This study combines new methods of fluorescence photoconversion to produce a dense reaction product that can be visualized at the electron microscopic level at the site of drug accumulation. In collaboration with G. Merlino, we have localized the human EGF receptor expressed in a transgenic mouse, an expression uniquely localized in the testis under the control of specific differentiation signals. The study of this expression may lead to a new understanding of the control of spermatocyte differentiation. We have also begun evaluation of methods to localize specific receptors for small peptides ligands, such as interleukin 6, using a histochemical approach that detects the presence of bound ligand added after cell fixation. One of the promising methods for this "ligand histochemistry" approach employs highly sensitive 1nm colloidal gold labeling methods.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08011-16 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Structures and Roles of Transformation-Sensitive Cell Surface Glycoproteins

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

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| Other: | S. Aota | Visiting Fellow | LMB, NCI |
| | T. Nagai | Guest Researcher | LMB, NCI |
| | S. K. Akiyama | Guest Researcher | LMB, NCI |
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Laboratory of Molecular Biology

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| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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| 3.7 | 2.8 | 0.9 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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

Fibronectin and other extracellular molecules play crucial roles in cell adhesion, migration, and invasion. Polypeptide recognition sequences necessary for cell interactions with fibronectin and other proteins are being characterized by synthetic peptide analyses and site-directed mutagenesis. A variety of synthetic peptide inhibitors from fibronectin, laminin, and collagen were compared as inhibitors of the migration of several types of human tumor cells. Peptides containing the Arg-Gly-Asp sequence were the most effective. Anti-integrin antibodies were particularly effective inhibitors. Anti-$\alpha_5$ and anti-$\alpha_2$ monoclonal antibodies displayed specificity depending on the ligand used for the migration substrate, i.e. for fibronectin and collagen, respectively. An anti-$\beta_1$ monoclonal antibody was the most general inhibitor on various substrates, as well as inhibiting tumor cell invasion at microgram concentrations. Collaborative studies are being completed on a cell-type specific adhesion site used by melanomas and some lymphocytes. The minimal critical sequence appears to be Leu-Asp-Val; this sequence is also present in other adhesion proteins. A novel heat-shock protein previously shown to be decreased after malignant transformation was found to bind specifically to fetuin as well as to collagen, indicating a broader function than previously known. Studies will continue on other crucial polypeptide sequences, on their roles in adhesion, migration, and invasion, and on developing novel inhibitors of their functions.

Major Findings:

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

We have continued to characterize the polypeptide recognition sequences necessary for normal and tumor cell interactions with fibronectin, using synthetic peptide analyses and site-directed mutagenesis. Six classes of synthetic peptide inhibitor representing putative adhesive recognition sequences in fibronectin, laminin, and collagen were compared for their abilities to inhibit the migration of several different types of human tumor cells. Peptides containing the Arg-Gly-Asp sequence effectively inhibited tumor cell migration on fibronectin, but not on laminin or collagen. In general, other peptide inhibitors of *in vitro* adhesion showed only minimal inhibition of tumor cell migration. In contrast, monoclonal antibody inhibitors of the binding of receptors to these ligands produced marked inhibition of tumor cell migration. The antibodies demonstrated ligand specificity, e.g. anti-$\alpha_5$ integrin monoclonals inhibited tumor cell migration on fibronectin, but not on collagen or laminin substrates. Conversely, anti-$\alpha_2$ monoclonals inhibited tumor cell migration in 3-dimensional collagen gels, but generally not on fibronectin or laminin. The antibodies were non-cytotoxic; in fact, substrates comprised solely of these monoclonals were capable of directly mediating cell migration by substituting for the normal ligand. An anti-$\beta_1$ monoclonal antibody was the most general inhibitor of tumor cell migration on various substrates. It also blocked *in vitro* invasion at concentrations of only 1-5 μg/ml.

Studies on general and cell-type specific adhesive recognition sequences in fibronectin are continuing. Site-directed deletion analyses using fibronectin cDNA sequences expressed in *E. coli* suggest the existence of two sub-sites within a "synergistic" sequence we had identified previously. These sub-sites are being defined by extensive fine-structure deletion mapping; they are important because they appear to provide ligand specificity and adequate binding affinity to the Arg-Gly-Asp sequence found in a large number of cell surface interaction proteins.

We are completing collaborative work to identify the minimal active sequence of the CS1 cell-type specific adhesion region in fibronectin, which is now known to be used by neural crest derivatives such as melanomas and by certain lymphocytes. Quantitative comparisons of the adhesive activities of overlapping peptides from this region and homologues with local substitutions or scrambled amino acid sequences reveals that the minimal active sequence is Leu-Asp-Val, a novel adhesive recognition sequence. Interestingly, this sequence is present in a number of other adhesion proteins: its role in other cell surface interaction proteins warrants investigation.

A 47,000 dalton heat-shock protein previously shown to be decreased after malignant transformation was found to bind specifically to fetuin as well as to collagen. This dual specificity further supports the impression that this protein is not a collagen receptor, but may instead play a more general role in protein processing.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08710-14 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

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| 1.0 | 1.0 | 0.0 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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

Two major questions of DNA replication are being studied: First, what are the molecular mechanisms involved in the initiation of replication? Second, how is DNA replication regulated? To address these questions, I have been using an *in vitro* replication system that replicates plasmid DNA carrying the bacteriophage P1 origin of replication. I have determined many host functions that participate in the replication reaction, including DnaA, DnaB, DnaC, 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. I have found that DnaK and DnaJ activate the specific DNA binding function of RepA by about 100-fold. The activation is ATP-dependent and DNA-independent.

I am also using this *in vitro* system to study the molecular mechanisms involved in the regulation of replication, since P1 normally exists stably as a unit copy plasmid. I have found that with reaction conditions that allow the replication of DNA carrying the origin region, plasmid DNA carrying in addition the control region is not replicated. Replication *in vitro* is restored when seven or more of the nine RepA binding sites are deleted from the control region or when wild-type RepA protein is substituted with RepA protein isolated from a *repA* mutant that has an increased copy number *in vivo*. I am currently trying to determine if DNA looping between the origin and the control locus completely explains regulation or if other factors are involved.





GPO 914-918

Major Findings:

The mini-P1 replicon consists of an origin of replication, a replicon specific initiator protein, RepA, and a control locus. The origin contains 5 direct repeats of a 19 bp sequence to which the P1 initiator protein, RepA, binds. About 100 bp to the left there are two direct repeats of the 9 bp DnaA protein binding site. Plasmids containing this origin are maintained at high copy number. The replication control region, located 1 kb to the right of the origin, is required to maintain mini-P1 plasmids at one copy per *E. coli* chromosome. This region contains nine repeats of the RepA binding site. The obvious mechanism of regulation by titration of RepA by the control locus is apparently ruled out by the finding that RepA is autoregulated.

We have developed an *in vitro* DNA replication system that replicates plasmid DNA containing the P1 origin of replication. The reaction requires two origin specific DNA binding proteins, DnaA and RepA. RNA polymerase, DNA gyrase, DNA polymerase III, DnaB, DnaC and DnaG are also required. I have found that three *E. coli* heat shock proteins, DnaJ, DnaK and GrpE are essential. Furthermore, DnaJ and RepA can be isolated from cells as a protein complex. The DnaJ-RepA complex can be reconstituted *in vitro*. The complex contains equimolar amounts of the two proteins and the native molecular weight of the complex suggests that it contains a dimer of each protein.

I observed that both RepA and the DnaJ-RepA complex bound specifically, but poorly to P1 origin DNA. Since heat shock proteins are known to be involved in protein folding and renaturation, I tested (in collaboration with K. McKenney and J. Hoskins, National Institute of Standards and Technology) whether DnaK, DnaJ and GrpE might in some way activate RepA protein, thus increasing its DNA binding activity. The major conclusion of our work is that DnaK and DnaJ, in an ATP-dependent reaction, activate RepA in the absence of DNA such that it is then capable of binding to P1 origin DNA with about a 100-fold higher affinity. GrpE is not essential for RepA activation. ATP is required. This reaction provides the first simple *in vitro* system for determining the function of DnaK and DnaJ. Our results best fit with the models implicating hsp70-like proteins in protein folding or renaturation. I am currently biochemically characterizing the activated form of RepA.

P1 plasmid DNA carrying the control region in addition to the origin is not replicated by our in *vitro* system even when RepA is added in a 10-fold excess relative to binding sites. We have seen by electron microscopy that RepA binds to the origin and control region simultaneously, causing the intervening DNA to loop. Our interpretation is that this looping inhibits initiation. In collaboration with K. McKenney and J. Hoskins, we have found that DNA synthesis is restored when the distal seven or more RepA binding sites are deleted from the control region or when wild-type RepA protein is substituted with RepA protein isolated from a *repA* mutant that has an increased copy number *in vivo*. I am currently trying to understand what conditions or factors regulate looping.

References:

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PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 08714-13 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Bacterial Functions Involved in Cell Growth Control

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

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|---|---|---|---|
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Laboratory of Molecular Biology

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Biochemical Genetics Section

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| 5 | 3 | 2 |

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☐ (a1) Minors
☐ (a2) Interviews

B

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

We have been studying the role that protein degradation plays in regulating cell growth control, through the study of the proteases which carry out ATP-dependent proteolysis in *E. coli*, The Lon protease degrades RcsA, a positive regulator of capsular polysaccharide synthesis, and SulA, a cell division inhibitor. We have demonstrated that RcsA interacts in the cell with another positive regulator, RcsB to promote efficient capsule synthesis. The Clp protease, a second major ATP-dependent protease, contains a large subunit, ClpA, with ATPase activity; this subunit has been well conserved in both other prokaryotic cells and eukaryotic cells. The smaller subunit, ClpP, contains the active site of the protease, and undergoes a self-processing reaction. The small subunit is also conserved in many cells. The structure and general organization of the Clp protease suggests that it has many similarities to the eukaryotic energy-dependent proteases which degrade ubiquitin-conjugated proteins. Alp, a third energy-dependent protease activity, which can substitute for Lon when overproduced, consists of a set of closely linked genes and a positive regulator. In another approach to the study of bacterial cell growth control, mutants which may be defective in partition of the bacterial chromosome have been selected, mapped and characterized.





GPO 914-918

Major Findings:

1. We have continued our analysis of the regulation of capsular polysaccharide synthesis. This year, this analysis has focused on the role of the unstable positive regulator, RcsA. RcsA and RcsB were purified from overproducing cells and used to raise antibodies. Studies with the anti-RcsA antibody have demonstrated that the basal level of RcsA in wild-type cells is exceedingly low. We have confirmed the effect of *lon* mutants in stabilizing RcsA. rcsA*, a mutation which increases capsule synthesis even in the presence of the Lon protease, does not have a dramatic effect on the stability of RcsA, but may increase the interaction of RcsA with either DNA or other factors. Since the other positive regulator, RcsB affects the stability of RcsA (RcsA is more stable when RcsB is overproduced and is less stable when it is absent), we believe RcsA interacts directly with RcsB. This interaction may allow RcsB to bind better to DNA, or may increase the half-life of the activated form of RcsB. RcsA has a possible DNA binding domain at its N-terminus. Both RcsA and RcsB share homology with a class of regulatory proteins whose mode of action has not yet been well-defined.

2. In preparation for the *in vitro* reconstruction of the capsule regulatory system, we have defined a fragment carrying a promoter which is regulated in common with the chromosomal cps-lac fusions which we have used in the past. Binding to this fragment of RcsA and RcsB, alone and together, will be tested.

3. We have continued our studies on the Clp energy-dependent protease of *E. coli*. Using site-directed mutagenesis of the ClpP subunit, we have changed both the previously identified active site serine and a conserved histidine, and shown that both are necessary for Clp activity *in vivo* and *in vitro*. Therefore, it seems likely that Clp proteolysis involves the catalytic triad found in other serine proteases, although the sequence context appears to be unique. ClpP is synthesized with an amino-terminal leader of 14 amino acids which is rapidly cleaved off. This cleavage depends on active ClpP *in vivo*, although it does not need to proceed by an intramolecular reaction. This processing is not dependent on the ATPase subunit, ClpA. The discovery of this ClpP-specific processing reaction suggests the possibility that ClpP may be involved in some ATP-independent processing events. The significance of the processing for Clp activity is not yet clear.

4. Purified ClpP assembles into donut-shaped particles which are reminiscent of the 20S proteosomes found in eukaryotic cells. Analogs of ClpP are found in the genome of chloroplasts. In addition, proteins which cross-react with ClpP can be found in many eukaryotic cells, and others have shown that one subunit of the proteosome cross-reacts with ClpP. The proteosome is also known to assemble, in the presence of larger ATPase subunits, into a larger structure which carries out the energy-dependent degradation of ubiquitin-conjugated proteins. This assembly is reminiscent of our findings with Clp, where ClpA, the ATPase subunit, assembles with the ClpP particles to form an ATP-dependent protease.

5. We have identified a third energy-dependent proteolytic system, Alp, by the ability of plasmids carrying Alp to suppress *lon* mutants. We suspected the existence of other components of the Alp system, and these have now been identified. Chromosomal mutations in genes called *slp* inactivate the Alp system. Furthermore, a *lac* fusion to a *slp* gene was isolated. Using this

fusion, we were able to show that the plasmid-borne function, *alp*, acts as a positive regulator of the *slp* genes. *slp* and *alp* map close to each other on the chromosome. We believe that *alp* is the regulator for this protease, and the *slp*
mutants define the protease genes. In collaboration with J. Trempy (Oregon State University), the *slp* genes have been cloned and are in the process of being sequenced.

6. We have selected *E. coli* mutants which have increased chromosome number, as a way of identifying genes involved in chromosome partitioning. These mutants map to four clusters. Most mutations are dominant to wild-type, which is consistent with a change in function or increased function, rather than loss of function. One locus was found to define a new class of mutations in rpoB, the gene for an RNA polymerase subunit. The other loci seem to define new genes.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08717-12 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

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

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| | W.-T. Chen | Guest Researcher | LMB, NCI |
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Laboratory of Molecular Biology

SECTION

Membrane Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.3 | 3.3 | 1.0 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Receptors for key extracellular proteins such as fibronectin, collagen, and laminin are thought to mediate adherence, migration, and invasion by normal or tumor cells. The functions and distributions of integrin receptors for these molecules were explored using several subunit-specific monoclonal antibodies. The $\alpha_5\beta_1$ fibronectin receptor was found to play central roles in cell adhesion, extracellular matrix assembly, and actin cytoskeletal organization. It was found to synergize with the T-cell receptor in lymphocyte proliferation. Other members of the $\beta_1$ integrin family were found to be involved in cell-cell as well as cell-matrix interactions, e.g., in the maintenance of cell contacts and adjacent cytoskeletal organization in keratinocytes. Inhibition of $\beta_1$ integrin function disrupted fibronectin matrix assembly in several systems, and blocked normal gastrulation in embryonic development. Biosynthesis and localization of integrin receptors was found to differ in certain tumor cells. For example, the rate of maturation of $\beta_1$ subunits was accelerated in transformed human cells, and localization was disrupted in these cells and in squamous cell carcinomas. Another integrin receptor termed $\alpha_4\beta_1$, which binds to a cell-type specific sequence in fibronectin, was isolated from metastatic human melanoma cells. The distribution of integrin receptors reflects, and may also affect, their function: when localized to adhesion plaques, they appear to be part of an extracellular matrix assembly system and to help to retard cell migration. In rapidly migrating embryonic cells or certain tumor cells, they can be diffusely organized and more directly involved in cell migration. Further analyses of the regulation and function of these integrin receptors for extracellular proteins will provide further insights into the mechanisms of cell adhesion, migration, and invasion, and should provide novel approaches to inhibiting them more specifically.





GPO 914-918

Major Findings:

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

The functions of $\beta_1$ integrin receptors such as the classical fibronectin receptor have been examined using several subunit-specific monoclonal antibodies. The $\alpha_5\beta_1$ fibronectin receptor was found to play central roles in cell adhesion, extracellular matrix assembly, and actin cytoskeletal organization of normal human fibroblasts. In addition, it was found to synergize with anti-T cell receptor antibodies to induce proliferation of CD4+ T lymphocytes.

Localization of integrin receptors were compared *in vivo* and *in vitro*. In human skin, levels of integrin receptors for fibronectin, collagen, and laminin were similar in normal skin and basal cell carcinomas, but were variably decreased in squamous cell carcinomas. In various cultured human fibroblasts, contrary to published studies with rodent cells, there were no decreases in total levels of this receptor. Instead, its localization was disrupted from a clustered to a diffuse distribution, and the ratio of immature to mature $\beta_1$ subunits was markedly altered. The latter maturation process was found to involve changes in glycosylation, converting the receptor from an inactive precursor to an active fibronectin-binding molecule. These studies suggest that integrin quantities, distribution, biosynthesis, and function can be altered depending on the tumor, and that it is important to focus on human rather than animal models.

In a collaborative study, the receptor for an alternatively spliced, cell-type specific sequence in fibronectin was isolated by affinity chromatography. This $\alpha_4\beta_1$ integrin receptor is present on metastatic human melanoma cells and contributes significantly to their adhesion to fibronectin.

Although $\beta_1$ integrins are generally viewed as receptors for extracellular matrix molecules, new evidence points to important roles for them in cell-cell interactions as well. In keratinocytes, several members of this receptor family were found to be located primarily in cell-cell contact sites rather than in cell-substrate adhesions. Antibodies against the $\beta_1$ integrin subunit produced disruption of cell-cell contacts and dispersal of colonies of cells *in vitro*. In addition, these antibodies disrupted the organization of intracellular microfilament bundles normally associated with cell-cell contact regions. Our studies indicate that this class of receptor plays novel roles in cell-cell interactions as well as in cell-substrate adhesion.

Cells produce and shape the extracellular matrix, e.g. by regulating patterns of matrix protein assembly. Antibodies against $\alpha_5$ or $\beta_1$ integrin subunits inhibited fibronectin matrix assembly by mechanisms that only partially overlapped their effects on adhesive interactions. In collaborative studies, these findings were extended to other model systems for matrix assembly, showing the generality of this assembly mechanism. In embryonic development,

the importance of $\beta_1$ integrins in assembling the fibronectin matrix was evaluated using antibodies effective against either intracellular or extracellular domains of these receptors. The $\beta_1$ integrins were found to be essential for matrix assembly and for normal gastrulation.

According to immunological analyses of function, the $\alpha_5\beta_1$ integrin receptor appeared to play differing roles in different cells, depending on its distribution. If localized to adhesion plaques, it acted as a part of a matrix assembly system and tended to retard cell migration; in normal human fibroblasts, migration on fibronectin instead appeared to be mediated most effectively by a vitronectin receptor. In contrast, integrin receptors were diffuse in rapidly migrating embryonic cells or mesenchymally-derived tumor cells, where they were more often directly involved in cell migration, but not in matrix assembly. Insights into the mechanisms that control receptor distribution and function may provide means to develop specific inhibitors of each of the processes they mediate.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB08719-10 LMB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development and Uses of Eukaryotic Vectors

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

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The primary focus of work in the Molecular Genetics Section continues to be on control of cell proliferation in mammalian cells, with investigation of negative growth regulatory pathways emphasized within this framework. Several results of interest have been obtained. i) The short sequence motif GAGGCNGAGGC, which is partially responsible for growth regulatory properties of 7SL RNA and Alu elements, has been shown to bind a previously undiscovered protein containing tandem $C_2H_2$ class zinc fingers of the TFIIIA/*Krüppel* type. Characterized members of this family are transcription factors and master control genes in early embryogenesis. ii) A serious technical obstacle to use of RNA mobility shift assays in crude nuclear extracts has been overcome: high non-specific binding activity (which in the past completely masked specific RNA-protein interactions) was eliminated by high levels of the homopolymer poly(rG). This finding opens the way for the investigation of small RNA polymerase III-transcribed RNAs as nuclear regulatory factors. iii) Recombinant circle polymerase chain reaction (RCPCR), a novel method for site-specific mutagenesis and other molecular cloning procedures, was developed.





GPO 914-918

Major Findings:

I. New methods to improve DNA-mediated transformation efficiencies. By coupling an electroporation-cell synchronization-butyrate procedure (previously developed in this laboratory) with a cytomegalovirus promoter-containing vector, it was possible for the first time to successfully transfect quiescent and senescent normal human embryo fibroblasts. Transfection of these cells in a non-dividing state is a prerequisite to their future use in studies on both oncogenes and anti-oncogenes.

II. Application of gene transfer methods to detection of genes that regulate cell growth.

A. Development of transfection/FACS assay: Results of this project have been published.

B. Construction of cDNA expression vector libraries. Inactive. The post-doctoral responsible for this project left the laboratory.

C. Inhibition of HeLa cell replication: The role(s) of RNA polymerase III (pol III)-transcribed genes, in particular the 7SL RNA/Alu family, in regulating mammalian cell growth has continued to be the central focus of research in the the MGS. Earlier it was shown that two small DNA sequence motifs in these genes, the B block RNA polymerase III (pol III) promoter element and the GAGGCNGAGGC papovavirus origin homology region, are required for perturbation of HeLa cell growth in transient gene transfer assays. Work in this area over the past year has concentrated on identifying a cellular factor which, because it interacts with the latter ("ori") sequence motif, is a potential competitor for the DNA binding activity of simian virus 40 T antigen.

To further pursue this putative cellular T antigen homolog, a HeLa λgt11 bacteriophage cDNA library was screened for clones encoding proteins with GAGGC-binding activity. Six positive clones were detected; four of these clones contained inserts of 3.5 kb while the other two clones contained inserts of 2.8 kb. Lysates derived from these clones reacted with a GAGGC-containing oligonucleotide, but not a control oligonucleotide. Identical gel shift results were obtained with lysates from clones with either 3.5 or 2.8 kb inserts, suggesting that the clones encoded identical or closely related proteins.

The DNA sequence analysis of these inserts revealed that both contain an open reading frame encoding 338 amino acids with the same $NH_2$ terminus; however, the only ATG near the start of this open reading frame is out of phase and terminates at a UAA after 15 bp. Accordingly, it seems most likely that these cDNA clones represent only a partial copies of the cognate mRNA. The UAA stop codon following amino acid residue 338 is probably the correct translation stop codon, since DNA sequences of the 2.8 and 3.5 kb inserts are more divergent downstream from that point.

Inspection of the predicted polypeptide indicated the existence of two zinc finger motifs of the $C_2H_2$ class, as well as a third degenerate zinc finger. The interval between the two intact motifs is 28 amino acids, whereas the third partially conserved zinc finger is 37 amino acids downstream from its nearest neighbor. The distance from the N-terminal zinc finger to the predicted stop codon is 107 amino acids.

Comparison of the polypeptide with the translated GenBank/EMBL data bank revealed that the gene encoding this protein, which is provisionally referred to as the OBP gene, has not been previously described. At the same time, the translated GenBank/EMBL data base search confirmed strong similarity of the predicted polypeptide to other members of the $C_2H_2$ zinc finger class of which TFIIIA is the prototype. As in most members of this family, phenylalanine and leucine residues within the finger tip are conserved. There is no clear sequence similarity to other $C_2H_2$ zinc finger proteins outside of the finger motifs.

In light of what is known concerning other members of this zinc finger protein subclass, the identity of the gene isolated in this study has quite interesting implications. The Drosophila members of this family, including *Krüppel*, *hunchback*, and *serendipity*, are gap class genes involved in controlling segmentation during embyronic development. *Krüppel* and *hunchback* are transcription factors, as are the best characterized eukaryotic family representatives, TFIIIA and Sp1. Thus, the OBP gene is a candidate for the second $C_2H_2$ zinc finger pol III transcription factor to be described and, in view of the exquisite sensitivity of Alu expression to changes in cell differentiation or proliferation, (one of) the first developmentally regulated mammalian $C_2H_2$ zinc finger protein for which a target gene family can be proposed.

In considering the potential growth regulatory properties of the Alu gene family, one framework in which to view Alu family elements is as components in an input/output network. Following this line of reasoning, the OBP gene may contribute to the differentiation-related input mechanisms responsible for the known developmental- and transformation-dependent variations in Alu expression levels. With respect to output mechanisms, there are at least two plausible ways by which Alu repeats might affect cellular metabolism: i) as cis-acting elements modulating nearby single-copy transcription units or DNA replication origins, and ii) as elements which encode trans-acting small RNAs.

To further explore the possibility that pol III-transcribed Alu RNAs may function as trans-acting regulatory molecules, it was necessary to develop an assay for Alu RNA-protein interactions in crude nuclear extracts. This was not a trivial problem, because, unlike DNA-protein mobility shift assays, RNA-protein assays have been plagued by extremely high non-specific binding activities. The only known solution to this problem required destruction of the intact RNA molecule with T1 RNase. In investigating alternative approaches, it was discovered in this laboratory that high concentrations of the homopolymer poly(rG) effectively block non-specific nuclear binding activit(ies). Poly(rC) and poly(rU) are completely ineffective and poly(rA) only partially blocks non-specific binding.

Use of the poly(rG)-modified RNA mobility shift assay in conjunction with Alu 3α1 RNA (produced in a T7 polymerase *in vitro* transcription) has allowed identification of Alu RNA-nuclear protein interactions common to both HeLa cells and SV40-transformed human embryo fibroblasts, as well as an interaction specific to HeLa cells.

D. Regulation of growth in cell types other than HeLa. Project completed; results published.

III. Development of new molecular cloning techniques.

A major concern in many molecular genetic studies is the rapid and efficient generation of site-specific mutations. To this end, a method termed recombinant circle polymerase chain reaction (recombinant circle PCR or RCPCR) was developed. Recombinant circle PCR involves recombining products from two separate PCR amplifications: one reaction employs a pair of divergent primers from a position clockwise to the desired mutation site on a circular template; the second reaction employs a pair of divergent primers from a position counterclockwise to the desired mutation site on the same template. This method requires no restriction enzyme digestions and has proven to be very efficient.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08750-10 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

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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.)*

Cyclic AMP binds to its receptor protein CRP, and induces a conformational change (allosteric transition) in the protein. Cyclic AMP•CRP complex, and not free CRP, binds at or near many promoters of *E. coli* in a sequence-specific way and either represses or activates transcription. We are investigating the structural changes in CRP, induced by cAMP, that are required to make the former proficient for gene regulation and the points of contact between cAMP•CRP complex and RNA polymerase needed to modulate transcription. We have isolated and characterized several classes of mutations in the *crp* gene, which identify the amino acids participating in the cyclic AMP-induced allosteric shift in the protein. Various substitutions for Asp138 located at the apex of the hinge connecting the cAMP-binding domain to the DNA-binding domain of CRP cause biochemically distinct phenotypes. For example, Ala138 is defective in the cAMP-induced allosteric change, Asn138 functions in the absence of cAMP and Phe138 is defective for DNA binding. Thus, the hinge connecting the two domains of CRP is a key area for the transmission of the cAMP-induced allosteric change. In addition, this region is directly involved in DNA interaction. We are also studying the mechanism by which CRP activates transcription. We are analyzing this problem genetically by looking for CRP or RNA polymerase mutants that have lost the ability to interact with each other. In this regard, we have isolated mutations of *E. coli* which co-transduce with the β and β' genes encoding two RNA polymerase subunits. These mutations are being analyzed now. The mechanism by which a DNA-bound CRP communicates with RNA polymerase to activate it is also being studied. This is monitored by *in vitro* transcription using DNA templates with altered location or orientation of the CRP binding site with respect to the promoter or with a single stranded gap introduced between the CRP binding site and the promoter. We have found that OxyR, another transcriptional activator, is active only under non-reducing conditions.

Major Findings:

CRP:

We are studying two DNA binding proteins of Escherichia coli. The cAMP receptor protein (CRP) binds to DNA and can activate or repress transcription of many operons of *Escherichia coli*. The OxyR protein similarly can activate or repress transcription at different operons. Although seemingly functionally similar CRP and OxyR are different in many respects. Study of such a protein provides a model system for investigations of transcriptional activators and repressors, and provides information on protein-protein interactions, DNA-protein interactions, and protein conformation.

We are studying two aspects of CRP:

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

1. The amino acid at position 138, at the apex of the hinge, appears to be a key residue in the cAMP-induced allosteric change. By substitution of different amino acids for the wild type aspartic acid, we can obtain three classes of mutants: cAMP-independent (CRP*), cAMP-induced conformational change defective (Allo$^-$), or DNA binding defective. We have performed *in vitro* transcription using purified mutant protein on a supercoiled *lac* template and have shown that the defects in the conformational change or the DNA binding translate into activity defects for the proteins. Two of these mutant proteins - one CRP* and one Allo$^-$ - are being crystallized for x-ray diffraction analysis of their structures by Irene Weber of the Frederick Cancer Research Facility.

2. We are analyzing the cAMP-induced allosteric change by determining the minimal energy structures of CRP with two cAMPs bound, with one cAMP bound per dimer and without cAMP bound. We are using the CHARMM program for the determination of the structures. We have found there is no gross structural change of CRP when one or both cAMPS are removed, but several subtle very likely important changes. There are expected changes in the cAMP-binding pocket, and in the hinge. The relative positions of the DNA-binding $\alpha$-helices are altered in the absence of cAMP, providing a possible explanation as to why CRP can bind to DNA with high affinity only in the presence of cAMP.

B. How CRP activates transcription.

CRP may activate transcription either by contact with RNA polymerase at the promoter, or by altering the DNA conformation in the promoter. We feel the first is more likely, but to gain more evidence to distinguish between these two models, we have developed a plasmid for in vitro transcription where we can introduce a nick (single-stranded cut) between the CRP binding site and the promoter. If CRP activation is mediated through protein-protein interaction, transcription should occur despite the nick. If CRP activation is accomplished by altering the DNA in adjacent regions (including the

promoter), then transcription should not occur when a nick is present. We are also using these plasmids to alter the location and orientation of the CRP binding site with respect to the promoter.

We have taken a genetic approach to study this topic by isolating RNA polymerase mutants which are inactivable by CRP. We have found such a mutant that maps at least near one of the RNA polymerase genes. We are analyzing this mutation and have found suppressor mutations in the *crp* gene that allow the mutant to function again. These suggest that there may be at least a functional interaction between CRP and RNA polymerase.

OxyR

OxyR regulates the expression of genes involved in the repair of damage caused by oxidizing agents. We have found that the transcription activation protein OxyR functions for activation, *in vitro*, only under oxidizing conditions (in the presence of $O_2$). Under reducing conditions, the protein can bind to DNA, but does not activate transcription. Precisely how OxyR is activated remains unknown. In a study complementary to that on CRP, we are looking for RNA polymerase mutants which are no longer activable by OxyR.

References:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08751-10 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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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| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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


The gal operon of *E. coli* is negatively controlled by two regulatory proteins: Gal repressor and a newly discovered factor termed UIF (ultrainducibility factor). Both repressor and UIF act by binding to the same DNA sequences, $O_E$ and $O_I$, providing an example of cellular regulation by different factors acting through common DNA elements. While repressor inhibits *gal* transcription under normal conditions, UIF down regulates *gal* only in cells devoid of Gal repressor. Both proteins are inactivated by Galactose. Inactivation of UIF, in the absence of repressor, causes extreme derepression which is termed ultrainduction.

The mechanism of repression by Gal repressor has been studied in detail. Apparently, repressor does not act by sterically hindering RNA polymerase binding to promoter. Repression involves formation of a DNA-multiprotein complex of higher order structure, comprising DNA, one molecule of RNA polymerase, one molecule of cyclic AMP receptor protein and two molecules of repressor. In this complex, two repressor molecules, bound to two spatially separated sites, interact with each other looping out the intervening DNA segment to which the other proteins are bound. We have proposed that repression of transcription is achieved by a novel mechanism in which repressor freezes the bound RNA polymerase by a direct contact with the latter. The formation of DNA loop with a higher order structure facilitates such contact(s). We are currently investigating the various protein-protein contact sites and the state of transcription initiation (isomerization or promoter clearance) at which RNA polymerase is frozen.





PHS 6040 (Rev 1/84)

GPO 914-918

Major Findings:

In previous years we have shown that negative control of the *gal* operon is achieved by binding of Gal repressor to operators $O_E^G$-$O_I^G$ which are spatially separated by 114 bp and encompass the promoters. Repression requires interaction between the two DNA-bound repressors forming a DNA loop. We have also shown that Lac repressor can also repress the *gal* operon, provided that $O_E$ and $O_I$ have been converted to *lac* operator sequences ($O_E^L$ and $O_I^L$). DNA loop formation and its involvement in repression is demonstrated now by several experiments:

1. Lac repressor bound to $O_E^L$ and $O_I^L$ form the predicted DNA loop as observed under electron microscopy.

2. Lac repressor binds to $O_E^L$ and $O_I^L$ highly cooperatively with protein-protein interaction energy ($\Delta G_{EI}$) of -2KCals as determined by quantitative DNase foot-printing experiment as well as gel electrophoretic mobility retardation assay.

3. Lac repressor binding creates DNase I hypersensitive sites in the DNA segment encompassed by two repressor-occupied sites.

4. A mutant Lac repressor, which binds to DNA with affinity equal to that of wild type Lac repressor, does not form DNA loop as studied by electron microscopy.

5. The mutant repressor does not show cooperative binding with a $\Delta G_{EI}$ of less than -0.2 KCals and does not create DNase I hypersensitive sites.

6. The mutant repressor fails to repress the operon.

7. Gal repressor shows cooperative binding to $O_E^G$ and $O_I^G$ only at temperatures of 4°C or less. The cause of the inability of cooperative Gal repressor binding at higher temperatures are now being investigated.

We have proposed that RNA polymerase, cyclic AMP receptor protein and repressor bind to *gal* DNA simultaneously forming a higher order structure. The two operator-bound repressors interact with each other and with RNA polymerase inducing a conformational change in RNA polymerase that keeps the promoters inactive.The following observations are consistent with the above model of negative control of gene expression:

1. We have shown that DNA loop formation does not block binding of RNA polymerase and cyclic AMP receptor protein to their corresponding binding sites. We are currently investigating the simultaneous occupation of repressor, RNA polymerase and cyclic AMP receptor protein on DNA, the premier feature of the model, by *in vivo* foot-printing.

2. Gel mobility retardation assay has shown that both Gal and Lac repressors induce strong bending in DNA; such bending presumably helps further DNA-protein or protein-protein contact(s) in the formation of higher order structure proposed in the model.

3. We have isolated and have done preliminary characterization of a mutant Gal repressor, whose activity seems to be influenced by alpha subunit of RNA polymerase implicating an interaction between repressor and RNA polymerase.

We are currently conducting a thorough investigation of establishing and identifying contact sites between different protein molecules by isolating and characterizing contact defective mutants of repressor and RNA polymerase.

We have also discovered a second negative control element for *gal* operon. In a strain completely devoid of Gal repressor, the *gal* operon can be induced further by another three-fold. This phenomenon, termed ultrainduction, is epistatic to the presence of Gal repressor. Construction and characterization of gene and operon fusions between the *gal* control DNA segment and a reporter gene have shown that the second regulatory protein, termed UIF (ultrainducibility factor) acts by binding to the same two operator DNA sequences to which Gal repressor binds, thus illustrating that cellular regulation mediated by different factors can act through common sites. We have isolated and are currently characterizing mutants of the UIF.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08752-10 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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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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 |

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

A. The effects of $Zn^{+2}$ on the binding of 3,3',5-triiodo-L-thyronine ($T_3$) to the purified human $T_3$ nuclear receptor (h-TRβ1) was studied. In the presence of $Zn^{+2}$, $T_3$ binding to h-TRβ1 was reduced in a concentration- and temperature-dependent manner. The inhibition of $T_3$ binding was within minutes and fully reversible. Analysis of the binding data indicated that $Zn^{+2}$ is a non-competitive inhibitor of $T_3$ binding to h-TRβ1. The inhibitory activity of other divalent cations was found to be in the following order: $Co^{+2}>Cd^{+2}>Ni^{+2}>Zn^{+2}> Cu^{+2}>Mn^{+2}$. However, $Mg^{+2}$ and $Ca^{+2}$ had no effect. The rapid and reversible effects of $Zn^{+2}$ suggest that $Zn^{+2}$ may play a role in modulating hormone binding to h-TRβ1 *in vivo*.

B. Three monoclonal antibodies (mAbs) recognizing different domains of h-TRβ1 were developed. J51, J52, and J53 are specific against the β form of thyroid hormone nuclear receptor. J51 is human specific and J52 and J53 cross reacted with rat TRβ1 with ~1% and ~30% of the activity, respectively. The epitopes for J51 and J52 and J53 are located in the DNA binding domain, D domain and hormone binding domain, respectively. These mAbs are useful to understand the structure and the inter-relationship of domains of TRβ1 at the molecular level.

II. Regulation of thyroid hormone binding to its cytosolic binding protein (p58) by L-α-alanine. The human cytosolic thyroid hormone binding protein (p58) was recently shown to be a monomer of pyruvate kinase, subtype $PKM_2$, and have intrinsic pyruvate kinase activity. The effect of L-α-alanine on the binding and enzymatic activity of p58 was evaluated. Analysis of the competitive binding data indicated that alanine, at the physiological concentration, is a non-competitive inhibitor of $T_3$ binding to p58. Furthermore, alanine was found to be a "mixed" inhibitor of the substrate phosphoenol pyruvate. These data suggested that alanine might play a role in the regulation of $T_3$ binding to p58 *in vivo*.

Major Findings:

I. Molecular mechanism of thyroid hormone nuclear receptor-thyroid Hormone-DNA interaction.

A. Studies on the structure in relationship to the complex biological function of the $T_3$ nuclear receptor at the molecular level have been impeded by the lack of purified $T_3$ nuclear receptor. Recently, however, we have expressed large amounts of h-TRβ1 in *E. coli* and purified it to apparent homogeneity. The purified h-TRβ1 binds to $T_3$ and its analogs with affinity and specificity similar to those reported for the $T_3$ nuclear receptors identified in tissues and cultured cells. Using affinity labeling with [3',5'-$^{125}$I]-L-thyroxine, the hormone binding site was identified to be in a domain beginning at $Phe_{240}$ and ending at $Asp_{456}$. With the availability of large amounts of purified active receptor, it has become possible to address the question of how hormone binding activity could be modulated. We reasoned that for a hormone to be a functional regulator, flexibility of the structure in the receptor should exist for the hormone to bind and release rapidly as dictated by physiological signals. We, therefore searched for effectors which could possibly fulfill such a role.

Examination of the cDNA deduced amino acid sequence revealed a cluster of histidines and cysteines between $His_{400}$ and $Asp_{456}$ in the important region of the hormone binding domain. Analysis of the distribution patterns of histidines and cysteines identified several motifs which could bind $Zn^{+2}$. The effect of $Zn^{+2}$ on the binding of the purified h-TRβ1 was studied. In the presence of $Zn^{+2}$, $T_3$ binding activity was reduced in a concentration- and temperature-dependent manner. The apparent half-maximal inhibitory concentrations of $Zn^{+2}$ for $T_3$ binding were 8 μM, 200 μM and 500 μM at 37°C, 22°C, and 4°C, respectively. The inhibition of $T_3$ binding was rapid; at 22°C, the inhibition occurred within two minutes. The inhibition was reversible; upon addition of 5 mM EDTA, the $T_3$ binding activity was fully restored. Analysis of the binding data indicated that $Zn^{+2}$ is a non-competitive inhibitor of $T_3$ binding to h-TRβ1. Binding of $Zn^{+2}$ to h-TRβ1 resulted in a reduction of affinity for $T_3$ by ten-fold without affecting the number of $T_3$ binding sites. The effect on $T_3$ binding by other divalent cations was also evaluated; the inhibitory activity was found to be in the following order: $Co^{+2}>Cd^{+2}>Ni^{+2}>Zn^{+2}>Cu+^{2}>Mn^{+2}$. However, $Mg+^{2}$ and $Ca^{+2}$ had no effect. The rapid and reversible effects of $Zn^{+2}$ suggest that $Zn^{+2}$ may play a role in modulating hormone binding to h-TRβ1 *in vivo*.

B. The mRNAs of the various isoforms of c-*erb*As are expressed with different abundance in different tissues. In human, the mRNA of h-TRα1 is most abundantly expressed in brain and prostate. For h-TRβ1, it is most abundantly expressed in spleen and for h-TRα2, it is in brain. At the present time, however, it is unknown whether there are regulatory steps involved at the level of translation for the expression of c-*erb*A proteins. We, therefore, developed monoclonal antibodies specific for h-TRβ1 to study the tissue distribution and possible translational regulation of h-TRβ1.

The purified h-TRβ1 was used to produce monoclonal antibodies. Three hybridomas, secreting mAb J51, J52 and J53, were isolated. All of these mAbs recognized h-TRβ1. J51 and J52 belong to the $IgG1_{-\kappa}$ subclass; J53 is an IgM. To evaluate cross-reactivity with other classes of c-*erb*As, the three mAbs were used to immunoprecipitate the *in vitro* translation products of human TRα1, TRα2, rat TRβ1, TRα1 and TRα2. None of these three mAbs reacted with human or rat

TRα2 or TRα1. J51 did not react with rat TRβ1, but J52 and J53 reacted with rat TRβ1 with ~1% and ~30% of the activity of h-TRβ1, respectively. To localize the epitopes in the h-TRβ1 molecule, [$^{35}$S]methionine-labeled and truncated h-TRβ1 continuing the hormone binding domain E ($Lys_{235}$-$Asp_{456}$), domain D ($Ala_{170}$-$Asp_{456}$), part of domain D ($Lys_{201}$-Asp-$_{456}$; $Asp_{211}$-$Asp_{456}$) or the DNA binding domain C ($Glu_{100}$-$Asp_{456}$) were expressed in *E. coli* and purified. Immunoprecipitation of the above truncated h-TRβ1 with mAbs indicated that the epitopes for J51, J52 and J53 were located in the DNA binding domain C ($Glu_{100}$-$Met_{169}$), part of domain D ($Ala_{170}$-$Gln_{200}$) and the hormone binding domain E ($Lys_{235}$-$Asp_{456}$), respectively. $GH_3$ cells were metabolically labeled with [$^{35}$S]methionine and nuclear extracts were prepared. Using J52, two proteins with the molecular weights of 62K and 46K were specifically immunoprecipitated. The 62K was the h-TRβ2 and the 46K protein could be its degradation product. Furthermore, using immunofluorescence method, a nuclear localization of the antigen for J52 was visualized. These results indicated J52 recognized the endogenous T3 nuclear receptor and would be a useful mAb to study tissue distribution, translational and hormonal control of the $T_3$ nuclear receptor.

II. Regulation of thyroid hormone binding to the human cytosolic thyroid hormone binding protein (p58). - Effects of L-α-alanine.

We have recently cloned a cDNA for a human cytosolic thyroid hormone binding protein (p58). Sequence analysis indicated that p58 is a monomer of the pyruvate kinase (PK), subtype $M_2$. p58 has ~5% of the enzymatic activity of the tetrameric PK. Binding of $T_3$ resulted in the inhibition of its intrinsic pyruvate kinase activity. Conversion of p58 to the tetramer is reversible and is under the control of fructose 1,6-bisphosphate (Fru-1,6-$P_2$). The conversion is inhibited by $T_3$ in a dose-dependent manner. Since pyruvate kinase is a key enzyme in regulating cellular ADP, ATP and pyruvate, p58 was postulated to be involved in mediating some of the cellular metabolic effects induced by thyroid hormones.

L-α-alanine has been shown to inhibit the kinase activity of $PKM_2$. This inhibition is competitive with respect to the substrate phosphoenol-pyruvate. $PKM_2$ was found to increase in many tumors and the amount of $PKM_2$ correlated well with the degree of inhibition by alanine. Therefore, alanine conceivably could be a regulator for PK activity *in vivo*. However, the effect of alanine on the monomer (p58) is unknown because the monomer in its native form has not been isolated and purified until recently. We, therefore, examined whether alanine also binds to p58. Furthermore, we also evaluated its effect on the conversion of p58 to the tetrameric PK.

Examination of the PK activity of A431 cytosolic extracts in the presence of increasing concentration of alanine indicated that alanine is an inhibitor of PK. The extent of inhibition, however, depends on the concentration of Fru-1,6-$P_2$. Below 0.1 mM of Fru-1,6-$P_2$, alanine inhibits nearly all of the PK activity. When Fru-1,6-$P_2$ is higher than 1 mM, the inhibitory effect of alanine is prevented. Evaluation of the effect of alanine on the PK activity of the monomeric p58 indicated that increasing concentrations of alanine were found to decrease, but not completely abolish the PK activity. Initial rate measurements on the hydrolysis of the substrate phosphoenol pyruvate in the presence of increasing concentrations of alanine showed that alanine is a "mixed" inhibitor to phosphoenol pyruvate in the enzymatic activity of p58.

Analysis of the competitive binding data indicated that alanine, at the physiological concentration, is a non-competitive inhibitor of $T_3$ binding to the monomer p58. The following affinity constants were obtained: $K_1 = 3.1 \times 10^5$ $M^{-1}$, $K_2 = 7.3\ 10^7\ M^{-1}$ and $K_3 = 1.9 \times 10^7\ M^{-1}$, where $K_1$ is the affinity constant of binding alanine to p58, $K_2$ is the affinity constant of binding $T_3$ to p58 and $K_3$ is the affinity constant of binding $T_3$ to p58 with bound alanine.

The present study showed that alanine is a "mixed" inhibitor of p58 by either binding to free p58 or p58-PEP complex. Thus, alanine could regulate PK activity not only at the level of tetrameric PK, but also at the level of monomeric p58. The binding of physiological concentration of alanine to p58 led to the reduction of affinity in the binding of $T_3$ to p58. Even though the precise physiological consequence of binding to T3 to p58 is unknown at the present time, it is reasonable to postulate that alanine would play a regulatory role to that function, once it is identified.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08753-08 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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:

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LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Biology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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| 12.0 | 9.0 | 2.0 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

*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 TGFα, IL2, IL4, IL6, 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. Such molecules may be useful in the elimination of tumor cells or other cells responsible for several human diseases. Mutational analysis has been used to delineate the function of the various domains of PE. Mutations at positions 276 and 279 of PE block cytotoxicity probably by preventing proteolytic processing of PE. Mutations at the carboxyl end of PE abolish cytotoxicity by interfering with translocation of a processed form of PE. The activity of CD4-PE40 has been further evaluated and the chimeric toxin has been found to block the spread of HIV *in vitro* and to be active against several different strains of HIV as well as SIV.

Other Personnel:

| | | |
|---|---|---|
| S. Adhya | Chief, DGS | LMB, NCI |
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| C. Siegall | Biotechnology Fellow | LMB, NCI |
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| T. Prior | Visiting Fellow | LMB. NCI |
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| S. Seetharam | Special Volunteer | LMB, NCI |
| M. Gallo | Microbiologist | LMB, NCI |
| E. Lovelace | Biologist | LMB, NCI |
| J. Batra | Visiting Associate | LMB, NCI |

Major Findings:

*Pseudomonas* toxin (PE) is a single chain bacterial toxin which has been genetically modified so that it selectively kills cells which bear specific surface receptor molecules. Using an *E. coli* expression system, various mutant forms of PE have been prepared in order to determine which portions of the molecule are required for optimal cell killing activity. These studies have shown that deletion of all of domain I (amino acids 1-253) yields a molecule (PE40) which has very low cytotoxic activity, because it no longer binds to the PE receptor present on all cells. Therefore, domain I has been replaced with different cell targeting molecules to create novel chimeric toxins which specifically kill different types of cells. These include TGFα-PE40 which kills many cancer cells derived from epithelia containing EGF receptors, IL2-PE40 which kills cells including leukemia cells with IL2 receptors, as well as activated T cells, IL4-PE40 which kills cells with IL4 receptors, and IL6-PE40 which kills certain myeloma and hepatoma cells. To kill HIV infected cells, domain I was replaced with CD4 to create CD4-PE40 which was shown to kill HIV infected cells by binding to the envelope protein GP120 present on the surface of cells producing different strains of the AIDS virus.

New Cytotoxic Molecules:

Previously, antibody toxin fusion proteins were made by chemically attaching modified forms of *Pseudomonas* toxin to antibodies. We have now made a chimeric toxin composed of two antibody variable domains linked to *Pseudomonas*. The first molecule of this type we made is anti-Tac(Fv)-PE40. It binds to the p55 subunit of the IL2 receptor present on human and monkey cells; the chimeric toxin is very active in killing these types of cells. Anti-Tac(Fv)-PE40 has recently been found to kill many leukemic cell lines expressing the IL2 receptor as well as human and primate activated T cells. This agent is being developed in collaboration with Protein Design Labs for the possible treatment of allograft rejection and of leukemias containing IL2 receptors on their surface. To simplify the construction of single chain antibody toxin fusion proteins, a new method was developed to directly clone the antibody variable region DNAs from RNA. To do this, RNA was prepared from hybridoma OVB3 and a chimeric toxin created using DNA primers and PCR to generate cDNAs encoding the antibody regions; the cDNAs were then cloned into a PE40 expression vector. This method was used to create a new chimeric toxin OVB3-PE which specifically kills cells bearing the OVB3 antigen. Several other chimeric toxins have recently been made using this technology.

Mechanism of Action of *Pseudomonas* Toxin:

To understand how *Pseudomonas* toxin works and to devise more active and specific chimeric toxins, genetic and biochemical experiments have been carried out on the toxin. It was previously shown that domain I and particularly lysine 57 in domain I was important for cell binding. To determine which amino acids in domain II are required for toxin action, we began by changing each arginine residue to a glycine or other residue. These experiments showed that arginine 276 and arginine 279 were very important for toxin action. Mutation at either of these positions greatly diminished the activity of the toxin. Biochemical studies by M. Ogata and D. FitzGerald have shown that in the target cell the wild type toxin is cleaved near arginine 279, but the mutant toxins are not cleaved at this site. This processing step generates a 37 kd fragment which is translocated into the cytosol. Thus, proteolytic cleavage near arginine 279 is required for intoxication and cell death.

Another region required for the activity of *Pseudomonas* toxin lies at the carboxyl end. It has been shown that the last five amino acids of PE, REDLK, comprise a recognition sequence required for intoxication. Deletion of this region leads to non-toxic molecules which still have ADP-ribosylation activity. Mutations in this region still allow *Pseudomonas* exotoxin to be processed to the 37 kd fragment but no translocation of the fragment occurs. The REDLK sequence can be replaced by KDEL which is the canonical endoplasmic reticulum sequence found at the end of proteins that are destined to remain within the endoplasmic reticulum of eucaryotic cells. Thus *Pseudomonas* exotoxin (and some other toxins) use a biochemical pathway containing the same elements that are used to retain proteins within the endoplasmic reticulum. The nature of this pathway is currently under investigation.

Because intoxication with *Pseudomonas* exotoxin is blocked by agents that raise intravesicular pH, it is thought that the acidic pH found in endocytic vesicles is important for *Pseudomonas* exotoxin action. To study the effect of pH on the molecule, whole *Pseudomonas* exotoxin and fragments of the toxin made in *E. coli* were exposed to acidic pH and the ability of these fragments to enter a lipid environment measured. Unexpectedly, all three of the domains of *Pseudomonas* exotoxin were found to become soluble in lipids when the pH was lowered to 5 or below. Thus, a pH induced change induced in *Pseudomonas* exotoxin conformation probably generates a fragment which binds tightly to lipids as part of the translocation step. Domain Ib is a small portion of the toxin that lies between amino acids 364 and 400. To define the role of domain Ib, several deletion mutants were created. It was found that almost all of domain Ib (amino acids 365 through 380) could be deleted without a significant loss of activity. This region can be used to insert foreign (non-PE) sequences for introduction into the cytosol. Finally, the amino acids required for ADP-ribosylation activity have been determined by making deletions at the 5' and 3' end of this domain. These studies have shown that the ADP-ribosylating domain begins around amino acid 400 and ends shortly after amino acid 600.

Further Studies on the Activities of Chimeric Toxins:

The activity of TGFα-PE40 has been tested on many different types of tumor cell lines. It has been found to be toxic to many ovarian cancer cell lines, many kidney cancer cell lines, many epidermoid carcinomas, and several hepatomas. Several of these cell lines are extremely sensitive to the

chimeric toxin suggesting they may be selectively killed in immunodeficient mice bearing tumors derived from these cell lines. Such studies are currently in progress. Several agents have been evaluated which appear to alter the processing of the toxin and enhance its activity. Several of these are derivatives of verapamil. One of these, D-792, is very active in cell culture enhancing the activity of both ricin A chain and *Pseudomonas* toxin containing immunotoxins.

Animal Experiments:

Lys-PE40 was coupled to an antibody that reacts with the human transferrin receptor and this agent tested against human tumors growing in immunodeficient mice. Antitransferrin-LysPE40 was found to completely suppress the growth of an epidermoid carcinoma cell line, A431, and to cause regression of large rapidly growing A431 tumors in nude mice. This study shows that a chimeric toxin with a molecular weight of 200,000 can penetrate large tumors and cause tumor cell death. It is envisioned that immunotoxins will be used with chemotherapy. To evaluate the activity of such agents, immunotoxin OVB3-PE directed against ovarian and some colon tumors was tested in combination with cyclophosphamide against a human colon carcinoma, HT29. The combination of OVB3-PE and cyclophosphamide was clearly better than either agent given alone.

IL2-PE40:

To evaluate the use of chimeric toxins directed at the IL2 receptor, several disease models have been investigated. One of these is experimental autoimmune uveoretinities (EAU) which is induced in Lewis rats by active immunization with retinal S-antigen. Rats with EAU were treated with IL2-PE40 beginning 7-10 days after immunization. The chimeric toxins specifically suppressed development of the disease and also suppressed the development of IL2 receptor bearing T cells in lymph nodes near the antigen injection site. These studies suggest the agents like IL2-PE40 may be useful in certain autoimmune diseases.

HIV and AIDS:

In collaboration with E. Berger and B. Moss, Lab. of Viral Diseases, NIAID, we have constructed the chimeric toxin CD4-PE40 directed at HIV infected cells. CD4-PE40 has now been tested against cells expressing GP120 from several different HIV isolates. CD4-PE40 has been found to be active against all these isolates including SIV. In addition, CD4-PE40 has been shown to suppress the spread of HIV when a small number of infected cells are mixed with a large number of uninfected cellls. Several different types of CD4-PE containing molecules have been made to determine if any are more active than CD4-PE40. A CD4-PE40 molecule ending in KDEL has been found to be more active. In addition, mutations have been introduced into CD4-PE40 which could possibly affected the survival of the molecule in animals. However, no significant increase in half-life as yet been obtained.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 08754-07 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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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| Co-PI: | M. M. Gottesman | Chief, Laboratory of Cell Biology | NCI |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Resistance to multiple drugs is major impediment to the successful chemotherapy of human cancers. One mechanism of multidrug-resistance is the expression of a 170,000 dalton energy-dependent drug efflux pump (P-glycoprotein or the multidrug transporter, the product of the human *MDR*1 gene) which confers resistance to colchicine, adriamycin, vincristine, vinblastine, puromycin, and actinomycin D. Several lines of investigation concerning the multidrug transporter have been pursued: 1) Evidence for ATP-dependent transport activity of P-glycoprotein in vesicles and across epithelial monolayers has been obtained; 2) Novel expression vectors which utilize a full-length *MDR*1 cDNA as a dominant selectable marker are used to introduce and amplify non-selectable genes in cultured cells and *MDR*1 retroviral vectors have been developed; 3) Many human tumors express *MDR*1 RNA, and this expression may predict drug-resistance in some cancers, and correlate with the development of drug-resistance in others; 4) Transgenic mice have been constructed in which the human *MDR*1 mRNA is expressed in the bone marrow at levels comparable to those found in human tumors. This level of *MDR*1 expression is sufficient to confer resistance to leukopenia induced by MDR drugs; 5) Increased expression of *MDR*1 RNA has been demonstrated in regenerating rat liver, in cultured rodent cells after exposure to chemotherapeutic agents, and in kidney cancer cells after heat shock; 6) The intron-exon structure of the human *MDR*1 gene has been determined and supports a model of independent evolution of the two halves of the multidrug transporter. Similarly, a deletion analysis of the *MDR*1 cDNA is consistent with important functions being contributed by both the amino and carboxy-terminal halves of the molecule as is a study demonstrating photoaffinity labeling of both halves of P-glycoprotein by the hydrophobic drug $^{3}$H-azidopine; and 7) Evidence that P-glycoprotein acts by pumping hydrophobic drugs out of the lipid bilayer has been obtained.





GPO 914-918

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Major Findings:

1. Vesicles which transport $^{3}$H-vinblastine have been prepared from human multidrug-resistant KB cells. Analysis of the nucleotide requirement for this transport shows that ATP > GTP and that UTP, CTP, and NAD do not support transport.

2. Epithelial monolayers formed from MDCK cells into which the human *MDR*1 cDNA has been introduced by retroviral infection transport vinblastine, actinomycin D, vincristine, daunorubicin and verapamil. These data support a model in which P-glycoprotein expression in kidney, liver and intestine results in excretion into urine, bile and intestinal lumen of toxic hydrophobic compounds.

3. A unique cloning site downstream from an SV40 promoter has been introduced into a retroviral vector in which the *MDR*1 cDNA is under control of a Harvey sarcoma virus promoter. An IL-2 receptor cDNA has been introduced into this vector. After transfection into NIH 3T3 cells and selection for colchicine-resistance conferred by the *MDR*1 cDNA, IL-2 receptor is expressed in the recipient cells. Selection for increased colchicine-resistance results in high levels of IL-2 receptor expression. Thus, this vector can be used to introduce and amplify expression of non-selectable cDNAs.

4. The human *MDR*1 gene has been introduced via retroviral infection into mouse bone marrow *in vitro*. Infected GM-CSF show up to 30% colchicine and vinblastine-resistant colonies. Expression of the human cDNA persists for several weeks after transplantation of infected marrow into mice, suggesting that this may be a promising approach for gene therapy.

5. A protein chimera between the multidrug transporter and adenosine deaminase (ADA) has been produced by fusing an ADA cDNA to the 3'-end of an *MDR*1 cDNA in a retroviral vector. When packaged as a retrovirus, this can be used to infect cultured NIH and KNIH cells which are colchicine-resistant and express ADA activity. Tumors derived from MDR-ADA infected KNIH cells stably express the functional chimera which can be detected in cell cultures derived from tumors grown in nude mice. These experiments demonstrate the feasibility of using the MDR-ADA retrovirus for gene therapy experiments.

6. We have continued to analyze human cancers for expression of *MDR*1 mRNA with a sensitive and quantitative slot blot technique. The data base of human cancers has been expanded to over 500 different tumors. A high percentage of kidney, adrenal, colon and liver cancers express *MDR*1 mRNA. *MDR*1 RNA is expressed less commonly in leukemias and lymphomas at first presentation, but when expressed in acute non-lymphocytic leukemia it predicts poor response to chemotherapy. High levels of *MDR*1 RNA are found in many chronic lymphocytic leukemias in blast crisis, and in recurrent ovarian cancer and neuroblastoma, which correlates well with failure of these tumors to respond to chemotherapy with MDR drugs.

7. The human *MDR*1 cDNA has been expressed in transgenic mouse bone marrow and spleen under control of a chicken β-actin promoter. RNA levels comparable to those found in human cancers confer resistance to leukopenia induced by many different MDR drugs. Verapamil, an inhibitor of the multidrug transporter, sensitizes the bone marrow of these MDR-transgenic mice to the toxic effects of MDR drugs. This MDR transgenic mouse model will be useful for testing dose intensification chemotherapeutic regiments, the efficacy of inhibition of the multidrug transporter, and therapies based on gene transfer of the *MDR*1 cDNA followed by selection of resistant bone marrow.

8. Model systems in which levels of *mdr* RNA are regulated have been developed. In regenerating rat liver, increases in *mdr* RNA occur without proportionate increases in transcription, suggesting that RNA levels in this system are regulated by mRNA stabilization. In rodent cells in culture, treatment with chemotherapeutic drugs also raises *mdr* RNA levels, but again, increases in transcription by nuclear run-off assays cannot be demonstrated. Most human cells do not show regulation of *MDR*1 RNA levels; however, in response to heat shock, up to 10-fold increases in *MDR*1 RNA are seen in renal adenocarcinoma cells without concomitant increases in transcription. These results suggest that in rodent and human cells *mdr* RNA levels are regulated by different factors, and that mRNA stabilization may play an important role in regulating these levels.

9. The complete *MDR*1 gene has been isolated on a set of overlapping cosmids and lambda phage clones. The positions of all the introns and exons has been determined. Despite the superficial similarities in structure and sequence of the amino and carboxy-terminal halves of P-glycoprotein, the positions of the introns in these two halves are different, suggesting independent evolution, and, possibly, independent function.

10. Structure-function relationships in the *MDR*1 gene have been analyzed by generating deletion, substitution, and insertion mutations in an *MDR*1 cDNA. Only small deletions in the carboxy-terminus are tolerated; all substantial deletions or substitutions result in loss of function in both the amino and carboxy-terminus. These results demonstrate the necessity for two functioning halves of P-glycoprotein, and help define the limits for allowable alterations in this molecule.

11. $^{3}$H-azidopine, an inhibitor of the function of P-glycoprotein which can be used as a photoaffinity label of the transporter, has been used to analyze which regions of P-glycoprotein interact with the drug. Using a combination of proteolytic digestion and antibody precipitation, regions in both the amino and carboxy-termini of P-glycoprotein have been shown to be labeled by $^{3}$H-azidopine, consistent with an important function for both halves of the molecule.

12. Specific antisera against several different domains of P-glycoprotein have been raised in rabbits. Antigens were prepared after expression of partial *MDR*1 cDNAs in *E.coli* using an inducible T7 expression system.

13. A baculovirus vector expressing P-glycoprotein has been constructed and used to infect insect cells. These cells produce large amounts of P-glycoprotein which binds hydrophobic drugs, but is not glycosylated. This recombinant P-glycoprotein may prove useful for physical or functional studies of the transporter.

14. The non-specific hydrophobic membrane label $^{125}$I-INA (iodinated naphthalene azide) detects P-glycoprotein as a major transmembrane protein in drug-resistant cells. INA can be exploited as a specific label for P-glycoprotein if it is activated by energy transfer from the chromophore (and transport substrate) doxorubicin. By energy transfer from doxorubicin or rhodamine 123, INA labels many proteins in drug-sensitive cells, but predominantly P-glycoprotein in drug-resistant cells. This result suggests that P-glycoprotein is able to extract doxorubicin and rhodamine 123 from the lipid bilayer of resistant cells and supports a model of P-glycoprotein as a "hydrophobic vacuum cleaner."

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08756 03 LMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

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| | M. M. Gottesman | Chief, Laboratory of Cell Biology | NCI |
| | M. Willingham | Chief, Ultrastructural Cytochemistry Sect. | LMB, NCI |
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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 use of transgenic mice constitutes a powerful approach to the study of gene function and regulation. We are using this technology to investigate several important aspects of cancer progression: the ability of malignant cells to evade destruction by chemotherapy, and the role various growth factors and oncogenes play in the initiation and development of the transformed phenotype.

Transgenic mice bearing a human gene (*MDR*1) encoding a multidrug transporter (P-glycoprotein) capable of conferring multidrug resistance have been generated. As a direct result of expression of the human *MDR*1 gene in the normally drug-sensitive bone marrow of these transgenic animals, the marrow becomes resistant to the cytotoxic effects of a number of commonly used chemotherapeutic drugs. Since bone marrow suppression is a major drawback to the use of chemotherapy, these transgenic mice are being used as a model to test the efficacy of new chemotherapeutic agents, and to determine if elevated and/or more toxic doses of drugs can cure previously unresponsive cancers.

Transforming growth factor alpha (TGFα), epidermal growth factor (EGF) and related peptides stimulate cellular proliferation by binding to, and activating the tyrosine kinase of, the EGF receptor. Perturbation of this signal transduction pathway can lead to the transformation of cells in culture, and has been implicated in the development of human cancer. To elucidate the role of EGF receptor and its ligands in various physiologic and pathologic processes, transgenic mice were made bearing DNA fragments encoding either the human EGF receptor or the human TGFα gene. Analysis of EGF receptor and TGFα transgenic mice have led to significant discoveries about spermiogenesis and mammary gland development. Furthermore, we have found that overexpression of TGFα induces hepatocellular carcinoma, mammary adenocarcinoma, and a lesion resembling chronic pancreatitis.

Major Findings:

Transgenic mice are made in this laboratory by the microinjection of purified DNA fragments into a pronucleus of single-cell mouse embryos derived from either outbred CD1 or inbred FVB mice. Embryos surviving microinjection are implanted into pseudopregnant CD1 foster mothers. Founder transgenic mice are identified by Southern blot analysis of genomic DNA isolated from tails of two-week old offspring. Mice possessing transgenes are expanded to establish heterozygotic and homozygotic lines. Using this approach, a number of transgenic mouse lines have been established to pursue questions about the function and regulation of specific genes.

A human gene (*MDR*1) 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 08754-07 for details). Transgenic mice were generated carrying a DNA fragment consisting of the chicken β-actin gene promoter upstream of a human *MDR*1 cDNA. Human P-glycoprotein RNA and protein were detected in the transgenic bone marrow, which is normally exquisitely sensitive to chemotherapy. These transgenic animals have now been exposed to a plethora of chemotherapeutic agents to determine if the bone marrow becomes drug resistant in these transgenic mice, including daunomycin, doxorubicin, taxol, vincristine and vinblastine. The data clearly demonstrate that relative to control, the transgenic bone marrow does indeed become resistant to the expected drug-induced leukopenia, and that bone marrow suppression is either greatly ameliorated or eliminated, depending on the specific drug and dosage. Furthermore, drug resistance in these transgenic mice is defeated by the administration of reversing agents, such as verapamil. These mice should prove to be extremely useful as an *in vivo* model system to test the efficacy of novel chemotherapeutic drugs or regimens, and of agents which inhibit activity of the multidrug transporter.

We and others have shown that overexpression or inappropriate expression of the EGF receptor or its ligands can contribute to the appearance of the transformed phenotype in cultured cells. To determine the *in vivo* consequences of perturbing this signal transduction pathway, we made transgenic mice using TGFα and EGF receptor genes. In one series of experiments, a DNA fragment was used containing the mouse metallothionein-1 promoter upstream of the human TGFα cDNA. Mice bearing this transgene expressed human TGFα RNA and protein in a number of tissues, including the liver, pancreas and breast. Furthermore, elevated levels of TGFα were detected in the blood and urine of transgenic mice. TGFα transgenic mice were characterized by the appearance of a number of specific lesions. A high incidence of hepatocellular carcinoma was found in male transgenic mice over ten months old, multiparous female mice became susceptible to mammary adenocarcinoma, and all mice developed severe interstitial fibrosis and ductular metaplasia of the pancreas. Furthermore, abnormal development of the mammary gland was noted in these TGFα transgenic animals. The specific role of TGFα in the appearance of these lesions is presently being studied.

In another series of experiments, transgenic mice were made bearing a DNA fragment containing the chicken β-actin promoter upstream of the human EGF receptor cDNA. In one unique line of mice expression of the human EGF receptor was found to be restricted to the mouse testis, suggesting that the transgene integrated near or into a gene containing a testis-specific enhancer element. Immunohistochemical analysis demonstrated that the human EGF receptors appeared postmeiotically at the round-spermatid stage of spermiogenesis. Approximately 300,000 receptors were produced per cell. At

the end of spermiogenesis, these receptors were found to be sequestered in the residual bodies, and excluded from the sperm by some compartmentalization mechanism. Ultimately, the transgenic receptors were absorbed by the Sertoli cells. Evidence suggested that the EGF receptor transgene had integrated into, and inactivated, a necessary testicular gene. Two out of two homozygote male mice were sterile, while a homozygote female was fertile. Furthermore, sperm motility was severely impaired in the homozygote male animals. Experiments are underway to elucidate the human EGF receptor compartmentalization mechanism, and the nature of the inactivated testicular gene.

Transgenic mice have also been made using DNAs encoding growth factors related to EGF. The gene encoding one such peptide (Int3) is associated with mammary tumor activation by the mouse mammary tumor virus (MMTV). Preliminary evidence suggests that in transgenic mice the virally activated form of this peptide causes mammary and salivary adenocarcinomas, and interferes with the ability of male animals to produce functional sperm and female animals to efficiently lactate. The relationship between Int3 and these dramatic phenotypic manifestations is being examined.

In a related project intended to determine the transcriptional mechanism by which cellular oncogenes like the EGF receptor gene are regulated, the receptor gene promoter was isolated and characterized by protein-binding assays, mutagenesis analysis, and a cell free transcription system (see Annual Report Number Z01 CB 08000-20 for details). To validate transfection assays and to determine how the promoter functions in vivo, DNA fragments containing all elements known to be necessary for optimal gene activity were placed upstream of the chloramphenical acetyltransferase (CAT) reporter gene, and the resulting hybrid DNAs used to make transgenic mice. Surprisingly, in virtually every line of mice made from this construct (nine lines) the EGF receptor gene promoter was most active in the thymus, as determined by CAT activity in the tissues of the transgenic mice. Cell separation techniques were used to demonstrate that the promoter was only active in the thymic epithelial cells, and not in the thymocytes. Based on these intriguing results, we are now examining the role of the EGF receptor in thymic epithelial function and thymocyte differentiation.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08757-03 LMB

PERIOD COVERED

October 1, 1989 to September 20, 1990

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

Development of Immunotoxins for Cancer

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

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

COOPERATING UNITS *(if any)*

J. Pearson, FCRF — J. Shiloach, BioPilot Plant, LCDB, NIDDK

D. Longo, FCRF — R. Heinrikson, Upjohn Company

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Ultrastructural Cytochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.0 | 3.0 | 1.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

*Pseudomonas* Exotoxin (PE) incubated with cells was proteolytically cleaved to produce an N-terminal 28 kDa and a C-terminal 37 kDa fragment, which is composed of a portion of domain II and all of domain III (the ADP-ribosylating domain). Cleavage required the presence of $Arg^{276}$, $Pro^{278}$, and $Arg^{279}$. Cleavage was evident at 10 minutes after toxin addition and endosome preparations contained the processed fragments. Cytosol from toxin treated cells was greatly enriched in the 37 kDa fragment which is essential for toxicity since mutant PE molecules that do not produce this fragment or deliver it to the cytosol, fail to kill cells. Cell membranes were shown to have a calcium-dependent protease that cleaved PE with a pH optimum of 5.5. This activity was solubilized with 1% NP-40. PE40 was cleaved between domains II and III by two retroviral proteases. HIV protease hydrolyzed the Leu-Leu bond at position 389-390 while the corresponding AMV protease cleaved at the Asp-Val bond at position 406-407.

Major Findings:

Intracellular Proteolytic Processing of Pseudomonas Exotoxin (PE).

To kill cells, PE is internalized via coated pits and endocytic vesicles. However, before it reaches the cell cytoplasm and shuts down protein synthesis, PE must be proteolytically cleaved to produce two fragments; one is an N-terminal fragment of 28 kDa and the other a C-terminal fragment of 37 kDa. The N-terminal fragment contains the toxin's binding domain while the C-terminal fragment has the ADP-ribosylating activity and most of the translocating domain. Initially the two fragments are joined by the disulfide bond linking cysteines 265 and 287. When the fragments are reduced the C-terminal fragment is freed to translocate to the cytoplasm and ADP-ribosylate elongation factor 2. When cytoplasm fractions were prepared, the C-terminal fragment was found there highly enriched compared to native toxin or the 28 kDa fragment. The translocation of the 37 kDa fragment was later confirmed by showing that non-toxic PE mutants failed to deliver this fragment to the cytoplasm.

Analysis of the proteolytic process has revealed that $Arg^{276}$, $Pro^{278}$ and $Arg^{279}$ are required to be present to allow the generation of the appropriate 28/37 kDa pair of fragments. These fragments were not produced when $Arg^{276}$ was changed either to glycine or lysine. Similar results were seen when $Arg^{279}$ was changed to the same two amino acids. When $Pro^{278}$ was changed to methionine, proteolysis was reduced greater than ten-fold. Other substitution mutations introduced between residues 274-284 did not interfere with proteolysis. Thus the motif Arg, X, Pro, Arg is required to make PE an appropriate substrate for cell-mediated proteolysis.

Cells were treated with compounds known to alter certain aspects of endocytosis and intracellular traffic and then assayed for their ability to cleave PE. When cells were incubated with sodium azide and 2-deoxyglucose, compounds known to prevent endocytic uptake, there was no cleavage of PE. This provided indirect evidence that proteolysis occurred intracellularly. This was confirmed by fractionating cells and preparing endosomes and showing that cleaved PE could be recovered from these organelles. Interestingly, cleavage was inhibited when cells were treated with monensin but not with ammonium chloride. Both monensin and ammonium chloride protected cells from the toxic effects of PE. While monensin can prevent the generation of the active 37 kDa fragment, ammonium chloride must protect cells by some other mechanism.

Identification of important residues in the C-terminal fragment that mediate PE toxicity.

The generation of the C-terminal 37 kDa fragment is not sufficient by itself to mediate translocation to the cytoplasm. Three regions in this fragment were identified as being necessary for translocation - either directly or indirectly. When trytophan 281 was changed to methionine, high quantities of the 37 kDa fragment were generated but cells were not killed. When the C-terminal sequence RDELK was changed or deleted, likewise, there was a normal amount of the 37 kDa fragment made but none reached the cytoplasm. Finally, the result was the same when alanine at either position 339 or 343 was changed to glutamine.

Preliminary characterization of the cellular protease that mediates PE cleavage.

When cells were homogenized and separated into a high speed supernatant and high speed pellet, the protease was recovered in the pellet. When membranes from the pellet fraction were incubated with radioactive PE, it was found that proteolysis

was calcium dependent but not ATP dependent. When PE and membranes were mixed at a variety of pHs, from 5.0 to 7.5, the greatest amount of the 28/37 kDa product. was generated at pH 5.5. It remains to be determined whether this reflects a conformational change in the toxin or the pH optimum of the protease. Membranes were treated with either high salt or detergent. Extraction of the protease was seen with 1% NP-40 but not with NaCl up to 1.0 M. This suggests that the protease is an integral membrane protein.

PE40 as substrates for retroviral proteases.

Virally encoded proteases from human immunodeficiency virus (HIV) and avian myeloblastosis virus (AMV) were compared in their ability to hydrolyze Lys PE40. The HIV protease hydrolyzed two peptide bonds in Lys PE40, a Leu-Leu bond in the interdomain region (residues 389-390) and a Leu-Ala bond in a non-structured region three residues from the N-terminus. Neither of these sites was cleaved by the AMV enzyme. Instead, hydrolysis occurred at the Asp-Val (residue 406-407) bond in another part of the $NH_2$-terminal region of domain II. Synthetic peptides corresponding to these cleavage sites were hydrolyzed by the individual proteases with the same specificity displayed toward the protein substrate. However, peptide substrate for one protease were neither substrates nor competitive inhibitors for the other.

Publications:

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SUMMARY STATEMENT

LABORATORY OF BIOCHEMISTRY

DCBDC, NCI

OCTOBER 1, 1989 TO SEPTEMBER 30, 1990

INTRODUCTION

Despite severe budget restrictions and a decrease in personnel this has been an extremely good year for the Laboratory of Biochemistry. With the cloning, purification and characterization of several factors involved in the regulation of gene expression and DNA replication, the demonstration of a LINE-1 encoded protein in human cells and the identification of the role of prothymosin $\alpha$ in cell division, to name some of the highlights, the laboratory's achievements have remained at the forefront of several fields of central interest in biochemistry and molecular biology.

The outstanding achievements of two members of the laboratory have been recognized by the creation of two new sections: Dean Hamer, a leader in the field of the regulation of gene expression, is the chief of the Section on Gene Structure and Regulation. Shelby Berger, an accomplished molecular biologist and protein chemist, is the chief of the Section on Genes and Gene Products. Upon the unanimous recommendation of the Section Chiefs, a nation-wide search was initiated to fill the vacancy left after the retirement of Warren Evans. It is encouraging that many first class young applicants have already applied for this position.

Carl Wu, who has been teaching a biology course at Harvard University for the last six months, and Bruce Paterson, who is spending a sabbatical year at the Biozentrum, Basel, Switzerland, will return to the laboratory this summer, bringing back new ideas and expertise.

The entire staff is looking forward to a productive year provided that no additional reduction in the budget obliges us to reduce further the excellent group of post doctoral fellows and technical assistants whose ideas and hard work are essential to reach our goals.

With the exponentially growing number of proteins identified, sequenced and synthesized in large quantity with the help of DNA recombinant technology, the role and mechanism of action of proteins in the regulation of cellular processes has become the focus of general interest. It is no wonder that most molecular biologists are becoming interested in protein chemistry and that protein chemists are using the techniques of molecular genetics. These common approaches have strengthened the links between the different groups which compose the laboratory. The broad range of complementary expertise brought to the laboratory by the previous Chief of the laboratory, Maxine Singer, has greatly facilitated this transition. The Laboratory of Liochemistry, in collaboration with other groups at NIH, is in an ideal position to study the complex network of factors controlling normal cell growth and differentiation and ultimately to elucidate the alteration of the network responsible for neoplasic transformation.

REGULATION OF GENE EXPRESSION

The regulation of gene expression remains one of the major topics of interest in the laboratory with five different groups studying gene regulation in different systems.

Dr. Wu and his colleagues study the structure, regulation and mode of action of two classes of sequence-specific DNA-binding proteins in *Drosophila*. A major achievement this year was the characterization of the transcriptional activator of the heat shock genes (Heat Shock Factor, HSF). HSF was cloned and expressed in *E. coli*. Functional domains of the HSF protein responsible for DNA binding, oligomerization, and transcription activation were identified. The recombinant HSF which is constitutively active, lacking the negative regulation to which HSF is subjected to *in vivo*, can now be used to isolate the inhibitory factor responsible for the inactivation of HSF. Studies of the interaction between HSF and the inhibitory factor will allow this group to elucidate the mechanism by which the inactive form of HSF acts as a sensor of cellular stress. A second project under investigation by this group deals with the regulation of temporal and spatial expression of the segmentation gene *fushi tarazu* during early embryogenesis. The transcriptional activator of this gene, FTZ-F1, was cloned and found to encode a new member of the steroid receptor family. A transcriptional repressor of *fushi tarazu*, FTZ-F2, was also cloned and found to encode a new "zinc finger" protein. Thus, the complex expression pattern of a developmental gene is mediated by a combination of positive and negative transcription factors. A particularly revealing outcome of this work is that both FTZ-F1 and FTZ-F2 were missed by classical genetic screens for embryonic lethals with a segmentation phenotype, underscoring the need for biochemical screens wisely used by Dr. Wu to complement genetic studies.

Major progress has also been made by Dr. Hamer's group in the elucidation of the basic mechanism of metallothionein gene regulation in yeast. Dr. Hamer and his colleagues showed that metal ions directly interact with a regulatory protein, ACE1, altering its conformation so that it can bind to the metallothionein gene control sequences and thereby activate transcription. Remarkably, the predicted structure of the regulatory protein is similar to that of metallothionein itself. The isolation of a metallothionein regulatory factor from mouse cells, a logical extension of this work, is under way.

Another aspect of the regulation of gene expression is studied by Bruce Paterson and his colleagues, who are focusing their attention on the regulation of a group of genes involved in determining the muscle phenotype in both vertebrates and invertebrates. They have isolated two different myogenic genes from chicken (CMD1 and Cmgn1) and a potential homolog from *Drosophila*. The avian genes not only activate a cotransfected muscle specific promoter/reporter gene but are also capable of the phenotypic conversion of mouse 10T1/2 fibroblasts whereas the *Drosophila* gene can only activate the muscle specific reporter gene. The stimulation of the mouse genes by their avian homologs suggests a complex regulatory network. All the proteins encoded by these genes share a common structural motif which consists of a basic domain adjacent to a putative "helix-loop-helix" element. This structural motif is conserved from fly to man. The long term goal of Dr. Paterson's group is to elucidate the mode of action of these proteins.

The role of DNA methylation in the modulation of gene expression is a 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 along with a constellation of trans-acting factors. The genetic basis for selective demethylation and expression of IAP provirus is being investigated in normal mouse lymphocytes while relevant transcription factors are being studied in nuclear extracts of various transformed rodent and primate cells. IAP elements are also used in this group by Kira Lueders to isolate putative glucose-responsive elements flanking the IAP sequences that are expressed at high level by pancreatic β cells of diabetic-susceptible but not resistant mice, upon glucose administration in order to study their extent of methylation and further define the role of DNA methylation in the control of gene expression.

The two current research projects of Dr. Peterkofsky are an outgrowth of her previous work on the regulation of the synthesis of extracellular matrix components. As a first step in elucidating the cause of altered collagen synthesis in a chemically transformed hamster fibroblast cell line, her group has obtained strong suggestive evidence that the selective suppression of synthesis of the proα1 subunit of type I procollagen in the transformed cells is the result of a post-transcriptional defect. Her second project deals with the mechanism of inhibition of collagen and proteoglycan synthesis in scurvy. She presented last year evidence that regulation of growth by vitamin C involves
IGF-1 and an inhibitor of IGF-1. These conclusions are now supported by the demonstration that increased levels of unliganded IGF-1 binding proteins in scorbutic and fasted sera are responsible for the inhibition of IGF1-dependent functions. Preliminary characterization of three IGF-1 binding proteins suggests that one of them is specifically increased during vitamin C deficiency while the two others are increased in both fasting and scurvy.

## PROTEINS AND THE CONTROL OF CELLULAR PROCESSES

The structure of biologically important proteins and their role in the regulation of cell function is the focus of interest of three different groups.

Dr. Berger and her colleagues are studying the newly discovered role of prothymosin α in cell division. Their previous studies with prothymosin α mRNAs, synthetic and natural prothymosin α, and transfected cDNAs and genes led them to reassess the significance of the extracellular role for the proteolytic derivative of prothymosin α, thymosin α, postulated by others. Instead, the Berger group has now obtained convincing evidence that prothymosin α is itself an active cellular component performing an intracellular function in proliferating cells. This highly acidic protein contains a nuclear targetting signal which is both necessary and sufficient for its transport to the nucleus and its synthesis is regulated in a cell cycle-dependent manner. Cell division requires sufficient quantities of prothymosin α in order to take place.

There are few biological systems which are not regulated by $Ca^{2+}$. In all eukaryotic cells $Ca^{2+}$ regulation is at least partially mediated by calmodulin. Two major aspects of the mechanism of action of calmodulin in stimulus response coupling are studied by Dr. Klee and her colleagues: 1) How does $Ca^{2+}$ alter the structure of calmodulin thereby increasing its affinity for its target proteins by 2-3 orders of magnitude and 2) the role of calmodulin in protein dephosphorylation. Whereas the crystal structure of the $Ca^{2+}$-saturated calmodulin has been elucidated, very little is known about the structure of

$Ca^{2+}$-free calmodulin. Dr. Klee and colleagues have used limited proteolysis and calmodulin mutants to demonstrate that $Ca^{2+}$ destabilizes and $Mg^{2+}$ stabilizes the central helix of calmodulin. The cloning this year of the two subunits of *Drosophila* calmodulin-regulated protein phosphatase, calcineurin, has revealed the extreme conservation of the catalytic and regulatory domains of this protein during evolution. The calmodulin-binding and autoinhibitory domains have been characterized and the structural organization of the functional domains at the gene level determined.

Dr. Wagner's long time interest in the role of protein phosphorylation in the regulation of cellular motility has led him to start studying the role of $Ca^{2+}$ and protein phosphorylation in the regulation of secretion. He has selected as a model system chromaffin and PC12 cells. Using a variety of protein phosphatase inhibitors his group has shown that norepinephrine secretion by PC12 cells is regulated by a $Ca^{2+}$-stimulated phosphorylation. Using permeabilized chromaffin cells which have lost the ability to secrete norepinephrine, they have demonstrated the presence in crude cytosolic extracts of factor(s) able to restore the $Ca^{2+}$-dependent secretion. The purification of this factor(s) is under way.

Dr. Mage's major interest is the interaction of T cell receptors with class I MHC molecules. While continuing to test multivalent conjugates of antigens for their ability to elicit a T cell-independent immune response Dr. Mage has started a new research project with the ultimate goal of improving presently available inhibitors of HIV infection. Multivalent conjugates of the CD4 receptor proteins that bind the viral gp120 protein and inhibit HIV infection *in vitro* will be tested for their increased affinity and stability over the preparations of the CD4 protein which are presently tested clinically. This collaborative project with Dr. Gershoni (NCI) is supported by the AIDS program.

ORGANIZATION OF THE HUMAN GENOME

Two different aspects of the organization of the human genome are studied by the groups of Drs. Singer and McBride.

Dr. Singer and her colleagues continue to carry out experiments designed to elucidate the mechanism of LINE-1 transposition in the human genome. This year substantial progress was made, and depended on prior investments of time and effort in constructing useful plasmids and in preparing antibodies to LINE-1 fusion proteins synthesized in *E. coli*. Their experiments fall into two types, conveniently described as 1) transcription and 2) translation of LINE-1 sequences in human cells. Both of these processes are required by current models for
LINE-1 transposition.

With respect to the teratocarcinoma-cell specific transcription, the following advances were made this year. A second human teratocarcinoma cell line (2102) was found to promote LINE-1 transcription at a high rate, confirming earlier conclusions. Previous work on the internal LINE-1 promoter, which measured expression of a reporter gene, was confirmed at the RNA level by analyzing transcripts directly. They also demonstrated that in addition to the presence of an essential DNA sequence motif in the first 100 bp at the 5' end of human LINE-1s, several other significant regulatory seqences occur between base pairs 100 and 600 from the 5' end. Antibody to the ORFI protein allowed the detection of a protein of the approximate expected size in the cytoplasmic fraction of human teratocarcinoma cell lines - NTera2D1 and 2102-EP. This is the first demonstration of a LINE-1 encoded protein in human or other mammalian cells.

The laboratory and NIH as a whole are fortunate to benefit from the expertise of O. Wesley McBride. His group has a long lasting interest in human chromosome mapping as a tool to identify genes involved in genetic diseases and human neoplasia. Complementary methods such as Southern analysis of DNAs from a panel of human-rodent cell hybrids developed in his laboratory, *in situ* hybridization of metaphase spreads with labeled probes, and genetic linkage analysis in the 40 large CEPH kindreds, as well as families with genetic diseases using fragment length polymorphism as markers, are being used for this purpose. Fifteen to twenty new genes have been mapped during the last year, their size, organization and copy number determined, and alternative splicing sites and amplification of entire chromosomal regions identified. The sensitivity of the *in situ* hybridization techniques has been improved to detect single copy genes and used to map the human thyroid peroxidase gene to chromosome 2p25.3 (telomere). Development of new and more precise cytogenetic techniques permitting banding of the chromosome after hybrization has allowed this group to localize hybridizing sequences to a specific band. Amongst the highlights of this year's achievements are the mapping of the hydroxyindole o-methyltransferase gene expressed by Drs. Klein and Donohue to the pseudoautosomal region of the X (and Y) chromosome (this is only the third gene assigned to this region). An alphoid satellite probe from a clone isolated by Dr. H. Ozer has been found to identify chromosomes 1, 5, and 19. This probe may have a clinical human cytogenetic application. A linkage map of four probes spanning a 15 cM region of chromosome 22q11.2, including a region involved in the DiGeorge Syndrome, has been completed and additional genes have been added to a cluster previously mapped in the laboratory at chromosome 22q12.

DNA REPLICATION

The mechanism and control of DNA replication is a major interest in the Laboratory. Four independent groups studying different but potentially overlapping aspects of this critical cellular event have made important contributions during the past year.

Dr. 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. Major findings include 1) the preliminary evidence that the precise control of β-polymerase expression is important for normal cell growth and the isolation 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, and 2) the demonstration that A1 hnRNP binding protein has annealing activity toward both RNA and ssDNA leading to the attractive hypothesis that the protein may play a role in mRNA splicing and DNA recombination.

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 two subunits of the dimeric enzyme has been identified and a peptide inhibiting the dimerization process is being tested as a specific inhibitor of the enzyme and as an antiviral agent; forthcoming information on the substrate binding domain will be used to identify or design more specific inhibitors of HIV-1 reverse transcriptase.

Also supported by the AIDS program are the comparative studies of the mechanism of action of the ribonuclease H activity of two viral reverse transcriptases by

Dr. Berger and her collaborators. Using enzyme kinetics, they have shown that only one binding site for the template:primer suffices for both polymerase and RNase H catalysis.

Research in the Microbial Genetics and Biochemistry Section is devoted to replication, recombination and redistribution of DNA.

Studies of P1 plasmid maintenance by Drs. Yarmolinsky and Chattoraj have been pursued along several lines by a combination of biochemical, genetic and physiological methods. Large (up to 104-fold) influences of accessory proteins in altering the efficiency with which proteins more directly involved in replicating or redistributing plasmid DNA perform their functions have been demonstrated. Of particular interest are findings that implicate the concerted action of three heat shock proteins in altering the binding of the P1 initiator protein to its sites of binding within the origin of replication and within a replication-control region. Other findings elucidate the multiple (even antagonistic) functions of the initiator gene products and the relation of plasmid replication and partition functions to each other.

Dr. Michael Lichten is studying the mechanism and regulation of meiotic recombination in the yeast *Saccharomyces cerevisiae*. Techniques previouly developed by his group have been applied to a study of the timing of various meiotic recombination events at the molecular level. Particular attention has been given to the relationship between double-strand chromosome breaks (thought to initiate meiotic recombination), formation of heteroduplex DNA, and appearance of mature recombinant molecules. The effects of chromosome structure on the timing and location of these events is being studied. Finally, cloned DNA fragments containing sequences that promote meiotic recombination in their vicinity have been isolated and are being characterized.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00366-19 LB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Structure and Expression of Endogenous Retroviral Elements

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

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

COOPERATING UNITS (if any)

E. Leiter, Jackson Laboratory, Bar Harbor, ME

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.0 | 3.0 | 1.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We are examining cellular mechanisms that regulate intracisternal A-particle (IAP) expression in normal and transformed mouse cells. As one aspect of the program, we are studying the interaction of cellular factors with cis-regulatory sequences in the IAP LTR. A novel enhancer-binding protein fraction has been isolated by DNA affinity chromatography from transformed human and mouse cells. The fraction, designated EBP-80, contains two protein components of 75 and 85 kDa. We have now shown that EBP-80 acts to stimulate transcription from IAP LTRs in a cell-free system. The EBP-80 binding domain in a number of recently expressed and/or transposed IAP elements contains a particular sequence variant which has higher affinity for the factor and confers greater promoter activity on the LTR. Methylation of a single CpG residue in the EBP-80 binding domain reduces both affinity for the factor and promoter activity of the LTR. EBP-80 has been isolated from cultured human cells in sufficient quantity to permit peptide separation, microsequencing and development of specific oligonucleotide probes for gene cloning.

We are also studying whether particular elements of the multicopy IAP gene family are selectively activated in different cell types, and the means by which this might be accomplished. Isolation and sequencing of multiple cDNA clones from BALB/c thymocytes and stimulated splenic B-cells reveals that the two cell types express very limited and largely overlapping sets of IAP transcripts. Only a few highly related IAP elements seem to be responsible for most of the IAP expression in these cells. Efforts to clone these elements from genomic DNA are in progress. We plan to follow IAP expression during the stages of plasmacytoma development since enhanced expression may be a very early marker of B-cell transformation.

Project Description

Major Findings:

A. Transacting Factors for IAP Expression (Dr. M. Falzon)

Last year's Annual Report described the isolation of a novel enhancer-binding protein fraction from nuclear extracts of both human (293) and mouse (myeloma) cell lines. The material was purified by affinity chromatography on an oligonucleotide representing one of several protein-binding domains in the U3 regulatory region of a cloned IAP LTR. This domain contained a 5 bp tract homologous to the SV-40 "enhancer-core" motif, and the isolated fraction, designated EBP-80, bound with similar affinities to regions of SV40, polyoma and MSV LTR that contained this same motif. Dr. Falzon has subsequently compared the promoter activity of the LTR from a recently transposed IAP proviral element (rc-mos) with that of the original genomic element (MIA14). The EBP-80 binding domain (designated Enh-2) in the rc-mos LTR contains a variant sequence which lacks the enhancer core motif. Nevertheless, EBP-80 binds to the rc-mos Enh2 domain more strongly than to the Enh2 domain of MIA14. Site-directed mutagenesis studies show that the Enh2 domain of rc-mos confers increased promoter activity on the LTR both in vitro and in vivo. Added EBP-80 stimulates in vitro transcription from both the rc-mos and MIA14 LTRs, but the rc-mos LTR remains the more active promoter at saturating levels of factor. The rc-mos Enh2 motif or a closely related sequence is found in the LTRs of most expressed and/or transposed IAP elements. Heightened levels of EBP-80 in certain cells, particularly those transformed by nuclear oncogenes, may favor expression of individual IAP elements containing this sequence motif.

The promoter activity of IAP LTRs in vivo is known to be inhibited by methylation at certain CpG positions in the U3 regulatory region (see earlier Annual Reports). The Enh2 domain contains one such site: the presence of $^{m}C$ at this position inhibits transcription from LTR:CAT constructs both in vitro and in vivo. Oligonucleotides representing the Enh2 binding domain show reduced affinity for EBP-80 (assayed by gel shift or filter-binding) when this site is made to contain $^{m}C$. The results indicate that site-specific methylation in the Enh2 binding site inhibits LTR promoter activity by reducing affinity for the EBP-80 transcription factor.

Dr. Falzon has continued the large scale preparation of EBP-80 from a transformed human cell line (293), and has in hand 5-10 µg of affinity-purified material which will be used to obtain microsequence of the protein components preparatory to cloning.

B. Endogenous Retrovirus Expression in Mouse Diabetes

Previous work with Dr. E. Leiter (Jackson Laboratory) suggested that IAP expression could have a possible role in the pathogenesis of genetically determined mouse diabetes. In mice of diabetes-susceptible strains, glucose-enhanced IAP expression was associated with the presence of circulating antibodies against the retroviral structural protein. However, it has not been possible to show that these antibodies have a primary role

in causing the characteristic β-cell degeneration seen in diabetic animals. Studies on this aspect of the problem are currently in abeyance in our laboratory.

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

We have shown by a 2-D gel electrophoretic procedure that a particular subset of IAP elements is demethylated, and presumably available for transcription, in normal mouse tissues such as thymus and spleen (see Annual Report 1989). J. Mietz is currently examining the number and variety of IAP elements actually expressed in normal BALB/c lymphocytes. She has prepared oligo(dT)-primed cDNA libraries from thymocytes and LPS-stimulated splenic B cells of young adult BALB/cAn mice, and selected 25 clones from each library using a probe from the 3' end of the IAP LTR. DNA from each clone has been sequenced through the R and U3 portions of the LTR and enough internal sequence to yield about 1 kb of data.

Previously isolated IAP elements have differed from one another by 2-5% in sequence; therefore cDNA clones that are identical over 1 kb are very likely to have arisen from transcripts of the same IAP provirus. By this criterion, the cDNA clones isolated from activated B-cells represent a numerically small and closely related set of IAP elements: all of the clones were identical over the internal region and the U3 portion of the LTR (about 900 bp in all). The U3 sequence was distinguished by certain variations from those of previously reported IAP elements. The set of B-cell clones could be subdivided on the basis of (a) the number of repeats of a 13 bp motif in the R region, and (b) alternative short sequence motifs just upstream of the polyadenylation signal. Five individual IAP elements appeared to be represented in the 25 B-cell clones and 8 in the set of cDNAs from thymus. The more abundantly transcribed elements were found in both cell types. The thymus set included 3 single cDNA clones representing IAP elements not encountered in B cells. Two 23 bp oligonucleotides specific for the major groups of expressed IAP elements in B-cells were used to probe blots of BamHI-digested BALB/cAn genomic DNA. Each probe detected about 20 bands. Two-dimensional gel electrophoresis was then used to determine which of these BamHI fragments contain oligonucleotide-reactive IAP elements that are demethylated at a HaeII site in their 5' LTRs. Several such fragments were identified and attempts are in progress to clone the demethylated (putatively active) elements.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 00945-17 LB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Factors Regulating the Synthesis of Collagen in Normal and Transformed Cells

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

| | | | |
|---|---|---|---|
| B. Peterkofsky | Research Chemist | LB | NCI |
| K. Takeda | Visiting Fellow | LB | NCI |
| E. Schalk | Guest Researcher | LB | NCI |
| V. Shah | Visiting Fellow | LB | NCI |
| K. Hadley | IRTA Fellow | LB | NCI |

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Biological Interactions Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 5.4 | 4.4 | 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 cartilage 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 human skin fibroblasts. The presence of the inhibitor in fasted and scorbutic sera was correlated with an increase in low molecular weight IGF-I binding proteins (30-50 kDa) that can inhibit binding of IGF-I to its receptor on 3T3 cells and human fibroblasts. A 30 kDa binding protein was increased in both scurvy and fasting while a 36 kDa binding protein appeared to be uniquely induced by vitamin C deficiency. 4-Nitroquinoline-1-oxide transformed Syrian hamster embryo fibroblasts (NQT-SHE) do not synthesize the pro 1 (I) subunit of type I procollagen but continue to synthesize altered forms of the other subunit, pro 2(I). The level of poly A+ pro 1(I) mRNA is approximately 1% of the level in normal hamster primary fibroblasts and 10% of the level in a normal hamster fibroblast line (BHK). These levels of the mRNA should lead to detectable synthesis of the pro 1(I) chain, which suggests that the mRNA cannot be translated into protein. In vitro nuclear run-off transcription assays showed that the rates of transcription of the pro 1(I) gene in nuclei from NQT-SHE cells was similar to that in the normal BHK fibroblasts, suggesting that the low steady state level of pro 1(I) mRNA in NQT-SHE fibroblasts is not caused by a defect in transcription. Thus, the failure of NQT-SHE cells to synthesize the pro 1(I) polypeptide may result from multiple defects in the transcription to translation pathway.





GPO 914-918

## Project Description

### Objectives:

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

### Major Findings:

I. Regulation of Collagen and Proteoglycan Synthesis During Scurvy and Fasting

Our previous results suggested that ascorbate-deficient and fasted, ascorbate-supplemented guinea pigs are equivalent with respect to the mechanisms by which collagen and cartilage proteoglycan synthesis are decreased. Furthermore, sera from these animals could transmit the defects in extracellular matrix synthesis to cultured 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 all of these functions 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. SDS/PAGE analysis of [$^{125}$I]IGF-I affinity cross-linked forms revealed that normal sera contained a major species of 34 kDa with minor species of 38 and 41 kDa. In scorbutic and fasted sera, the 38-kDa species increased dramatically with a lesser increase in the 34-kDa species. Scorbutic sera also contained a unique 44-kDa species, suggesting that there is specific regulation of this species in vitamin C deficiency. Gel filtration of unlabeled sera showed that total binding activity in the 30-50-kDa region increased about 10 times above normal in both deficiencies while there was no difference in the activity of the binding subunit of the large (150 kDa) IGF binding complex isolated by subsequent acidic gel filtration. Binding protein activity of the 30-50-kDa S200 fractions was correlated with their ability to inhibit binding of [$^{125}$I]IGF-I to its receptor, indicating that they have the potential to inhibit IGF-I-dependent functions. An IGF-I analog with normal affinity for the unsaturated 30-50-kDa binding proteins from fasted and scorbutic sera, but with reduced affinity for the IGF-I receptor, was equivalent to IGF-I in reversing the inhibition of collagen synthesis by scorbutic guinea pig serum in human fibroblasts. This result suggests that reversal of inhibition is due to saturation of the binding proteins with ligand and provides further evidence that the IGF binding proteins that are increased in scorbutic and fasted guinea pig sera may be the IGF-I-reversible inhibitor.

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

A. Modified Proα2(I) Chains

Our previous work showed that there were two polypeptides secreted by

NQT-SHE cells that could be separated by ammonium sulfate fractionation and they were designated as N33 and N50 collagens. They differed from the proα2(I) chain in type I procollagen secreted by normal Syrian hamster fibroblasts in that they migrated more slowly during SDS/PAGE. The modification was retained in the collagenous domain after limited pepsin digestion of the procollagen forms. It also was present in a cyanogen bromide peptide (CB-3,5) that spans about 60% of the C-terminal end of the chain (60 kDa) and in two V8 protease-derived peptides (V8-1 and 2) of approximately 30 and 40 kDa, respectively, which also are derived from the C-terminal domain. Several lower molecular weight V8 protease-derived peptides from these chains were identical to those from the normal chain, indicating that the modification was localized in the C-terminal half of the molecule. The modification is present only in the secreted N-collagens and not in the intracellular polypeptides, indicating that it occurs post-transitionally. It is a novel type of modification since it occurs independently of proline and lysine hydroxylation. Preliminary experiments using isoelectric focusing suggest that only one of the modified V8 protease-derived peptides (V8-2) has acquired a negative charge so there may be more than one type of modification. Since secreted proteins may become sulfated under abnormal culture conditions, we labeled cells with [$^{35}$S] sulfate and determined whether label was incorporated into the N-collagens, but none was observed. Experiments in which the V8 peptides were treated with a specific glycosidase indicated that the modification did not involve O-glycosylation.

B. Mechanism for Lack of Expression of the Proα1(I) Subunit

1. Transcription of the Proα1 (I) Gene

Our previous results suggested that failure of NQT-SHE cells to synthesize the proα1(I) subunit of type I procollagen could be explained by the absence of proα1(I) mRNA, as measured by dot-blot hybridization of total cellular RNA with a cDNA probe and also by cell-free translation. In order to determine whether a low level of mRNA might be present and to quantitate the amount, poly A+ RNA was isolated and analyzed since sensitivity is greatly increased by this procedure. Proα1(I) mRNA was detectable and was present at 1% of the level in primary hamster fibroblasts and 10% of the level in a normal, established line of hamster fibroblasts (BHK). Synthesis of the proα1(I) polypeptide in BHK cells is easily detectable by SDS/PAGE so there would be no problem in detecting synthesis in NQT-SHE cells at the level of mRNA in these cells. Similarly, the cell-free translation assay used previously would have been capable of detecting the amount of proα1(I) mRNA that is present in NQT-SHE cells. Therefore, it appears that the proα1(I) mRNA in NQT-SHE cells is not translatable.

In order to determine whether the decreased level of proα1(I) mRNA in NQT-SHE fibroblasts was due to a defect in transcription, a nuclear run-off assay was carried out. The rate of transcription of the proα1(I) gene with nuclei from NQT-SHE fibroblasts was similar to the transcription rate of the proα2(I) gene in these cells. By

normalization of the transcription rates for proα1(I) obtained with nuclei from different cell lines relative to the transcription rates of actin, transcription rates for the proα1(I) gene could be directly compared in the different lines. Using this procedure, the transcription rate of the proα1(I) gene in NQT-SHE cells was calculated to be similar to that of the gene in BHK cells.

These results suggest that failure of transformed NQT-SHE fibroblasts to synthesize the proα1(I) polypeptide may result from several problems: 1) either there is defective processing of the initial transcript or there is increased degradation of the mRNA so that the steady state level of mRNA is decreased; 2) the mRNA that does accumulate may not be translatable.

2. Attempts to Increase Expression of the proα1(I) Gene

a. Transfection of the Human Gene Into NQT-SHE Fibroblasts

We previously found that several clones obtained after transfection of NQT-SHE cells with a cosmid containing the entire proα1(I) gene expressed increased levels of proα1(I) mRNA, as measured by dot-blot hybridization assays of total RNA. The level of expression, however, was still considerably less than in primary SHE fibroblasts or BHK cells. When poly A+ mRNA was measured, there was little difference in the levels of proα1(I) mRNA in the transfected or control NQT-SHE cells. These data suggest that there may be an increase in nuclear RNA in the transfected cells containing the human gene with no conversion to the fully processed transcript. Therefore the transcription rate in nuclei of transfected cells is being examined.

b. Effect of Transforming Growth Factor (TGF)-β on proα1(I) Expression

Many studies have shown that TGF-β stimulates type I collagen synthesis or increases the steady state level of the mRNAs for the proα chains. Increased steady state levels of proα1(I) mRNA induced by TGF-β have been reported to result from either increased transcription or increased stability of the mRNA. We carried out experiments to determine whether TGF-β could stimulate expression of the proα1(I) gene in NQT-SHE fibroblasts. Subconfluent cultures of NQT-SHE, BHK and SHE fibroblasts were treated with the growth factor. Human fibroblasts also were used since it has been reported that collagen synthesis is stimulated by TGF-β in these cells. Cytoplasmic RNA was isolated and analyzed by dot-blot hybridization for proα1(I) and proα2(I) mRNA. Alternatively, proteins were labelled with radioactive proline and the rate of collagen synthesis was measured.

TGF-β treatment led to an increase in the level of proα1(I) mRNA in SHE, human fibroblasts and BHK fibroblasts by factors of 5, 2.5 and 2 times, respectively, but there was no effect in NQT-SHE cells. Proα2(I) mRNA, however, was elevated significantly (4 times control) only in SHE fibroblasts. In spite of the large increase in the level of type I

collagen mRNAs in SHE fibroblasts, the relative rate of collagen synthesis was increased only two-fold under the same conditions, indicating the lack of stringent coupling between mRNA concentrations and translation rates. We cannot exclude the possibility that the rate of transcription of the proα1(I) gene was increased in NQT-SHE cells but that due to the post-transcriptional defect in these cells, there was no increase in mRNA levels. This possibility is being investigated.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 05202-23 LB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

| | | | |
|---|---|---|---|
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| M. Wang | Visiting Associate | LB | NCI |
| H-F. Yi | Visiting Fellow | LB | NCI |
| B. Watson | Graduate Student, Howard Univ. | LB | NCI |
| W. Fibison | Guest Researcher | LB | NCI |
| J. Clark | Laboratory Worker | LB | NCI |

COOPERATING UNITS *(if any)* NICHHD: W. Leonard, D. Rodbard, D. Klein, R. Klausner, I. Owens; NIAMS: P. Steinert; NCI: A. Fornace, P. Elwood; C. Klee, F. Gonzalez, S. Kimura, S. Tronick, N. Popescu, K. Kelly; NHLBI: R. Adelstein; NIDR: L. Fisher, M. Young, A. Notkins; NIDDK: L. Kohn, A. Burns; R. Pirtle, U. TX., B. Vold, SRI, D. Joseph, U.N.C., E. Strehler, Zurich, K. Uyeda, U. TX, SW Med. School, B.J. Song; NIAAA: A. Jaiswal, NYU, H. Ozer, NJ School of Med.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.5 | 3.0 | 0.5 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Several approaches have been used in this laboratory for the chromosomal localization of cloned human genes and for efforts to identify genes involved in genetic diseases and human neoplasia. The mapping approaches are complementary and include Southern analysis of DNAs from a panel of well-characterized human-rodent somatic cell hybrids, in situ hybridization of metaphase spreads with either 3H-labeled or biotinylated probes, and genetic linkage analysis in the 40 large C.E.P.H. kindreds as well as families with genetic diseases using restriction fragment length polymorphisms detected in the laboratory as markers. About 15-20 additional genes have been mapped by each method during the past year. The specific approaches have varied to provide information concerning gene size and organization, copy number, presence of alternative splicing, amplification of entire chromosomal regions, and other features. Our cytogenetic techniques have been improved to allow chromosomal localization of single copy genes by in situ hybridization with biotinylated cosmid probes and used to map the human thyroid peroxidase gene to chromosome 2p25.3 (telomere). Techniques have been developed to permit subsequent banding of the chromosomes after hybridization and propidium iodide staining thereby permitting chromosome identification and localization of the hybridizing sequence to a specific band. This represents a significant improvement in existing cytogenetic technology. Other highlights include the mapping of an expressed gene isolated by Drs. Klein & S. Donohue to the pseudoautosomal region of the X (and Y) chromosome and this represents only the third gene assigned to this region. An alphoid satellite probe from a clone isolated by Dr. H. Ozer has been found to identify human chromosomes 1, 5, and 19 and it may have a clinical human cytogenetic application. A linkage map of four probes spanning a 15cM region of chromosome 22q11.2 including a region involved in the DiGeorge Syndrome has been completed and additional genes have been added to a cluster previously mapped in the laboratory at 22q12.





GPO 914-918

## 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 an authentic thyroid stimulating hormone receptor (L. Kohn, NIDDKD), synexin (A. Burns, NIDDK), NM23H1 (a protein implicated in both tumorigenesis and metastasis)(P. Steeg, NCI), a small family of (intron containing) tRNATYR genes (B. Vold, Stanford Res. Institute), 3 members of a uridine monophosphate glucuronosyl transferase family (I. Owens, NICHHD), interleukin-2 receptor beta (W.J. Leonard, NICHHD), iron responsive element binding protein gene (R. Klausner, NICHHD), loricrin (P. Steinert, NCI), folate binding protein (MFBP) genes (P. Elwood, NCI), a calcium channel protein gene (H. Chin, NINDS), lymphokine P40 (B. Mock, NCI), histidine decarboxylase (D. Joseph, U. N.C.), an alphoid satellite (H. Ozer, N.J. School Med.), and hydroxyindole O-methyltransferase gene (D. Klein, NICHHD).

A large number of genes have been further regionally localized on human chromosomes during the past year by in situ hybridization with 3H-labeled and biotinylated probes. Some of these genes include IL-2RB, NMMHCA, synexin, NM23H1 and a related sequence, calcineurin A, calcineurin B, aspartyl tRNA synthetase, osteopontin, BSPI, UMPGTH1 and H2, MFBP, Ca ATPAses isoforms 1 and 3, an alphoid satellite sequence, pSP2.8, and thyroid peroxidase.

A third method for gene mapping has involved genetic linkage analysis in the 40 large C.E.P.H. pedigrees using RFLPs as markers. Twenty one probe-enzyme combinations have been used to map 18 genes during this period including thyroid peroxidase, HIOMT, NMMHCA, calmodulin gene on chromosome 2, loricrin, filaggrin, cardiac myosin, NM23H1, PDGFB, polymerase beta, CKBB, a polymorphic glycine tRNA gene (chr 1), opal suppressor tRNA gene, CaATPase isoforms 1 and 3, CYP4B1, CYPIID1, and IREBP.

These complementary methods have provided some particularly interesting results including the discovery that the HIOMT gene is located on the pseudoautosomal region of the X (and Y) chromosome. Two highly informative VNTR loci are present at this locus. The thyroid peroxidase gene has been localized to the telomere of chromosome 2p by in situ hybridization with biotinylated cosmid probes and 4 highly informative VNTRs at this locus have permitted extensive linkage analysis with these TPO probes. An alphoid satellite probe cloned by H.

Ozer has been found to specifically hybridize to the centromeres of only 3 human chromosomes. Use of a PCR amplified and $^{32}$P-labeled tRNATYR probe has permitted chromosomal localization of this small family of intron containing and highly conserved tRNA genes, and most or all family members appear to be present on a single human chromosome. Two human epidermal protein genes (loricrin and filaggrin) have been mapped to a region on chromosome 1q previously suggested as a potential locus for a genetic skin disease and family studies involving skin diseases are contemplated by P. Steinert. The mapping and elucidation of two high frequency RFLPs at the locus for the recessive anti-oncogene NM23H1 has permitted high resolution linkage mapping at this site and allowed its use for deletion studies in human tumors. This gene has now been found to be hemizygously, and sometimes homozygously, deleted very frequently in tumor tissue of several types. Our linkage studies of cardiac myosin and other genes in the region of 14q11 have aided in ordering these various genes and anonymous polymorphic DNA markers and the disease locus for hypertrophic cardiomyopathy in one large pedigree studied by C. Seidman, et al.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 05203-22 LB

PERIOD COVERED
October 1, 1989 to September 30, 1990

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 |

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Biosynthesis Section

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

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.00 | 2.00 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors B

☐ (a2) Interviews AIDS research: 50% (see item C in Major Findings)

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

Our laboratory has a long standing interest in development of immunochemical methods for cell separation, for studying receptor-mediated activation of T cells, and in the role of T cells in tumor rejection. With respect to immune activation, we are particularly interested in developing methods for the direct measurement of the interaction of the T cell receptor with MHC antigens. As an outgrowth of these interests, we have recently been developing methods for constructing semisynthetic multivalent macromolecular reagents, for which we have coined the term "polygens". Polygens are high molecular weight soluble polymers conjugated to proteins such as antigens, antibodies, toxins, or lymphokines. We believe that polygens, by increasing the effective valence of protein epitopes, may help to measure low affinity molecular interactions such as that between the T cell receptor and MHC antigens, and that such studies may help us understand the molecular requirements for activation of T cells.

During this year we have used our general reagent Polyacrylamide-Streptavidin (PASA) to elicit a T-independent response to the native epitopes of streptavidin. We continued to develop polyvalent conjugates of Class I MHC antigens for use in studying interactions with T cell receptors. In addition, we have prepared polyvalent conjugates of the CD4 receptor for HIV. Such conjugates can bind the viral gp120 protein, and can inhibit HIV infection as measured by the syncytium assay.



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

## 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. "PASA" (polyacrylamide-streptavidin) can elicit the T-independent formation of antibodies to native protein epitopes of streptavidin. Both nude and conventional mice responded over a wide dose range, by several routes of immunization.

   Antibody responses to native protein epitopes usually require T cell help. The ability to elicit T-independent responses to native protein epitopes may be useful when antigens have evolved to avoid T-dependent responses, as in malaria, or where helper T cells have been destroyed by disease, as in AIDS.

B. We have prepared "Poly-MHC", i.e. a multivalent soluble form of recombinant Class I MHC molecules, by first mixing PASA with biotinylated antibody to the alpha 3 domain of Class I MHC. The resulting conjugate in turn can bind soluble Class I MHC molecules to form a soluble multivalent form of these molecules. These conjugates, by reason of multivalency, may have higher affinity for MHC ligands such as peptides or T cell receptors. We plan to use the conjugates to study such interactions, which are central to most immune phenomena.

C. We have also prepared "Poly-CD4", i.e. a multivalent form of the soluble CD4 molecule, by binding of periodate-oxidized soluble CD4 to the hydrazide derivative of polyacrylamide. The resulting conjugate binds the viral gp120 protein, as measured by binding of $^{35}$S-labeled gp120, followed by immunoprecipitation, gel electrophoresis and autoradiography. The conjugate also can inhibit infection by HIV *in vitro*, as measured in a syncytium assay. Another variety of poly-CD4 has been prepared by biocytinylation of soluble CD4 followed by reaction with PASA. We plan to see if various poly-CD4 constructs show improved *in vivo* half life and improved HIV neutralization capacity, compared to monomeric CD4.

### Publications:

Nardelli B, McHugh L, Mage MG. Polyacrylamide-streptavidin: a novel reagent for simplified construction of soluble multivalent macromolecular conjugates, J Imm Methods 1989;120:233-9.

Grohmann U, Ullrich SJ, Mage MG, Appella E, Fioretti MC, Puccetti P, Romani L. Identification and immunogenic properties of an 80-kDa surface antigen on a drug-treated tumor variant: relationship to MuLV gp70, Eur J Immunol 1990;20:629-36.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 05214-19 LB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

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

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

| | | | | |
|---|---|---|---|---|
| S. H. Wilson | Medical Officer | LB | NCI | |
| B. Zmudzka | Visiting Associate | LB | NCI | 6 mos. |
| P. Kedar | Visiting Fellow | LB | NCI | 6 mos. |
| S. Widen | IRTA Fellow | LB | NCI | |
| P. Becerra | Guest Research | 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, DCBD

SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.9 | 3.5 | .4 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We continued biochemical studies of mammalian DNA replication proteins. A cDNA for β-polymerase has been subcloned in expression vectors; the protein has been overproduced in E. coli, purified in mg quantities and sequenced. Structure-function aspects and in vitro DNA repair activities of the recombinant enzyme are being studied. Genomic DNA spanning the gene for human β-polymerase was further characterized. The promoter region was studied by detailed deletion and site mutagenesis using a transient expression system. In other work, we purified a transcription factor for the 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.





GPO 914-918

## Project Description

### Objectives:

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

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

### Major Findings:

A. Structure and Function of Mammalian DNA Polymerases

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

1. Human β-Polymerase

Background:

DNA polymerase β (β-pol) is a housekeeping enzyme considered to be involved in 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 and 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 AAAF; 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 E1A/E1B and

ras. Finally, we found that promoter activity is down-regulated by the HTLV-I regulatory protein, Tax. This palindrome shares sequence homology with oligonucleotide sequences in many viral and cellular promoters known as the ATF binding element or CRE.

b. Proteins in crude nuclear extract capable of binding to the human ß-pol promoter were surveyed by DNase I footprinting with the core promoter as probe. The results indicate a similar footprinting pattern for nuclear extract from all rodent tissues or from various cell lines representing differences in mRNA expression or ability to support fusion gene expression. The predominant footprinting corresponds to a decanucleotide palindromic element at -49 to -40 in the ß-pol promoter, plus several flanking residues on each side. From mutagenesis experiments, this decanucleotide palindromic element (GTGACGTCAC) is known to be involved for activity of the ß-pol-CAT fusion gene and is required for response to the effects of MNNG treatment or E1A/E18 expression or ras expression.

To purify protein(s) binding specifically to the palindromic element, we employed a gel mobility shift assay; an oligonucleotide corresponding to the palindrome and flanking residues was used as probe. Palindrome binding protein was purified from a bovine testes nuclear extract, and cDNA cloning and biochemical studies of the purified palindrome binding protein are underway. The purified protein strongly stimulates in vitro transcription of the ß-pol promoter and, as expected, binds tightly to the palindrome region of the core promoter. In equilibrium binding assays using the nitrocellulose filter method for collecting the protein-promoter complex, we found that binding is extremely tight ($K_d \simeq 10^{-13}$); binding is second order overall and is ionic strength dependent. About 70% of the free energy of binding is contributed by ionic interactions. The purified factor is able to bind tighter to the ß-pol promoter than several other palindrome sequence-containing promoters, indicating that the protein may have specificity for the ß-polymerase gene, compared with other genes.

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 $NH_2$-terminal 20% of the protein, and this $NH_2$-terminal domain (of about 75 residues) has binding affinity for ssDNA-agarase equal to that of the intact protein. The other protease-resistant segment, representing the COOH terminal domain of ~250 residues, does not bind to ssDNA, but has substantial DNA polymerase activity. The results are consistent with the idea that the $NH_2$-terminal domain is responsible for DNA binding activity of the intact protein and can interact with DNA in a "modular" fashion, whereas the C-terminal is responsible for polymerization.

d. DNA polymerase beta (ß-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 expression vectors in which cDNA for human ß-pol is inserted under the control of a metallothionein promoter (pMT) in the sense and antisense orientation, respectively, and these vectors then were used for stable transformation of mouse 3T3 cells. Vectors 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 levels of sense and antisense transcripts are strongly increased by culture of transformants in medium with 65 µM $Zn^{2+}$, although some expression is detected even without $Zn^{2+}$ induction. After 5 days of induction, the ß-pol level was ~3-fold higher in sense cells and ~10-fold lower in antisense cells than in parallel cultures without induction. The antisense line has a 3-fold increased cell doubling time in the presence of 65 µM $Zn^{2+}$ compared with the absence of $Zn^{2+}$.Zinc (65 µM) induction for the sense line results in normal growth for the first 3 days and, thereafter, a complete cessation of growth. Yet, these blocked cells remain fully viable. The results indicate that sudden deregulation of ß-pol expression alters cell growth in mouse 3T3 cells.

Publications:

Kumar A, Widen SG, Williams KR, Kedar P, Karpel RL, Wilson SH. Studies of the domain structure of mammalian DNA polymerase ß: identification of a discrete template binding domain, J Biol Chem 1990;265:2124-31.

Jeang KT, Widen SG, Semmes OJ IV, Wilson SH. HTLV-I-trans-activator protein, Tax, is a trans-repressor of the human ß-polymerase gene, Science 1990;247: 1082-4.

Englander EW, Wilson SH. Protein binding elements in the human ß-polymerase promoter, Nucleic Acids Res 1990;18:919-28.

Zmudzka BZ, Wilson SH. Deregulation of DNA polymerase ß by sense and antisense RNA expression in mouse 3T3 cells alters growth rate, Somatic Cell Molec Genet, in press.

Kedar PS, Lowy DR, Widen SG, Fornace AJ, Wilson SH. Transfected human ß-polymerase promoter contains a ras responsive element, Mol Cell Biol, in press.

Kedar PS, Widen SG, Englander E, Fornace AJ (Jr.), Wilson SH. DNA damage responsive element in cloned ß-polymerase promoter, Proc Nat Acad Sci USA, in press.

Kumar A, Abbotts J, Karawya E, Wilson SH. Identification and properties of the catalytic domain of mammalian DNA polymerase beta, Biochemistry, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 05231-16 LB

PERIOD COVERED
October 1, 1989 to September 30, 1990

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 |
| Wei Xi Qin | Visiting Fellow | LB | NCI |
| M. P. Strub | Visiting Fellow | LB | NCI |
| P. Stemmer | IRTA | LB | NCI |
| F. Suprynowicz | NRC Fellow | LB | NCI |

COOPERATING UNITS *(if any)*
Drs. A. Bax and M. Ikura, NIDDKD; Dr. Craig Montell, Johns Hopkins University; Dr. O. Wesley McBride, Laboratory of Biochemistry, National Cancer Institute; Dr. P. Cohen, University of Dundee, Dundee, Scotland

LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Protein Biochemistry

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

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 7.0 | 6.5 | 0.5 |

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

B

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

The mechanism of stimulus-response coupling mediated by $Ca^{2+}$ and calmodulin was studied at three levels: the modulation of the $Ca^{2+}$ signal by calmodulin, the regulation of the calmodulin-stimulated protein phosphatase, calcineurin, and the role of protein dephosphorylation in the control of mitosis. 1) Using limited proteolysis as a probe for calmodulin structure, we identified domains I and III and the central helix of calmodulin as domains whose conformation is specifically altered upon the stepwise titration of the calcium sites. A synthetic peptide corresponding to the calmodulin-binding domain of calcineurin increases the affinity of calmodulin for $Ca^{2+}$ 100-fold and modifies the binding pathway. 2) The high degree of structure conservation of the functional domains of the two subunits of calcineurin has been demonstrated by a comparison of the sequences of the mammalian proteins and the sequences of the two subunits of Drosophila melanogaster calcineurin predicted from the DNA sequences of the recently isolated cDNA and genomic clones. Synthetic peptides and site directed mutagenesis are being used to study the mechanism of action of the calmodulin-binding, inhibitory and catalytic domains of calcineurin which had previously been identified by limited proteolysis. 3) The involvement of $Ca^{2+}$-regulated protein dephosphorylation, possibly mediated by the calmodulin/calcineurin complex, in the control of the progression of mitotic cells from metaphase to telophase is being investigated by Frank Suprynowicz.



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

## 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

These studies were undertaken to gain insight into the molecular mechanism of the $Ca^{2+}$-dependent activation of many different enzymes by calmodulin. We demonstrated previously the $Mg^{2+}$-facilitated cooperative binding of $Ca^{2+}$ to the two halves of calmodulin. Julia Mackall has now used the susceptibility of calmodulin to proteolysis to probe the conformational changes accompanying this cooperative $Ca^{2+}$ binding to calmodulin and to analyze the structure of calmodulin in the $Ca^{2+}$-free and partially liganded states. Binding of two $Ca^{2+}$ stabilizes the F-helices of the $Ca^{2+}$-binding domains I and III, and destabilizes the helix connecting the two halves of the molecule. Thus, the two halves of the calmodulin molecule are not independent of each other and the central helix may play an active role in the concerted structural change of calmodulin upon $Ca^{2+}$ binding. Elucidation of the tertiary structure of calmodulin in solution, and in particular that of the central helix, determined by 2-D and 3-D NMR techniques by Drs. Bax and Ikura (NIDDKD) may permit the precise characterization of these $Ca^{2+}$-induced structural transitions.

The effect of target proteins on $Ca^{2+}$-binding to calmodulin is being investigated by Paul Stemmer. In the presence of a synthetic peptide corresponding to the calmodulin-binding domain of calcineurin the affinity of calmodulin for $Ca^{2+}$ is increased by two orders of magnitude as expected from the large $Ca^{2+}$-dependent increase in affinity of calcineurin for calmodulin. This large increase in affinity for $Ca^{2+}$ allows calmodulin to act as a exquisitely sensitive sensor of increased intracellular $Ca^{2+}$ concentrations. The study of the binding of $Ca^{2+}$ to calcineurin, which itself contains a $Ca^{2+}$ binding protein as an integral subunit, is under way and will be followed by a more complete study of the $Ca^{2+}$-binding and regulation of the calcineurin-calmodulin complex. The ultimate goal is to understand the roles played by the two multivalent $Ca^{2+}$ regulatory proteins in the $Ca^{2+}$-mediated control of protein dephosphorylation.

B. Interaction of Calmodulin with Target Proteins

The target protein under study is the $Ca^{2+}$/calmodulin-stimulated protein phosphatase, calcineurin. Like calmodulin it is a ubiquitous protein with

a predominant distribution in neural tissues. The enzyme from all eukaryotic organisms has preserved its characteristic subunit composition which consists of one catalytic, calmodulin-binding, 59,000 kDa subunit (calcineurin A) and one $Ca^{2+}$-binding regulatory subunit (calcineurin B). The cDNAs of the two subunits of human and Drosophila melanogaster calcineurin have been cloned and sequenced by Danilo Guerini. The primary structure of calcineurin B shows 88% identity between the human and the Drosophila proteins. As part of her study of the role of sulfhyldryl groups in the mechanism of action of calcineurin, Marie Paule Strub has shown that bovine calcineurin B, like its human counterpart, contains two cysteinyl residues at positions 11 and 153 instead of the methionine and serine erroneously identified in the previously reported sequence of calcineurin B. In agreement with the observations of Marie Krinks that different tissues contain immunologically different forms of calcineurin A with different substrate specificity, cloning of calcineurin A has shown the existence of at least four different isoforms. The two forms (now called βI and βII), described in last year's annual report, were, in collaboration with Dr. McBride, shown to be the result of alternative splicing. The cDNAs for two other forms (α and Y) have been reported by others. The α, β, and Y forms of calcineurin A appear to be the products of three different genes. Bovine brain calcineurin A has been shown by protein sequencing to be a mixture of 80% α and 20% βII. The α form is the major calcineurin component in brain, the β-II is found in specific areas of brain and in skeletal muscle; the β-I form has not yet been detected but low levels of β-I mRNA were visualized in brain RNA preparations by amplification with the polymerase chain reaction.

The catalytic and calmodulin-binding domains of calcineurin A, previously identified by limited proteolysis, are highly conserved in all the calcineurin A isoforms, including the enzyme from Drosophila. The variability of the inhibitory domains in the different forms makes them likely candidates to explain the different substrate specificities observed earlier. The inhibitory properties of synthetic peptides corresponding to these inhibitory domains are being studied by Marie Krinks with the hope of developing specific calcineurin inhibitors to facilitate the identification of the physiological roles of calcineurin. Danilo Guerini has developed a baculovirus expression system to synthesize the two subunits of the human β-II form of calcineurin. He is designing mutants to clarify the roles of the myristylation of calcineurin B and of the polyproline stretch at the amino terminus of the β-II form of calcineurin A in the folding, cellular localization and activity or regulation of the phosphatase.

The cloning of calcineurin from Drosophila was undertaken to explore the possibility that the identification of Drosophila mutant(s) will permit us to take advantage of the genetic approach to the study of the role of calcineurin in vivo in collaboration with Craig Montell at Johns Hopkins University. Analysis of genomic clones for both subunits has shown that while calcineurin B is encoded by an intron-less open reading frame, calcineurin A is a product of a complex gene with eleven introns dispersed over the 6-7000 bp genomic clone.

C. Role of Protein Phosphatases in Mitosis

The importance of protein phosphorylation/dephosphorylation for entry and progression of cells through mitosis is well established, and much attention has focused on the involvement or protein kinases in these events. Dr. Suprynowicz has initiated a study of the role of protein phosphatases in mitotic regulation, with emphasis on the progression from metaphase to telophase. Three specific events associated with metaphase/telophase transition were followed in vitro and in vivo:

1) dephosphorylation of mitosis-specific phosphoproteins recognized by the monoclonal antibody MPM-2, 2) assembly of nuclear lamins and 3) inactivation of the $p34^{cdc2}$ protein kinase, known to be essential for establishing and maintaining the mitotic state. In a cell free system, both $Ca^{2+}$-dependent and independent dephosphorylation of mitotic proteins are accompanied by lamin assembly without inactivation of $p34^{cdc2}$. The finding that $Ca^{2+}$-dependent phosphatase(s) can affect events associated with the metaphase/telophase transition suggests a role for cytosolic $Ca^{2+}$ in the control of mitosis in vivo.

Publications:

Guerini D, Klee CB. Cloning of human calcineurin A: evidence for two isozymes and identification of a polyproline structural domain, Proc Natl Acad Sci USA 1989;86:9183-7.

Guerini D, Krinks MH, Sikela JM, Hahn WE, Klee CB. Isolation and sequence of a cDNA clone for human calcineurin B, the $Ca^{2+}$-binding subunit of the $Ca^{2+}$/calmodulin-stimulated protein phosphatase, DNA 1989;8:675-82.

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Jean T, Heppel LA, Klee CB. Evidence for a GTP-dependent increase in membrane permeability for calcium in NG108-15 microsomes, Biochem Biophys Res Commun 1989;164:1060-8.

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Guerini D, Hubbard MJ, Krinks MH, Klee CB. Multiple forms of calcineurin, a brain isozyme of the calmodulin-stimulated protein phosphatase. In: Nishizuka Y, ed. Advance in second messenger and phosphoprotein research. New York: Raven Press, in press.

Klee CB, Guerini D, Krinks MH, de Camilli P, Solinema M. Calcineurin: a major $Ca^{2+}$/calmodulin regulated protein phosphatase in brain. In: Guidotti, A. ed. Neurotoxicity of excitatomy amino acids, Fidia Research Foundation Symposium Series. New York: Raven Press, in press.

Ikura M, Krinks MH, Torchia DA, Bax A. An efficient NMR approach for obtaining sequence-specific resonance assignments of larger proteins based on multiple isotopic labeling, FEBS Lett, in press.

Ikura M, Marion D, Kay LE, Shih H, Krinks MH, Klee CB, Bax A. Heteronuclear 3D NMR and isotopic labeling of calmodulin: towards the complete assignment of the $^{1}H$ NMR spectrum, Biochem Pharmocol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 05244-13 LB

PERIOD COVERED: October 1, 1989 to September 30, 1990

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

Organization of Repeated DNA Sequences in Primates

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

| | | | |
|---|---|---|---|
| M.F. Singer | Scientist Emeritus | LB | NCI |
| R.E. Thayer | Chemist | LB | NCI |
| G. Swergold | Biotechnology Fellow | LB | NCI |
| D. Leibold | Biotechnology Fellow | LB | NCI |
| L. Mizrokhi | IRTA | LB | NCI |
| J. McMillan | Visiting Fellow | LB | NCI |

COOPERATING UNITS (if any)

Thomas Fanning, Armed Forces Institute of Pathology

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: |
|---|---|---|
| 5.25 | 5.00 | 0.25 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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


Human LINE-1 DNA elements (L1Hs) belong to a recently recognized, distinctive class of transposable elements ubiquitous in eukaryotes. Called class II or non-LTR, or polyadenylated retrotransposons, these elements have no long terminal repeats, one or more open reading frames, one of which predicts a polypeptide similar to known reverse transcriptases, and an A-rich 3' end on the strand with the open reading frames. Models describing the mechanism of transposition of these elements have been proposed but none has yet been demonstrated. All the models require the transcription and translation of the elements.

Several years ago we demonstrated specific and full length L1Hs transcripts in human teratocarcinoma cells. We have now demonstrated, using appropriately constructed vectors and transient transfection, that 1) L1Hs contains internal transcriptional regulatory signals sufficient to promote transcription from the 5' end of an L1Hs element; 2) the first 100 bp from the 5' end contain essential regulatory sequences, and other significant regulatory sequences occur between bp 100 and 600 from the 5' end - the remaining 300 bp preceding the start of ORF1 appear to be unimportant for transcription; 3) transcription in 2 human teratocarcinoma cell lines is approximately as efficient as that directed by the SV40 early promoter; 4) transcription from L1Hs sequences is much less efficient in HeLa cells, CV1 (monkey cells) and 3T3 (mouse cells).

Using an antiserum prepared against the putative product of the L1Hs ORF1 we have detected a polypeptide of the appropriate size in the cytoplasmic fraction of human cells. Thus, both transcription and translation of L1Hs occur, consistent with the proposed models. Moreover, it is possible that these functions, as well as transposition, are most active in early embryonic cells.

The Drosophila melanogaster class II retrotransposon jockey has been shown to occur, in active form, in the distantly related species, D. funebris. This suggests the horizontal transfer of jockey within the Drosophila genus.

## Project Description

### Objectives:

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

Our current experiments approach these aims mainly through analysis of the transcription and translation of human LINE-1 elements, both of which are processes predicted by the model. Significant progress was made this year in both aspects. In addition, we initiated studies on the LINE-1-like element jockey, which occurs in Drosophila. The experimental advantages of Drosophila should help in studying the transposition mechanism of class II retrotransposons, including the human LINE-1 elements (L1Hs).

### Major Findings:

A. Line-1 Family Transcription

During this year we continued to investigate the transcription of L1Hs by studying artificial L1 constructs in transiently transfected tissue culture cells. Plasmid pLC1ZA, which we constructed and described last year, contains intact L1Hs 5'- and 3'-untranslated regions (UTRs) surrounding a fusion between the L1Hs ORF1 (the 5' ORF) and the bacterial reporter gene lacZ. Transcriptional activity was measured indirectly by assaying for beta-galactosidase (β-gal) activity in extracts from transiently transfected cells; expression of β-gal from the L1Hs constructs was compared with β-gal expression from a plasmid in which the SV40 early promoter drives the lacZ gene.

Previously we noted that expression of β-gal from pLC1ZA was virtually abolished when the first 100 base pairs were deleted from the 909 bp long 5' untranslated region. More detailed deletion analysis of the 5'-UTR has now revealed that other sequences within the first 660 bp contribute strongly to the activity of the intact 5' untranslated region, but none is essential, as are the first 100 bp. We have also now demonstrated directly that the steady state level of the L1-lacZ mRNA in cells transfected with the deletants correlates well with the reporter gene activity. Thus, the effects measured are on transcription, not translation.

In two transfected human teratocarcinoma cell lines, NTERA-2D1 and 2102-EP, the pLC1ZA-derived β-gal activity was 76% and 26% of that found for the SV40 promoter derived β-gal activity, respectively. In contrast, in HeLa cells the L1-derived β-gal activity was only 1% the SV40 derived activity. These data indicate that transcription of pLC1ZA is cell-type specific, consistent with our earlier finding on endogenous transcription of genomic L1Hs.

We have also further analyzed the start site of transcription from pLC1ZA using the polymerase chain reaction (PCR) and Northern blot analysis. These experiments indicate that most transcripts from transfected pLC1ZA begin at or near the beginning of the L1Hs 5' untranslated region. A small fraction of the transcripts (< 5%) begin upstream of the L1Hs untranslated region, within the vector. These longer transcripts could result either from imprecise L1Hs transcriptional starts, or from cryptic plasmid promoters. Deletion of upstream plasmid sequences from pLC1ZA reduces β-gal expression several fold indicating that upstream sequences influence the transcription rate but are not necessary for pLC1ZA transcription.

A new construct, pLC3ZA, was prepared in which the L1Hs 5' untranslated region is followed by the L1Hs ORF1 and inter-ORF regions derived from cD11, and by the lacZ gene fused to the cD11 ORFII. Transient transfection of pLC3ZA into NTERA-2D1 cells reveals that a low but detectable level of expression from the ORFII fusion occurs in these cells.

B. Translation of L1Hs Open Reading Frames

Earlier we showed that some, but not all, of the cDNAs we cloned from the cytoplasmic, polyadenylated, full length L1Hs transcripts in NTera2D1 cells yield RNAs that can be translated in vitro to give the predicted ORFI polypeptide. We have now demonstrated the occurrence of ORFI polypeptide in human cells.

We prepared an antiserum to the putative ORFI product by injecting rabbits with a fusion protein synthesized in E. coli. The E. coli carry a plasmid in which the trpE gene is fused, in frame, to L1Hs ORFI. This antiserum specifically recognizes, by Western blotting, an approximately 38 kDa protein, the size expected for the ORFI product. The protein is most abundant in human teratocarcinoma cell line 2102-EP, somewhat less abundant in the teratocarcinoma cell line NTera2D1 and the choriocarcinoma cell line JEG-3, and barely detectable in HeLa and 293 human cells. No reactive material was found in GMO327 or HL60 extracts. After transient transfection with pLC3ZA (see A above), a second reactive band was observed. The pLC3ZA ORFI polypeptide appears to be about 2 kDa larger than the endogenous protein; earlier sequence analysis of the cD11 ORFI incorporated into pLC3ZA predicted a 39.8 kDa polypeptide. It appears that the endogenous ORFI product is either encoded by an L1 (or L1s) with a shorter ORFI or is modified in a way that the pLC3ZA product is not.

C. Studies on the Jockey and Gypsy Transposable Elements of Drosophila

Jockey, a class II retrotransposon is similar to the human LINE-1 elements. In contrast, the gypsy element is a class I retrotransposon with LTRs. Both

jockey and gypsy were discovered in Drosophila melanogaster. This year we detected homologous elements in distantly related Drosophila species: jockey in D. funebris and gypsy in D. virilis. The two elements are transcribed and are mobile in the distantly related species. D. virilis gypsy elements and D. funebris jockey elements were cloned and sequenced. They contain open reading frames that correspond to those in the related D. melanogaster elements. Conserved regions occur in the 5'-untranslated DNA sequences and are likely to be involved in transcription regulation; in one case (a 6 bp conserved core in the jockey elements) this was proved directly.

Our studies strongly suggest a horizontal transmission of gypsy and jockey between distant Drosophila species. This is because species evolutionarily closer to D. melanogaster than D. virilis or D. funebris have no detectable jockey or gypsy elements, respectively.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 05258-11 LB

PERIOD COVERED
October 1, 1989 to September 30, 1990

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)*

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| J. Eldridge | Biochemist | LB | NCI |
| Z.-Y. Lin | Visiting Fellow | LB | NCI |
| C. Dechesne | Visiting Fellow | LB | NCI |
| Q. Wei | Visiting Fellow | LB | NCI |
| R. Masood | Visiting Fellow | LB | NCI |
| S. Nakamura | Visiting Scientist | LB | NCI |

COOPERATING UNITS *(if any)*
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LAB/BRANCH
Laboratory of Biochemistry, DCBD

SECTION
Biochemistry of Gene Expression Section

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

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

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

B

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

We have isolated and characterized a number of muscle-specific and "house-keeping" genes that are differentially expressed during myogenesis (muscle formation) in vitro. These include the alpha cardiac (embryonic muscle and cardiac specific isoform), alpha skeletal (adult skeletal muscle isoform) and beta cytoplasmic actin genes, and the myosin light chain 1f/3f gene. The alpha actin isoforms and the myosin light gene are only expressed in muscle whereas the beta actin gene is expressed in practically all undifferentiated cells and is transcriptionally suppressed in muscle. We have defined the cis regulatory regions of these genes responsible for this regulation in muscle and have made preliminary attempts to study the trans-acting proteins that interact with these regions. Recently we have isolated two different avian genes (CMD1 and CMGN1) that are involved in myogenic determination since the expression of either one of these genes in fibroblasts coverts the cells to a stable muscle phenotype. Cotransfections with either of these genes and a muscle-specific promoter, such as the alpha cardiac actin promoter, joined to a reporter gene like CAT (chloroamphenicol acetyltransferase) or LUC (luciferase) activates the reporter expression. We now have in hand two specific muscle factors and a series of differentially regulated genes from muscle and we are studying the molecular basis of this factor regulation of muscle gene expression/suppression.



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

## Project Description

Objectives:

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

Major Findings:

A. Regulation of Actin Isoform Expression

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

fragment joined to the CMD1 cDNA will convert fibroblasts to muscle. We are trying to identify the regions in the gene involved in autoregulation.

C. Characterization of a Possible Insect Myogenic Factor from Drosophila

Using a PCR fragment for the basic/helix-loop-helix 61 amino acids of CMD1, we screened a 0 to 16 hour embryonic cDNA library from Drosophila and isolated a cDNA, called DMD1, that encodes a polypeptide of 332 amino acids (longest open reading frame) and contains the characteristic basic/helix-loop-helix motif that is approximately 80% identical to CMD1. DMD1 will coactivate a muscle specific promoter in fibroblasts but will not convert the cell to the muscle phenotype. Chimeric proteins are being made between CMD1 and DMD1 to try and localize the conversion and activation domains. The cDNA was used to isolate the gene and both are under investigation in Dr. Walter Gehring's lab in the Biozentrum in Basel, Switzerland (Dr. Paterson is on sabbatical there for one year to study the role of DMD1 in Drosophila myogenesis).

D. 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.

E. Studies on the Yeast Myosin Gene

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

effect. We are trying to isolate the myosin I yeast gene promoter but this has not proved successful.

Publications:

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Van den Pol AN, Decavel C, Levi A, Paterson B. Hypothalamic expression of a novel gene product, VGF; immunocytochemical analysis, J NeuroSci 1989;9:4122-37.

Pash JM, Bhorjee JS, Paterson BM, Bustin M. Persistence of chromosomal proteins HMG-14/17 in myotubes following differentiation-dependent reduction of HMG mRNA, J Biol Chem 1990;265:4197-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 05262-10 LB

PERIOD COVERED: October 1, 1989 to September 30, 1990

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

Eukaryotic Gene Regulation and Function: The Metallothionein System

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

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| M. Ernoult | Visiting Fellow | LB | NCI |
| P. Furst | Guest Researcher | LB | NCI |
| R. Hackett | Staff Fellow | LB | NCI |
| S. Hu | Chemist | LB | NCI |
| V. Culotta | Guest Researcher | LB | NCI |
| C. Xu | Visiting Fellow | LB | NCI |
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COOPERATING UNITS (if any)

Mike Summers and Jose Casas-Finet, U. of Maryland at Baltimore; Dennis Winge, U. of Utah

LAB/BRANCH: Laboratory of Biochemistry, DCBDC

SECTION: Gene Structure and Regulation Section

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

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 8.75 | 8.5 | .25 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Cu ions induce yeast metallothionein gene transcription by altering the conformation and DNA binding activity of the ACE1 transcription factor. We have 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. In support of this model, we have shown that ACE1 binds Cu(I) cooperatively, that the Cu-binding cysteine residues and DNA-binding basic residues are interdigitated within a single domain, and that a purified ACE1 peptide is luminescent. An in vitro transcription system responsive to ACE1 has been developed, and a genetic search for additional metallothionein gene transcription factors has been initiated.



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

Projection Description

Objectives:

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

Major Findings:

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 are trying to understand how ACE1 works at the molecular and atomic level.

A. DNA and Cu Binding Domain: the "Copper Fist" Model

1. ACE1 binds to Cu cooperatively. There are several fascinating structural similarities between the DNA and Cu binding domain of ACE1 and metallothionein itself. Based on these similarities, we speculated that ACE1 forms a polynuclear Cu(I)-S cluster, or "copper fist", which organizes the amino-terminal domain into a specific DNA binding structure. One prediction of this model is that ACE1, like metallothionein, should bind to Cu cooperatively. In support of this, we showed that Cu activates ACE1 for DNA binding in an all-or-none fashion with a Hill coefficient of 4. Ag also activates ACE1 cooperatively, but the Hill coefficient is decreased and the affinity for DNA is weaker. The cooperative interaction between Cu and ACE1 allows the cell to respond to a small increase in metal concentration by a large increase in gene expression.

2. The DNA and Cu binding functions of ACE1 are interdigitated within a single domain. In all eukaryotic transcriptional regulatory factors studied to date, the DNA and small molecule binding functions are contained within discrete, separable domains. A critical prediction of the "copper fist" model is that the two functions are interdigitated within a single region of the protein. In support of this, we showed that mutations in all 11 cysteine residues within the minimal DNA and Cu binding domain are required for productive folding of the protein. In contrast, at least two basic residues are required for specific DNA recognition although not for Cu binding. These basic residues are located in between critical cysteine residues, providing strong evidence for the interdigitated nature of ACE1.

3. Overproduction and preliminary biophysical characterization of the DNA and Cu binding domain. Proof of the "copper fist" model will eventually require 3-dimensional structural data. As a first step, we used the E. coli phage T7 system to overproduce a soluble 110 amino acid peptide containing the DNA and Cu binding domain of ACE1. This peptide has been purified to homogeneity and characterized by UV, emission, and atomic absorption spectroscopy. The peptide contains multiple Cu(I) ions which are tightly bound through Cu(I)-S bonds. It also emits orange luminescence, a hallmark of polynuclear Cu(I)-S cluster proteins. Preliminary 2-D NMR experiments, performed by Mike Summers at U. of

Maryland, indicate a high degree of ordered structure. Crystallization attempts are under way with Paul Sigler's group at Yale.

B. Mechanism of Transcriptional Activation

1. ACE1 is functional in an *in vitro* transcription system. In order to understand how ACE1 activates transcription once it has bound to the template, it was critical to develop an *in vitro* transcription system responsive to the ACE1 protein. We developed such a system which was shown to require the following components: the complete ACE1 protein including its DNA binding and activation domains, a mouse extract to provide polymerase II and general factors, a template containing an ACE1 binding site, and Cu or Ag. We used this system to show that ACE1 is required for both the establishment and maintenance of a committed transcription complex.

2. TFIID is required for yeast metallothionein gene transcription. TFIID, the "TATA binding protein", is thought to play a critical role in RNA polymerase II transcription. We have investigated the role of TFIID in *CUP1* gene transcription both *in vivo* and *in vitro*. A single TATA sequence within the yeast promoter is required for efficient, Cu-inducible transcription both in yeast cells and in a mouse extract supplemented with the ACE1 regulatory protein. This sequence can be functionally replaced by a mouse TATA sequence in the *in vitro* system, and altering the position of this element alters the initiation site. Transcription in a TFIID-depleted mouse extract requires the addition of exogenous TFIID, which can be supplied either from mouse or yeast. Yeast TFIID, produced in bacteria, specifically binds to functional TATA sequences from both yeast and mouse metallothionein genes. Thus transcription of the yeast metallothionein gene requires a general transcription factor, TFIID, in addition to the specific activator protein ACE1.

3. Search for additional factors. We have initiated a genetic search for factors other than ACE1 and TFIID that are involved in the regulation of yeast metallothionein gene transcription. The search involves mutagenesis of an ACE1 deficient strain followed by selection for increased basal transcription of the *CUP1* gene. Over 100 such mutants have been collected, and one of the mutant genes has been cloned. Analysis of this clone should lend new insights into the varied pathways of metallothionein gene transcription.

C. Mammalian Projects

Because of the rapid progress made in yeast, interest in mammalian gene expression has decreased in the lab. One ongoing project is to clone mammalian metallothionein gene regulatory factors by complementation in a yeast strain carrying a metal responsive element linked to a reporter gene. This project is still in the stage of library construction. The search for such a factor by biochemical techniques is being continued by Jean Imbert, who has returned to his own laboratory in France; if clones are obtained, we will share in their characterization. The analysis of a zinc finger protein is being continued by Michelle Ernoult-Lange in her own laboratory, also in France.

Publications:

Furst P, Hamer DH. Cooperative activation of a eukaryotic transcription factor: interaction between Cu(I) and yeast ACE1 protein, Proc Natl Acad Sci USA 1989;86:5267-71.

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 05263-09 LB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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. The transcriptional activator of Drosophila heat shock genes (Heat Shock Factor, HSF) has been cloned. The HSF clone has been expressed in E. coli, and the properties of the recombinant HSF protein are being studied. Whereas the natural HSF protein in the cell exists in an inactive form that is incapable of binding to DNA, the recombinant protein was found to be active for binding DNA and activating transcription of heat shock genes in vitro. These results suggest that the HSF protein is subject to negative regulation within the cell. The availability of recombinant HSF now makes it feasible to design screens for the inhibitory factor (IF). Studies of the interaction between HSF and IF should elucidate the mechanism by which the inactive form of HSF behaves as a sensor of cellular stress. Two transcriptional regulators of the segmentation gene ftz, identified by biochemical screens, have been cloned and sequenced. FTZ-F1, an activator of the ftz gene, is a new member of the steroid hormone receptor superfamily. FTZ-F2 is a repressor of the ftz gene and a new zinc-finger protein. Both FTZ-F1 and FTZ-F2 genes were missed by genetic screens for embryonic lethals with a segmentation phenotype, underscoring the need for biochemical screens to complement genetic studies.

## Project Description

Objectives:

### I. Regulation of the hsp70 gene

A. Cloning the Drosophila HSF Gene

The Drosophila HSF gene was cloned by partial microsequence analysis of HPLC purified peptides derived by trypsin cleavage of purified HSF. Using oligonucleotide probes deduced from the peptide sequences, we have screened cDNA and genomic Drosophila libraries, and obtained the complete coding region for Drosophila HSF. The sequence of the Drosophila HSF reveals a 693 amino acid polypeptide that contains a leucine zipper (coiled-coil) motif, presumably involved in the HSF oligomerization. No obvious similarity to known DNA-binding motifs was found, suggesting the possibility that HSF may recognize its binding site by means of a novel structural motif. The Drosophila HSF gene maps to cytological location 55A on the Drosophila polytene chromosomes. Using sequence homologies between yeast and Drosophila HSF genes, we have obtained a partial sequence of human HSF, and are in the process of cloning the human HSF gene.

B. Expression of Drosophila HSF in E. coli

The Drosophila HSF gene was inserted into an expression vector (Studier T7 system) and expressed in E. coli. Recombinant HSF protein was synthesized at a high level, and was extracted in the soluble fraction of E. coli cell lysates. The recombinant HSF protein was found to bind to DNA specifically, and to stimulate transcription of a heat shock gene in an in vitro transcription assay. A deletion analysis revealed that the DNA-binding ability of HSF was contained within the N-terminal 164 amino acid residues, and the transcription activating ability to the C-terminal 325 residues. Further mutational studies are under way to map the DNA-binding and transcription activating domains more precisely.

C. Regulation of Drosophila HSF Activity

The natural Drosophila HSF protein is normally in an inactive state in the cell cytosol. However, the recombinant HSF protein isolated from E. coli was found to be active for DNA-binding and transcription activation even without heat shock. These results suggest that HSF is normally under negative regulation by a proposed inhibitory factor (IF). Biochemical and molecular genetic studies are under way to identify IF, and to study its mode of interaction with HSF.

D. Chromatin Reconstitution of the hsp70 Gene

The in vitro assembly of heat shock genes in chromatin has been achieved by the use of a Xenopus oocyte assembly system. The assembled chromatin displays the normal nucleosome spacing as gauged by micrococcal nuclease mapping. The presence of nucleosomes represses the ability of the heat shock gene template to be transcribed in a Drosophila embryo cell-free extract that supports vigorous transcription of naked DNA templates. The ability of the heat shock gene chromatin template to respond to HSF activation is now being studied. If successful, this will be the first system in which transcriptional activation

of a chromatin template by a transcription factor has been achieved. The system may provide a means to study under defined conditions in vitro how chromatin structure may play a role in gene regulation.

## II. Regulation of the Fushi Tarazu Gene

### A. FTZ-F1, a Transcriptional Activator of Ftz

We have previously purified FTZ-F1 to homogeneity and shown that it activates the ftz gene in transformed Drosophila embryos. The FTZ-F1 gene has now been cloned, using two parallel strategies. First, peptide microsequencing of purified FTZ-F1 produced a limited partial amino acid sequence of FTZ-F1 protein. Second, expression screening of cDNA libraries with the FTZ-F1 binding site was successful in isolating a putative FTZ-F1 cDNA. All sequences of the tryptic peptides of FTZ-F1 protein were found in the predicted FTZ-F1 protein sequence. Hence, the evidence strongly indicates that we have cloned the FTZ-F1 gene. The sequence of FTZ-F1 reveals a new member of the steroid hormone receptor family. The FTZ-F1 gene has been mapped cytologically to 75CD. Structure-function analyses are under way for this protein.

### B. FTZ-F1, a Transcriptional Repressor of the Ftz Gene

FTZ-F1 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 a transformed Drosophila embryo. The results suggest that FTZ-F2 functions as a transcriptional repressor of the ftz gene. A cDNA clone potentially encoding FTZ-F2 has been isolated, sequenced, and found to encode a new zinc-finger protein. The putative FTZ-F2 clone maps cytologically to 100D. We are currently studying the structure and function of this protein.

### C. Genetics of HSF, FTZ-F1 and FTZ-F2

None of the transcription factors we have cloned map to the locations of known Drosophila mutants. As a first step towards an analysis of the functions of these factors in vivo, we plan to construct dominant negative mutations for these proteins, and to introduce these mutant genes into Drosophila by P-element mediated germ-line transformation.

Publications:

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Ueda H, Sonoda S, Brown JL, Scott MS, Wu C. A sequence-specific DNA-binding protein that activates fushi tarazu segmentation gene expression, Genes and Dev 1990;4:624-35.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05264-09 LB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Intracisternal A-Particle Gene Expression in Pancreatic Beta Cells

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

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

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Biosynthesis Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.0 | 1.0 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

IAPs are constitutively expressed in pancreatic β-cells of certain diabetes-susceptible inbred mouse strains, and increase in response to hyperglycemic stress induced by the db gene in homozygous state. The difference in expression of IAP genes in islet cells of mice susceptible and resistant to induction of diabetes by the db gene provides a useful system in which to examine control of IAP element expression. Sequencing of pancreatic β-cell IAP cDNAs showed that five clones had identical sequences, suggesting that a major IAP transcript, derived from one or a number of identical genes, is present in these cells. In contrast, βTC cells, an established mouse cell line derived from a β-cell tumor, expressed a variety of IAP elements. An oligonucleotide probe specific for the IAP element expressed in pancreatic β-cells detected a subset of the total IAP elements with their flanking sequences in genomic DNA. Among these were two fragments in the DNA of a diabetes susceptible strain of mouse that were not present in a diabetes resistant strain. This probe will be used to determine whether there is a difference in genomic location of these IAP elements in the two strains.





GPO 914-916

## Project Description

### Objectives:

To study factors involved in control of expression of the endogenous proviral elements of the intracisternal A-particle (IAP) gene family and the effect of this expression on normal development and neoplastic transformation. Specifically, to determine the basis for constitutive IAP gene expression in strains of mice susceptible to induction of diabetes by the db gene.

### Major Findings:

IAPs are expressed in pancreatic β-cells of certain diabetes-susceptible inbred mouse strains, and the number of particles increases in response to hyperglycemic stress induced by the db gene in homozygous state. The difference in expression of IAP genes in β-cells of mice susceptible and resistant to induction of diabetes by the db gene provides a useful system in which to examine control of expression of IAP elements. Specifically, why are IAP genes in susceptible mouse strains constitutively expressed? Since the site of integration can have a profound effect on expression of endogenous proviruses, could expression in susceptible strains result from integration of an IAP element(s) in a distinct region of the genome that is constitutively transcribed in these strains? The aim of this project is to isolate the sequences flanking IAP elements which are expressed in glucose-stimulated β-cells from a susceptible mouse strain. The flanking sequences will be used to determine whether there is a difference in the genomic location of the expressed IAP elements in susceptible and resistant mouse strains.

A. cDNA Library of Expressed IAP Elements

A major problem in studying IAP gene expression in pancreatic β-cells of mice is the small amount of tissue and relatively low levels of expression. βTC1 cells, an established cell line derived from a β-cell tumor of C57BL/6J x DBA/2J mice, were used as a model for cDNA library preparation. A cDNA library representing about 1 kb of sequence from the 3' ends of expressed IAP genes was made from total RNA isolated from βTC cells. After conversion of DNaseI treated RNA to first-strand cDNA with oligo(dT) primer and reverse transcriptase, the single-stranded IAP cDNA was amplified by polymerase chain reaction (PCR) using primers from conserved regions of the IAP elements. The PCR product was cloned. Positive clones were selected with an IAP LTR probe. Comparable 950 bp regions from six clones were sequenced. The clones represented a closely related group, but no two were identical to one another. All displayed characteristic differences from previously sequenced IAP clones at a number of positions.

The same basic procedure was used to make a cDNA library from RNA of C57BL/Ks (BKs) (susceptible mouse) glucose-stimulated β-cells. RNA was provided by Dr. Edward Leiter's laboratory. Because of difficulties assuring that the small amount of total RNA was free of DNA, a modification of primer was required to assure that cDNA and not genomic sequences were amplified during PCR. A 3' primer that could only prime on a cDNA derived from a polyadenylated RNA was used. Five cDNA clones were isolated and

sequenced over 950 bp. The clones had identical sequences, suggesting that a major IAP transcript, derived from one or several identical genes, is present in pancreatic β-cells. A limited number of IAP elements has also been found to be expressed in normal mouse lymphocytes (see Kuff). This is in contrast to the variety of IAP elements expressed in tumor cells.

B. Detection of DNA Polymorphisms in Genomic DNA with an Oligo Probe Derived from Sequence of a Beta Cell IAP cDNA

The env region of the β-cell IAP cDNAs included a sequence that differed from the comparable region in previously sequenced IAP clones by 8 of 24 nt. A 24 nt oligo with the β-cell clone sequence, ISL, was used as a probe to analyze BamHI-cut genomic DNA from inbred strains of mice. Such a digest generates junction fragments containing IAP elements with their contiguous flanking sequences. The ISL oligo reacted with a subset of the total IAP fragments, representing approximately 20-50 elements. Among these the probe detected two restriction fragments (8 kb and 18 kb) in the DNA of susceptible strains of mice that were not present in DNA of mice resistant to the effects of the db gene. These two fragments will be cloned from size fractionated BamHI-digested BKs DNA. Unique sequences from the IAP-flanking regions will then be used as probes to determine whether there is a difference in genomic location of these IAP sequences in mice of susceptible and resistant strains.

Publications:

Lueders KK, Grossman Z, Fewell JW. Characterization of amplified intracisternal A-particle elements encoding integrase, Nucleic Acid Res 1989;17:9267-77.

Lueders KK. Transposition of intracisternal A-particle (IAP) genes, Bulletin du Cancer 1989;76:426.

Lueders KK. Genomic organization and expression of endogenous retrovirus-like elements in cultured rodent cells, Biologicals 1990, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 05265-08 LB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 |
| A. Rhoads | Guest Researcher | LB | NCI |
| Y. Yang | Visiting Fellow | LB | NCI |

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Protein Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.2 | 4.2 | 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.)*

To study the mechanism of secretion and its regulation by $Ca^{2+}$, we have been using digitonin-permeabilized PC12 cells and bovine chromaffin cells. The release of catecholamines from these permeabilized cells is both $Ca^{2+}$- and ATP-dependent. We have previously proposed that norepinephrine secretion by PC12 cells is regulated by a $Ca^{2+}$-stimulated phosphorylation. We have tested this model by adding protein phosphatases and phosphatase inhibitors to permeabilized PC12 cells. The effects of added phosphatases and phosphatase inhibitors on norepinephrine secretion are consistent with secretion in permeabilized PC12 cells being regulated by a $Ca^{2+}$-stimulated phosphorylation.

To identify proteins which are involved in the regulation of secretion we have been using digitonin-permeabilized bovine chromaffin cells. Prolonged incubation of permeabilized bovine chromaffin cells results in the 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 secretion. We are currently trying to isolate the proteins in this extract which are responsible for resorting secretory activity.

As reported last year, we find that GTP S induces $Ca^{2+}$-independent norepinephrine secretion in permeabililized PC12 cells. The GTP-binding protein responsible for this stimulation does not appear to be one of the well characterized heterotrimeric G-proteins. The addition of a crude mixture of GTP-binding proteins to permeabilized PC12 cells results in an enhancement of GTP S-stimulated norepinephrine secretion. We are currently characterizing this enhancement and starting to fractionate GTP-binding proteins to determine which is responsible for this stimulation.



PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Regulation of Secretion

Objectives:

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 catacholamines 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 previously used adenosine-5'-O-(3-thiotriphosphate), ATPγS, to examine the role of phosphorylation in the regulation of norepinephrine (NE) secretion in digitonin-permeabilized PC12 cells. On the basis of our results with ATPγS, we proposed that secretion in permeabilized PC12 cells is regulated by a $Ca^{2+}$-stimulated phosphorylation of some as yet unidentified protein. After this phosphorylation (or thiophosphorylation) has occurred, secretion no longer requires $Ca^{2+}$, but it is still ATP-dependent. Since ATPγS induced secretion in the absence of $Ca^{2+}$, we also proposed that there is a basal rate of phosphorylation which occurs in the absence of $Ca^{2+}$. Thus, when permeabilized cells are incubated with ATP in the absence of $Ca^{2+}$, some phosphorylation would occur, but the activities of $Ca^{2+}$-independent phosphatases are sufficient to keep the protein dephosphorylated, and consequently, there is little NE 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 increased levels of NE secretion. This model was based primarily on the effects of ATPγS. As ATPγS might induce secretion by some mechanism other than thiophosphorylation, we have tested this model by examining the effects of protein phosphatases and

phosphatase inhibitors. According to our proposal, phosphatase inhibitors should be able to inhibit dephosphorylation and, thereby, cause an increase in NE secretion from cells incubated with ATP in absence of $Ca^{2+}$. Conversely, addition of protein phosphatases should be able to dephosphorylate this protein and, thereby, cause a decrease in secretion from cells incubated with ATP in the presence of $Ca^{2+}$. Secretion in ATPγS should not be affected by these additions.

We have found that the addition of an exogenous type 2A protein phosphatase to digitonin-permeabilized PC12 cells caused as much as a 70% decrease in $Ca^{2+}$-dependent NE secretion. In the presence of okadaic acid, a potent inhibitor of type 2A protein phosphatases, phosphatase 2A had no effect on secretion. The addition of exogenous calcineurin, a $Ca^{2+}$-calmodulin-stimulated phosphatase, also caused decrease in $Ca^{2+}$-dependent secretion, but on a molar basis it was less effective than phosphatase 2A. Two phosphatase inhibitors, 1-naphthylphosphate and sodium pyrophosphate, caused 75-100% increases in the amount of NE secreted in the absence of $Ca^{2+}$ without affecting the amount of NE secreted in the presence of $Ca^{2+}$. This stimulation of $Ca^{2+}$-independent secretion by 1-naphthylphosphate and pyrophosphate suggests that there is a slow rate of $Ca^{2+}$-independent phosphorylation and that phosphorylation triggers secretion. Unlike the results obtained in the presence of ATP, secretion in the presence of ATPγS was not affected by the addition of type 2A protein phosphatase or by the addition of phosphatase inhibitors. These results are consistent with secretion in permeabilized PC12 cells being regulated by a $Ca^{2+}$-stimulated phosphorylation. Similar experiments are being carried out with digitonin-permeabilized bovine chromaffin cells. Preliminary experiments indicate that secretion in permeabilized chromaffin cells is also regulated by a $Ca^{2+}$-stimulated phosphorylation.

As noted above, prolonged incubation of permeabilized bovine chromaffin cells results in the loss of $Ca^{2+}$-dependent NE secretion. We have found that the addition of a cytosolic extract to these depleted cells restores $Ca^{2+}$-dependent secretion. This extract can be prepared either from isolated chromaffin cells or from the whole adrenal medulla. Digestion of this extract with trypsin or treatment with NEM results in a loss of its ability to restore secretion. Thus, proteins present in this extract are required for restoring secretory activity. NE secretion from depleted cells to which this extract has been added has the same $Ca^{2+}$- and ATP-dependencies as does NE secretion from cells which have not undergone a prolonged incubation. We are currently trying to isolate the proteins in this extract which are responsible for restoring secretory activity.

As reported last year, we find that GTPγS induces $Ca^{2+}$-independent secretion in permeabilized PC12 cells. The effect of GTPγS does not appear to be due to the release of $Ca^{2+}$, activation of protein kinase C, nor to the release of cAMP or cGMP. Cholera and pertussis toxins do not effect GTPγS stimulated NE secretion. These results suggest that the GTP-binding protein responsible for this stimulation is not one of the well characterized heterotrimeric G-proteins. In addition to the heterotrimeric G-proteins, a group of low molecular weight (20 to 30 kDa) GTP-binding proteins have been detected in a variety of mammalian tissues. While the functions of these proteins are not known, a GTP-binding protein of similar size (SEC4 protein) is required for exocytosis in yeast. We have identified four GTP-binding proteins with molecular weights between 22 and 27 kDa in PC12 cells. These protein are present both on the plasma membrane and

on the membranes of secretory vesicles. We have isolated crude mixtures of GTP-binding proteins from bovine brain and from bovine adrenal medulla. These preparations contain both heterotrimeric G-proteins and low molecular GTP-binding proteins. Addition of these mixtures of GTP-binding proteins to permeabilized PC12 cells results in an enhancement of the GTPγS-stimulated NE secretion. We are currently characterizing this enhancement and starting to fractionate GTP-binding proteins to determine which is responsible for this stimulation.

Publications:

Carroll AG, Wagner PD. Protein kinase C phosphorylation of thymus myosin, J Muscle Res and Cell Motility 1989;10:379-84.

Wagner PD, Vu ND. Thiophosphorylation causes $Ca^{2+}$-independent norepinephrine secretion from permeabilized PC12 cells, J Biol Chem 1989;264:19614-20.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 05267-06 LB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 | | | |
| B. Funnell | Visiting Scientist | LB | NCI | | | |
| K. Tilly | Sr. Staff Fellow | LB | NCI | R. Biggs | Student Trainee | |
| N. Treptow | Special Volunteer | LB | NCI | | LB NCI | |
| E. Maguin | Visiting Fellow | LB | NCI | | | |
| G. Mukhopadhyay | Visiting Fellow | LB | NCI | | | |
| S. Sozhamannan | Visiting Fellow | LB | NCI | | | |
| P. Papp | Visiting Fellow | LB | NCI | | | |

COOPERATING UNITS *(if any)*

Dr. S. Wickner, LMB, NCI; Dr. R. Rosenfeld, Hadassah Medical School, Jerusalem, Israel; H. Lehnberr, Biozentrum, Basel, Switzerland

LAB/BRANCH

Laboratory of Biochemistry, DCBDC

SECTION

Microbial Genetics and Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 6.2 | 6.0 | 0.2 |

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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.)*

Mechanisms responsible for the striking stability with which low copy number plasmids are inherited continue to be the subject of our research. In the period of this report, considerable understanding has been gained of the structure of ori and parS, the cis-acting sites involved respectively in plasmid replication and plasmid partition. The emphasis this year has been on how these sites change in shape and activity as a consequence of interactions with specific plasmid-determined and host-determined proteins or by interaction with regulatory DNA sequences. The development of means for recognizing and measuring structural changes that occur when the P1 initiator protein, RepA, interacts with its binding sites will enable the specification of RepA domains implicated in each of its several functions. Of particular significance is the genetic evidence suggesting that three heat shock proteins participate in concert to increase the affinity of RepA for its binding sites. At least one of these proteins, DnaK, is highly homologous to proteins of higher organisms. The most dramatic of the interactions studied, because of the unusual magnitude of its effect, is the binding of the accessory host factor IHF to parS. This binding induces a 10,000 fold increase in the affinity of the P1-encoded partition protein ParB for the plasmid centromere, parS, provided that the plasmid DNA is supercoiled.

## Project Description

Objectives:

We seek, through studies of bacteriophage P1, to understand the molecular basis for 1) the control of initiation of DNA replication, 2) the stable maintenance of replicons by a) their equitable distribution to daughter cells and b) prevention of the propagation of cells from which the replicon is lost. We also aim to provide a basis for understanding the overall genetic organization of this versatile organism which is providing powerful new techniques for generating and manipulating an exhaustive library of the human genome (Ref. 1,3).

A. Deletion Analysis of ori, the P1 Plasmid Origin.

The limits of ori required for efficient replication have been determined both in vivo and in vitro in a collaborative study with S. Wickner et al. (Ref. 4). Of five clustered binding sites for the P1 initiator protein, RepA, only three need remain intact in vitro, whereas the complete set appears essential in vivo. The basis for this difference remains to be determined.

B. incA-Insensitive repA Mutants With Which to Test a Model of Replication Control by Steric Hindrance.

Copy number control locus incA consists of nine clustered binding sites for RepA, located about one kb from the cluster of five similar sites at the origin. Steric hindrance to ori function by RepA-mediated pairing of incA with ori was previously suggested by electron microscopical observations in this laboratory. Mutations in repA that confer insensitivity to replication inhibition by incA have recently been isolated by Papp and Chattoraj. The mutations will be tested for their effects on RepA-mediated pairing using two newly developed in vivo and in vitro assays that are more reliable and more convenient than electron microscopy.

C. Influence of RepA on ori Conformation.

By use of a vector designed by S. Adhya (LMB) to measure DNA bending, Mukhopadhyay and Chattoraj determined that a single RepA binding site (of which there are five in ori) is significantly bent upon complexing with RepA. Bending increases with the participation of additional sites. A screen of mutant initiator proteins based on these findings should provide the means to determine the relevance of DNA conformational changes induced by RepA to RepA function in initiating DNA synthesis.

D. Location of a Second Replication Control Element Encoded Within repA.

Modest overexpression of repA transcription in vivo is inhibitory to RepA function as initiator. Genetic studies that bear on the nature of the inhibitory molecule and its possible role in preventing replication overshoot have been submitted by Muraiso, Mukhopadhyay and Chattoraj for publication.

E. Specification of Effects of Heat Shock Proteins on RepA Function.

Genetic evidence has been obtained by Tilly, Chattoraj, and Yarmolinsky for the participation of three heat shock proteins in RepA autoregulation, implicating these proteins in determining the affinity of RepA for its specific binding sites. These findings open the way to in vitro studies of heat shock protein involvement in protein folding and provide an explanation for a previous finding (Ref. 2) that P1 and F plasmid replication in vivo depend on these same proteins. In vitro studies of how heat shock proteins DnaJ and DnaK affect RepA-DNA complexes were initiated by S. Dasgupta and Chattoraj and are being continued by Chattoraj. A disaggregation effect of these proteins is under study.

F. Localization of Addictive Functions of P1

The localization of two genetic elements that together confer an apparent stability to P1 plasmid by causing death of cured segregants was undertaken by Maguin and Yarmolinsky in collaboration with H. Lehnherr. The constitutive lethal function was localized within 2.5 kb, the unstable antidote (responsible for the survival of P1 lysogens) to within 0.6 kb.

G. Assay for Partitioning in the Absence of Replication.

An assay has been developed by Treptow and Yarmolinsky that permits the demonstration of DNA partitioning between daughter cells that do not support replication of the partitioned DNA. The assay provides a test of partitioning models that are being evaluated by computer simulations performed in collaboration with R. Rosenfeld.

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

The continued characterization by Funnell of an early step in partition reveals that the P1-encoded ParB protein and accessory host protein IHF bind cooperatively with respect to each other: IHF increases the affinity of ParB for parS at least 10,000 fold; ParB increases the IHF affinity at least 100 fold. IHF introduces a strong bend at its binding site in parS. ParB binds in the following order of preference: supercoiled > circular > linear plasmid DNA. These observations indicate that the geometry of the complex, as well as the torsional stress provided by superhelicity, are important for its stability. The data support the view that in the complex ParB contacts all its binding sites (which flank the IHF binding site) simultaneously. Funnell's experiments continue to support the assertion that IHF-insensitive variants of parS differ only in their affinity for ParB, not in their specificity, despite recent claims from another laboratory of evidence for a specificity change as well.

Publications:

Yarmolinsky, MB, Hansen EB, Jafri S, Chattoraj DK. Participation of the lytic replicon in P1 plasmid maintenance, J Bacteriol 1989;171:4785-91.

Tilly K, Yarmolinsky M. Participation of Escherichia coli heat shock proteins DnaJ, DnaK, and GrpE in P1 plasmid replication. J Bacteriol 1989;171:6025-29.

Yarmolinsky MB. Bacteriophage P1. In: O'Brien SJ, ed. Locus maps of complex genomes. 5th ed. New York: Cold Spring Harbor Laboratory Press, 1990;(Book 1): 52-62.

Wickner S, Hoskins J, Chattoraj D, McKinney K. Deletion analysis of the mini-P1 origin of replication and the role of E. coli DnaA protein. J Biol Chem 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 05268-03 LB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Mechanisms of Meiotic Recombination

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

| | | | |
|---|---|---|---|
| M. Lichten | Senior Staff Fellow | LB | NCI |
| T-C. Wu | Chemist | LB | NCI |
| C. Goyon | Visiting Fellow | LB | NCI |
| M. Daly | IRTA Fellow | LB | NCI |

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry, DCBD

SECTION

Developmental Biochemistry and Genetics

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4 | 4 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors B

☐ (a2) Interviews

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

We have continued research aimed at understanding the molecular mechanism of meiotic recombination in the yeast Saccharomyces cerevisiae and the means by which yeast exerts control over meiotic recombination. We have examined the timing of various steps in meiotic recombination, using several different experimental approaches to detect recombinant DNA molecules and recombination intermediates. We have isolated several short regions of the yeast genome that contain sequences that stimulate meiotic recombination, and are continuing the analysis of the stimulator elements these regions contain.

## Project Description

### Objectives:

Our ultimate objective is an understanding of the molecular mechanism of meiotic recombination, using the yeast Saccharomyces cerevisiae as a model system. Towards this understanding, we have begun a series of experiments designed to describe, at the molecular level, the entire process of meiotic recombination, from initiating lesion, through intermediate structures, to production of mature recombinant products. As an initial step, we have examined the timing of formation of heteroduplex DNA during meiosis.

We have continued our examination of the means by which the level of meiotic recombination is controlled in different parts of the genome. Several clones have been isolated that contain portable promoters of meiotic recombination; characterization of these elements is in progress.

### Major Techniques Employed and Major Findings:

A. Timing of Recombination Events During Meiosis

Christophe Goyon has used physical methods to examine the timing of meiotic recombination events in synchronized yeast cultures. He followed the formation of heteroduplex DNA using denaturant-gel electrophoresis, and determined the time of appearance of the physical products of crossing-over using markers that confer restriction site polymorphisms. He also observed the appearance of double-strand DNA breaks, which have been suggested as initiators of recombination. To date, he has examined both the normal ARG4 locus and arg4 sequences in a plasmid inserted at the MAT locus. Heteroduplex DNA and crossover products appeared at the same time during meiosis, consistent with the suggestion that only a short period of time separates the formation of stable intermediate structures from their resolution as mature recombinant molecules. A double-strand break appeared at a specific site upstream of ARG4, as previously described (Sun et al., Nature 338:87). The timing of appearance and disappearance of this break was consistent with a role as a recombination precursor. Dr. Goyon is currently developing techniques to allow isolation and characterization of joint molecules that are recombination intermediates.

B. Analysis of Meiosis-Induced Double-Strand Breaks

As mentioned above, double-strand breaks have been suggested as the initiating lesion in meiotic recombination. During his study of the timing of recombination events during meiosis, Dr. Goyon also observed a double-strand break in sequences upstream of the arg4 gene inserted at MAT. Although time of appearance of this break was similar to that of the break upstream of the normal ARG4 locus, it was located about 1 kb upstream of sequences corresponding to the site of the break observed at the normal ARG4 locus. This observation implies that formation of double-strand breaks during meiosis does not involve simply the recognition of a short nucleotide sequence, but may rather involve recognition of a more complex chromosomal

structure. Dr. Goyon and Carol Wu are currently engaged in an examination of the exact nature of these structures.

C. Sequences That Modulate the Level of Meiotic Recombination

Michael Daly is continuing his search for sequences in the yeast genome that stimulate meiotic recombination in their vicinity, using a procedure (described in the previous report) that allows rapid screening of large numbers of cloned fragments for "hot spot" activity. He has, to date, identified four 2-3 kb fragments that contain recombination stimulators. These recombination stimulators are meiosis specific (they do not affect mitotic recombination), and are "portable", as they stimulate meiotic recombination when inserted at either of two different loci. He is currently subcloning portions of these fragments to further localize the stimulators, with the aim of determining the nucleotide sequence of each element. Carol Wu is also engaged in a search for sequences that stimulate meiotic recombination, but is taking a more directed approach. She has started with a 2.2 kb DNA fragment containing the LEU2 gene inserted at the URA3 locus; at this locus, LEU2 displays a low level of meiotic recombination. She is currently adding sequence that flank the normal LEU2 locus (which displays a high level of meiotic recombination) to the "cold" copy of LEU2 inserted at URA3, with the aim of localizing sequences responsible for the high recombination activity of the normal LEU2 locus. To date, she has identified a stimulator in the 1.3 kb of DNA immediately 3' to the normal LEU2 gene, and has also identified a short region 5' of LEU2 that appears to repress meiotic recombination.

Publications:

Lichten M, Haber JE. Position effects in ectopic and allelic mitotic recombination in Saccharomyces cerevisiae. Genetics 1989;123:261-8.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 05269-03 LB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
DNA Synthesis in Mammalian Cells: Studies of Nucleic Acid Binding Proteins

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

| | | | |
|---|---|---|---|
| S. H. Wilson | Medical Officer | LB | NCI |
| A. Kumar | Expert | LB | NCI |

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

LAB/BRANCH
Laboratory of Biochemistry, DCBDC

SECTION
Nucleic Acid Enzymology Section

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

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.6 | 0.3 | 0.3 |

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.)*

Physical biochemical studies of a single-stranded nucleic acid binding protein termed A1 were conducted. The protein binds cooperatively to either RNA or DNA and the full length protein binds much tighter than a truncated A1 protein lacking the glycine-rich COOH-terminal domain (residues 185-319). Our studies suggest the mechanism of A1 binding is, in some respects, similar to that of two well-known prokaryotic ssDNA binding proteins: Binding involves charge-charge interactions and close approach of aromatic amino acids to nucleotide bases. Our findings indicate that both the $NH_2$-terminal and COOH-terminal domains of the intact A1 protein make significant contributions to the overall free energy of binding of A1 to nucleic acids. Both the COOH-terminal fragment and intact protein have strand annealing activity for both RNA and ssDNA. This is of particular interest, since the results suggest that A1 could play a role in pre-mRNA splicing and/or homologous DNA recombination.

## Project Description

### Objectives:

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

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

### Major Findings:

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

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

   With a view toward understanding structure-function relationships of a protein component of the eukaryotic heterogeneous ribonucleoprotein complex, we have studied the interactions of recombinant A1 protein, its N-terminal two-thirds (UP1), and C-terminal domain with the fluorescent polynucleotide, poly (ethenoadenylic acid), poly($\varepsilon$A). The overall binding affinity of A1 for poly($\varepsilon$A) is significantly greater than that of UP1. This can be partly, but not entirely explained by the existence of cooperativity in A1-nucleic acid interactions: The cooperativity parameter, $\omega$, for A1-poly ($\varepsilon$A) interaction is about 30, whereas UP1 binds nucleic acids non-cooperatively ($\omega$=1). Gel retardation experiments performed with M13 DNA also show cooperative binding for intact A1 and non-cooperative binding for UP1. We now have found conditions for controlled proteolysis of A1 such that the COOH-terminal domain can be isolated in good yield. Binding experiments with the C-terminal domain show that this polypeptide is capable of binding to single-stranded nucleic acids. These results suggest that intact A1 has at least two potential binding regions for nucleic acids: one within the N-terminal domain and one within the C-terminal domain. Both intact A1 and the C-terminal domain are capable of protein-protein association.

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 incidently 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 very 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. This is of particular interest because it suggests a possible role of the annealing activity in pre-mRNA splicing. Indeed, we have shown that single-stranded oligonucleotides corresponding to the consensus mRNA splice injunction and the snRNA complimentary sequence are strongly hybridized by the A1 protein.

Publications

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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, UP1, with RNA and DNA, J Biol Chem, in press.

Kumar A, Casas-Finet JR, Luneau C, Karpel R, Merrill B, Williams KR, Wilson SH. Nucleic acid binding properties of the C-terminal domain fragment of mammalian A1 hnRNP, J Biol Chem, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 05270-03 LB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

DNA Synthesis in Mammalian Cells: Mechanism of HIV Reverse Transcriptase

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

| | | | | |
|---|---|---|---|---|
| S. H. Wilson | Medical Officer | LB | NCI | 40% of total effort |
| J. Abbotts | IRTA Fellow | LB | NCI | |
| P. Becerra | Guest Researcher | LB | NCI | |
| A. Kumar | Expert | LB | NCI | |
| S. Widen | IRTA Fellow | LB | NCI | |

COOPERATING UNITS *(if any)*

S. Boucage, FDA; S. Hughes, NCI-FCRF; S. Shiloach, NIH; P. Torrence, NIH, T. Kunkel, NIH; A. Gronenborn, NIH; S. Stahl, NIH; D. Davies, NIH, R. Suhadolnik, Temple Univ.; D. Hatfield, NIH; W. Egan, FDA.

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SECTION

Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.5 | 3.5 | |

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.)*

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 two 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 4 subfragments were overproduced from E. coli and purified. These recombinant enzymes and proteins are under study. HIV 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 interaction and have shown that a peptide inhibitor can block formation of the normal complex. Possible use of this peptide inhibitor as an antiviral agent is under study. Finally, we found that 3'-azido-dTTP (AZTTP) is a linear competitive inhibitor of DNA synthesis ($K_i$ = 20 nM) against substrate dTTP in the poly[r(A)]·oligo-[d(T)] system. Kinetic studies of DNA synthesis using AZTTP as substrate revealed that the $K_m$ is 3 μM. Using these values, a method was devised for calculation of rate constants for the enzyme-poly[r(A)]·oligo[d(T)] association($k_{on}$ = 2.6 x $10^8$ $M^{-1}s^{-1}$) and dissociation ($k_{off}$ = $39s^{-1}$), along with a KD value for dTTP binding (∿180 nM).

## 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 is described for overexpression and purification of the enzyme. The precise RT coding region of HXB2 proviral DNA is flanked by start and stop codons, and expression is driven by the temperature-inducible $\lambda$ $P_L$ 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 homodimer of the p66 polypeptide. p66RT, 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 trunctation 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, crystalized, 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.

B. Mapping of a Dimerization Domain in the HIV Reverse Transcriptase

1. The HIV reverse transcriptase routinely isolated from virions is a heterodimer 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 specifically and tightly ($K_d$=$10^{-12}$M) to a recombinant HIV RT p51 peptide, representing a C-terminal truncation of the p66 peptide. The resistance of this high affinity protein-protein binding to the presence of >1 M NaCl suggests that the interaction is largely hydrophobic. Further C-terminal truncation, yielding a p29 polypeptide, completely eliminates complex formation with p66, indicating that the central region of RT may be a dimerization domain responsible for this hydrophobic protein-protein interaction.

This idea was confirmed by conducting similar binding studies with the p20 peptide corresponding to the central region of p66 reverse transcriptase. p20 itself binds to p66 and also is able to block binding by the p51 peptide. The p15 peptide is unable to bind to p66. Thus, the p20 peptide contains a dimerization domain for reverse transcriptase. Analysis of possible antiviral activity of this p20 peptide is under way (collaboration with G. Pavlakis, NCI-FCRF).

2. Primer and dNTP recognition by purified HIV reverse transcriptase were further investigated. Earlier kinetic studies indicated that the reaction pathway for DNA synthesis is ordered, with template-primer and free enzyme combining to form the first complex in the reaction sequence (Majumdar et al., 1988). Through use of a substrate analogue for primer, further evidence was obtained for this first complex in the pathway, and rate values for enzyme-primer association and dissociation were calculated. We found that 3'azido-dTTP is a linear competitive inhibitor of DNA synthesis against the substrate dNTP (dTTP) in the poly [r(A)]-oligo [d(T)] replication system. This indicates that 3'azido-dTTP and dTTP combine with the same form of the enzyme in the reaction scheme, i.e., the enzyme-primer complex. Since the inhibition with 3'azido-dTTP is linear competitive, the dissociation constant is equal to the $K_i$ for inhibition (20 nM). Next, subtrate kinetic studies of DNA synthesis using 3'azido dTTP as substrate revealed that the $K_m$ is 2μM. Therefore, the $K_m$ for this substrate analogue is 100-fold higher than the $K_D$ for binding to the enzyme-primer complex. This difference is consistent with the proposed kinetic scheme and is due to the effect of enzyme-primer complex dissociation on the value of $K_m$. These results enable calculation of real time rate values for the enzyme-oligo dT association ($k_{on}$=$2.4\times10^{8}M^{-1}s^{-1}$) and dissociation ($k_{off}$=$35s^{-1}$).

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 (approximately 30%) using UV cross-linking under appropriate conditions. 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) and have found that 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 trypsin, and peptides have been resolved by HPLC. Peptides containing label are being sequenced (collaboration with K. Williams, Yale).

3. AZT and ddI exhibit a slight degree of toxicity toward normal cells, and one of the most likley targets for this effect is the cellular DNA repair enzyme DNA polymerase beta. We have overproduced active human DNA polymerase beta from E. coli and now are collaborating with other NCI 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.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08212-16 LB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
From Gene to Protein: Structure, Function, and Control in Eukaryotic Cells

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

| | | | |
|---|---|---|---|
| S. L. Berger | Research Chemist | LB | NCI |
| R. E. Manrow | Senior Staff Fellow | LB | NCI |
| M. S. Krug | Senior Staff Fellow | LB | NCI |
| A. R. Sburlati | Visiting Associate | LB | NCI |

COOPERATING UNITS (if any)
None

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

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.0 | 4.0 | |

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither B
☐ (a1) Minors
☐ (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 small, highly acidic protein originally thought to be a precursor for secreted thymic peptides. Our studies are not consistent with this view; they indicate that prothymosin α is regulated in a cell cycle dependent manner, and that cell division depends on sufficient quantities of prothymosin α. These results are based, in part, on a two step purification of prothymosin α which produces a virtually homogeneous protein. Methods have also been devised for localizing prothymosin α in the cell. Our results indicate that the protein is able to enter the nucleus and that a carboxyl terminal sequence is both necessary and sufficient for this process.

The mechanism of action of the ribonuclease H activity of reverse transcriptase from avian myeloblastosis virus and human immunodeficiency virus has been investigated using carefully constructed substrates which eliminate enzyme processivity. Under these conditions, the time course of the reaction is biphasic with a burst preceding a much slower steady state rate. The existence of a burst made possible the evaluation of three out of four rate constants that govern the hydrolysis of RNA; the rate limiting step is the regeneration of free enzyme. From the kinetics of the burst and the steady state rate, the values of $K_D$ and $K_M$ for the DNA:RNA hybrid in the RNase H reaction were determined and compared with the value of $K_M$ for the hybrid in the polymerase reaction. Further insight into substrate binding was obtained using inhibitors. The inhibition by ribonucleoside vanadyl complexes indicated that both the value of $K_i$ for the inhibitor and the complex pattern of inhibition were the same for the DNA:RNA hybrid regardless of which reaction was being studied. The combined data fit a model in which the DNA:RNA substrate is rigidly held in a single substrate binding site formed by the interaction of the two subunits of HIV reverse transcriptase. The binding site for deoxyribonucleoside triphosphates is separate and not affected by vanadyl complexes.

## Project Description

### Prothymosin α

#### Objectives:

The transition from quiescence to rapid growth and cell division is accompanied by dramatic changes at all levels of cellular organization. In normal lymphocytes, the initiation of cell growth and proliferation can be brought about by treatment with mitogens or antigens. Similarly, growth arrested cells undergo many of the same processes upon release from arresting conditions. Macromolecules responsible for regulating access to the cell cycle, progression through it, and entry into and exit from M phase are now beginning to be isolated and characterized.

A gene family that affects cell division has been identified in this laboratory. The encoded protein, prothymosin α, appears to be regulated in a cell cycle dependent manner. Upon stimulation of cells, prothymosin α levels increase as the cell cycle progresses; under conditions in which prothymosin α synthesis is curtailed, cells are able to synthesize RNA and protein, but they are unable to divide. It is our aim to understand the role played by prothymosin α in cell growth and division. Toward this end, it was necessary to determine the cellular location of the protein, the posttranslational modifications, if any, that it sustains, and the molecules with which it associates.

#### Major Findings:

Prothymosin α was first isolated from the thymus gland of the rat by Horecker and coworkers in 1984. Traditionally, it was believed to be a precursor for a variety of secreted hormone-like peptides, among which was thymosin $\alpha_1$. Thymosin $\alpha_1$ was reported to function in the immune system by enhancing immunoresponsiveness, promoting maturation of lymphoid cells and augmenting lymphokine production. In vivo, the peptide could also protect susceptible animals from opportunistic infections caused by Candida albicans. As more information accumulated, however, this position began to erode. When the thymus was extracted under strictly denaturing conditions, there was scant thymosin $\alpha_1$. Furthermore, material originally believed to be thymosin $\alpha_1$ in the peripheral circulation, on closer examination, turned out to be prothymosin α. Apparently, thymosin $\alpha_1$ was a relatively stable artifact of isolation derived from the first 28 amino acids of the supposed precursor.

Earlier, we presented evidence that prothymosin α was not a secreted protein, was not limited to the thymus gland, and was not involved in an immunological function. Studies of human prothymosin α cDNAs, mRNAs, cloned genes, transfected genes, and synthetic and natural proteins were not consistent with the proposed extracellular function. Rather, we classified prothymosin α as an intracellular protein involved in cell division.

In the past year, we have acquired a more detailed picture of the functional role of prothymosin α by preparing an antibody, by localizing the protein in the cell, and by devising a two step purification procedure. We have

also developed methods for manipulating prothymosin α levels in order to determine the cellular effects of prothymosin α deprivation.

Efforts to raise an antibody against prothymosin α, itself, or peptides derived from it have shown that the protein is virtually nonimmunogenic. Therefore, we have constructed a series of fusion proteins in order to produce a varied set, hopefully with increased antigenicity. The fusion proteins were synthesized in Escherichia coli transformed with pATH vectors containing sequences for anthranilate synthetase (the trpE gene) fused in frame with the following: the complete prothymosin α coding sequence; the sequence for 38 amino acids from the amino terminus; or the DNA encoding 19 amino acids from the carboxyl terminus. Proteins were evaluated and purified by SDS polyacrylamide gel electrophoresis. When injected into either rabbits, or animals less closely related to humans on the evolutionary scale, namely chickens, all animals produced a measurable response to the trpE moiety of the fusion protein. However, the serum from only one rabbit recognized prothymosin α sequences antigenically; that rabbit had received anthranilate synthetase fused with the complete sequence of prothymosin α. Although the antiserum does not retain specificity after dilutions of greater than 10-fold, it has nevertheless allowed us to recognize prothymosin α in crude samples of proteins isolated either from cells transfected with the prothymosin α gene, or from cells in culture expressing prothymosin α in a more regulated manner. These experiments show that prothymosin α is posttranslationally modified, but that it is not covalently attached to an RNA molecule as reported by Bogdanov and coworkers.

Since the antibody to prothymosin α can be used for qualitative evaluation of cellular proteins, but not for quantitative work, we have used two dimensional gels as an assay for prothymosin α. Prothymosin α is a highly acidic protein with 53 or 54 acidic residues out of a total of 109 or 110 amino acids, depending on which form of the alternatively spliced gene occurs in the experimental system under study. The unusual amino acid composition, together with its small size, make it easy to resolve the protein electrophoretically. Nevertheless, this approach is not ideal because proteins must be labeled in order to identify prothymosin α in samples containing amounts of material which do not exceed the capacity of the gel. To study total prothymosin α without the necessity for labeling, we have developed a purification scheme which requires only two steps to isolate prothymosin α in virtually homogeneous form starting from whole cells. This scheme will make possible cell cycle studies of the amount and location of all forms of the protein.

We have succeeded in localizing prothymosin α within the cell. In preliminary experiments with COS cells overexpressing the prothymosin α gene product, we found no prothymosin α in nuclei obtained by biochemical means. However, in these same experiments, approximately 70% of the T-antigen in COS cells leaked out of the nuclei. Since T-antigen is a known nuclear protein very much larger than prothymosin α, it is clear that such experiments are inconclusive. Nevertheless, Haritos and colleagues inferred that prothymosin α was a cytoplasmic protein based on a similar approach lacking in adequate controls. In order to avoid the problem of leakage of proteins during the isolation of nuclei, we enucleated COS cells in a more gentle manner. When treated with cytochalasin B and subsequently centrifuged, the nuclei bleb out of the cells surrounded by a sealed fragment of plasma membrane; small, weakly

bound proteins cannot escape under these conditions. When labeled proteins from cytoplasts and nuclei were evaluated electrophoretically, we discovered that fully half the prothymosin α in the cell was nuclear. Although these data strongly suggested that prothymosin α was a nuclear protein, we were not able to rule out the possibility that the overexpressed prothymosin α simply diffused throughout the cell, including the nucleus, during its perambulations. It is well known that the nuclear membrane and the nuclear pore complex do not pose a barrier to the diffusion of small proteins. The situation was resolved by constructing two prothymosin α/β galactosidase fusion proteins in pCH110, with the prothymosin α sequences located either at the amino or at the carboxyl terminus. Both constructs produced fusion proteins with molecular weights of approximately 130,000, when transfected into COS cells. The fusion proteins were detected by immunofluorescence using a mouse monoclonal antibody against β galactosidase and rhodamine-conjugated goat-anti-mouse IgG antibodies. In both cases, the covalently attached prothymosin α sequences directed the fusion protein to the nucleus, whereas β galactosidase, by itself, was excluded from the nucleus. These results prove that prothymosin α is a nuclear protein. We believe that the case against prothymosin α as a secreted protein is complete; there is no evidence to support an extracellular function.

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. The polymerase has been the object of several mechanistic studies and the target of many drug development projects. In contrast, virtually nothing is known about the RNase H activity except the location of the RNase H domain in the primary amino acid sequence of the enzyme. It is our aim to understand the mechanism of the RNase H activity, to distinguish features that are either common to or different from those of the polymerase activity, and to identify and characterize inhibitors.

Major Findings:

The RNase H activity of reverse transcriptase from human immunodeficiency virus and avian myeloblastosis virus has been examined with simplified substrates in order to dissect a complicated reaction into its component parts. The substrates consist of 3'-end labeled globin mRNA either hybridized to or covalently attached to oligo$(dT)_{15}$. Upon incubation with RNase H, the poly(A) tail of the globin mRNA is cleaved and released into the trichloroacetic acid soluble fraction during subsequent manipulations. Since each substrate molecule has only one labeled moiety, the enzyme must dissociate from the products, and reassociate with substrates before a second catalytic event can occur. In brief, we have forced the reaction to be distributive.

The time course of RNA hydrolysis was examined with a recombinant HIV reverse transcriptase. The reaction consisted of a very rapid component followed by a much slower steady state rate. The rapid phase of such a biphasic reaction is known as a burst; it occurs when the formation of products is not

rate limiting. In this case, the rate of dissociation of products from enzyme determined the steady state rate of the reaction. Thus, the existence of the burst made possible measurements of several kinetic constants as follows: since the products and the substrates of RNase H catalysis are almost identical, it became clear that the dissociation of products was qualitatively similar to the dissociation of substrates; hence, a second rate constant was ascertained. The rate at which the burst occurred provided yet another, namely, the rate constant governing the association of substrate with enzyme. The only rate constant that has eluded us governs the rate of hydrolysis of RNA in the hybrid.

From the burst and the steady state RNase H reaction, $K_D$ and $K_M$ for the DNA:RNA hybrid could be measured. These values are approximately 10-20 nM and 30 nM, respectively, with the difference reflecting the effect of rate constants on $K_M$. Upon comparing $K_D$ in the RNase H reaction with $K_M$ for the DNA:RNA hybrid measured during polymerization, a new concept emerged. The values were similar; $K_M$ for covalently attached globin mRNA-oligo(dT) is 12 nM in the polymerase reaction. This fact suggests that the DNA:RNA substrate is bound quantitatively in the same manner in both reactions.

A similar conclusion could be drawn from inhibitor studies. Ribonucleoside vanadyl complexes inhibit both the polymerase activity and the RNase H activity of reverse transcriptase, as well as the reaction catalyzed by the cellular RNase H from Escherichia coli. The inhibition is surprising because vanadyl complexes are transition state analogs for the 2',3' cyclic monophosphates that occur during catalysis by many ribonucleases including pancreatic ribonuclease A. Parenthetically, the products of RNase H reactions are 5'-phosphates and 3'-hydroxyl groups, neither of which resembles vanadyl complexes. Vanadyl complexes were tested as inhibitors in both the RNase H reaction and the polymease reaction with either the DNA:RNA hybrid or deoxyribonucleoside triphosphates (dNTPs), where appropriate, serving as the variable substrate. The complexes inhibited noncompetitively with respect to dNTPs in the polymerase reaction, but exhibited mixed inhibition with respect to the DNA:RNA hybrid substrate. A similar pattern of inhibition was observed when the complexes were tested in the RNase H reaction. In both reactions when globin:oligo(dT) was the variable substrate, the mixed inhibition was largely competitive, that is, there was a large effect on $K_M$, and a very small effect on $V_{MAX}$. The value of $K_i$ was 2-3 mM in all cases. These results suggest that the detailed binding of the DNA:RNA hybrid is identical regardless of the reaction in which reverse transcriptase is engaged. The data support a model in which the DNA:RNA hybrid is held rigidly in a single site.

Comparison of the kinetic constants governing the viral RNase H activity with those of the cellular enzyme from E. coli indicated that both $K_M$ and $V_{MAX}$ for the DNA:RNA hybrid were 10-fold higher for the cellular enzyme. Furthermore, vanadyl complexes inhibited the cellular enzyme with approximately the same value of $K_i$, but the pattern of inhibition showed a pronounced effect on $V_{MAX}$. From these results, it can be inferred that the E. coli enzyme has a more open, flexible active site than the viral enzymes. Earlier experiments which showed that E. coli RNase H, but not the viral enzymes, is able to cleave strained circular RNA molecules hybridized to DNA are consistent with this view.

Although the E. coli enzyme and the viral enzymes are similar, there is nevertheless reason to believe that specific inhibition of the viral enzymes can be accomplished. Our study of inhibitors suggests that the binding sites for the two substrates of the polymerase activity of reverse transcriptase are independent. Thus, mixtures of inhibitors which target both the template primer site and the dNTP site of the viral enzymes simultaneously might be potent inhibitors of viral function without having a substantive effect on the normal activities of the cell.

Publications:

None.

SUMMARY STATEMENT

ANNUAL REPORT
LABORATORY OF CELLULAR ONCOLOGY
DCBDC, NCI
October 1, 1989 through September 30, 1990

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 have made a series of chimeras between *ras* and the K*rev*-1 gene, which encodes a ras-like protein that can suppress ras-transformed cells. The results indicated that the *ras*-specific and K*rev*-1-specific amino acids immediately surrounding residues 32-44, which are identical between the two proteins, determined whether the protein induced cellular transformation or suppressed *ras* transformation. Since earlier observations by us and others have implicated this region in effector function, we suggest that K*rev*-1 may suppress *ras*-induced transformation by interfering with the interaction between ras protein and its effector. The influence of GAP (GTPase activating protein) on cell transformation by *ras* and other oncogenes was also examined. Overexpression of GAP in NIH 3T3 cells inhibited and suppressed transformation by c-*ras*, c-*src* and an activated c-*src* mutant, but not by v-*ras* or v-*mos*. We conclude GAP as a negative regulator is limiting in cells and overexpression of GAP can inhibit transformation by *ras*-dependent oncogenes. Our results make it unlikely that GAP by itself is the ras target, but they are compatible with the possibility that GAP may be part of a complex that forms the ras target.

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

This project is studying transformation by human and animal papillomaviruses. Certain genital human papillomaviruses (HPV), types 6 and 11, are frequently associated with benign condylomas but rarely with carcinomas, while other types, HPV16 and 18, are less frequently detected in condylomas but are the predominate types detected in genital carcinomas. Since the viral E6 and E7 genes are selectively retained and expressed in the carcinomas, we have undertaken a comparative analysis of these genes from the presumed low risk and high risk types. We have determined that the in vitro transforming activities of E6 and E7 from HPV6 are lower than those of E6 and E7 from HPV16 and 18; these results have also been correlated with lower efficiency of Rb binding and casein kinase II phosphorylation by the E7 encoded protein of HPV6, compared with E7 protein of HPV16 and 18. The data provide strong experimental support for the

hypothesis that the high risk types, in particular their E6 and E7 genes, play an important role in the genesis of human genital cancer and offer insight into the possible mechanisms of E6 and E7 induced carcinogenisis.

The E5 protein of bovine papillomavirus type-1, although only 44 amino acids in length, can independently transform rodent fibroblasts. Since it seemed unlikely that E5 has intrinsic enzymatic activity, we investigated the possibility that E5, which is a transmembrane protein, transforms by activating growth factor receptors. E5 was shown to potentiate the activity of co-transfected receptors in a ligand independent manner. Analysis of co-transfected EGF receptors suggested that E5 may transform by interfering with receptor processing.

Biology of *fps/fes* oncogenes

This project aims to elucidate the biological function(s) of the c-*fps/fes* proto-oncogene and to understand the molecular basis of its oncogenic potential. We have previously shown that the c-*fps/fes* proto-oncogene, when expressed at sufficiently high levels, can cause tumorigenic transformation of cells that do not express c-*fps/fes*, such as murine fibroblasts. We have now shown that introduction of c-*fps/fes* into a differentiated factor-dependent macrophage cell line where c-*fps/fes* is normally expressed, can relieve their dependence of CSF-1 for their growth. The release of these cells from CSF-1 dependence did not result from a direct action of the c-*fps/fes* product (NCP92) on targets of the CSF-1 receptor. Biochemical analysis showed that the phosphotyrosine substrates of NCP92 were different from those that are phosphorylated by CSF-1 receptor upon CSF-1 treatment of the cells. Neither was endogenous or ectopically expressed c-*fps/fes* a substrate of CSF-1 receptor kinase during ligand-activated signal transduction in these cells. Further analysis showed that in cells that overexpressed NCP92 there was no change in the rate of synthesis, the number of CSF-1 receptors at the cell surface, or in the ability of receptors to bind CSF-1. We conclude that 1) overexpression of c-*fps/fes* can subvert the mechanisms of normal growth control in mature macrophages by acting on targets that are different from those of the CSF-1 receptor pathway, 2) the c-*fps/fes* product does not mediate CSF-1-activated signal transduction, and 3) overexpressed c-*fps/fes* does not up- or down-regulate CSF-1 receptor expression, nor does it affect the binding of ligand to its receptor.

Role of protein kinases in modulating cell growth and malignant transformation

The focus of this project is to better elucidate the possible involvement of transmembrane signal transmission systems in the regulation of cell growth and as possible targets in the process of tumor initiation and tumor promotion. Phospholipase D-mediated hydrolysis of phospholipids may represent an important element in transmembrane signaling. It was determined that activators of protein kinase C (PKC), adenine and guanine nucleotides, and sphingosine all act to stimulate phospholipase D-catalyzed hydrolysis of phosphatidylethanolamine (PtdEtn). Studies performed with both intact cells and isolated membranes established that the prominent lipid peroxidation product 4-hydroxynonenal (HNE) significantly inhibited phospholipase D activity. In contrast, HNE stimulated a phospholipase C activity to enhance PtdEtn hydrolysis and increase DAG levels in NIH 3T3 cells.

Selective oxidative modification of the regulatory domain of PKC by the oxidant tumor promoter m-periodate was found to generate a constitutively active kinase no longer regulated by $Ca^{2+}$ or phospholipids. Oxidation of the catalytic domain resulted in the formation of an inactive PKC, and this inactivation may play a role in the process of down regulation. Activation of PKC, in turn, leads to the activation and subsequent redistribution of Raf-1 protein kinase (Raf-1PK) to the nucleus. Cerebral ischemia in Mongolian gerbils also was found to cause a redistribution of B-raf PK to the nucleus of hippocampal neurons. Together, the above results indicate a possible interrelationship between oxygen free radicals, lipid peroxidation products, and transmembrane signaling systems to modulate cell growth regulation, carcinogenesis, and tumor promotion.

Immunohistochemical evidence, kinase activity measurements, and Western blot analysis all indicate that PKC is significantly elevated in multiple drug-resistant MCF-7/ADR cells. High levels of PKC activity are found in the nucleus of MCF-7/ADR cells, and immunoblot analysis with PKC isotype-specific antisera indicate that nuclei isolated from MCF-7/ADR cells contain markedly elevated levels of a modified form of PKCα. These results suggest that an increase in a form of PKCα may play a role in modulating nuclear events to influence the development of multidrug resistance in MCF-7 cells.

Retrovirus replication and cellular oncogene expression

Using SK37 and SK39 primers in polymerase chain reactions (PCR), we amplified and cloned, from an HIV-1 seropositive mother and her child, a portion of HIV-related *gag* sequence (500 bp). The sequences cloned were very similar to each other, suggesting the possibility of vertical transmission. The few subtle differences between the two sequences suggests they may be considered a quasispecies. These clones showed a homology score of 82.7-87.2% with *gag* sequence of prototype HIV-1. The divergence was particularly prominent in the first 5' proximal 200 bp which gave only 79-80% homology with the corresponding domain of prototype virus sequences. Thus the clones represent variants that are markedly divergent from the prototype HIV-1 stains. There was an early appearance of stop codons in the cloned sequences, suggesting the presence of defective viral genomes.

The ability of cellular factors to transactivate the HIV-1 LTR was investigated in two promonocytic cell lines, U937 and HL-CZ, which represent cells whose differentiation has been arrested at different stages. The two lines differed in their constitutive transactivating abilities and the extent with which they responded to enhancement by TPA and TNF. They also showed differences in their response to deletion of a negative regulatory element from the LTR. These differences may be related to variations in the efficiency with which the latent HIV genome is activated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03663-14 LCO

PERIOD COVERED

October 1, 1989 through September 30, 1990

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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| | W. D. Ju | Biotechnology Fellow | LCO NCI |
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| | W. C. Vass | Biologist | LCO NCI |

COOPERATING UNITS *(if any)* Jackson Laboratory, Bar Harbor, Maine, Dr. J. Stone
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Hebrew University of Jerusalem, Jerusalem, Israel, Dr. A. Levitzki
Cetus Corporation, Emeryville, CA, Dr. F. McCormick

LAB/BRANCH

Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 6.2 | 4.6 | 1.6 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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


Oncogene studies have involved *ras* encoded proteins and the action of TGF-α on the epidermal growth factor receptor.

We have made a series of chimeras between *ras* and the K*rev*-1 gene, which encodes a ras-like protein that can suppress ras-transformed cells. The results indicated that the *ras*-specific and K*rev*-1-specific amino acids immediately surrounding residues 32-44, which are identical between the two proteins, determined whether the protein induced cellular transformation or suppressed *ras* transformation. Since earlier observations by us and others have implicated this region in effector function, we suggest that K*rev*-1 may suppress *ras*-induced transformation by interfering with the interaction between ras protein and its effector. The influence of GAP (GTPase activating protein) on cell transformation by *ras* and other oncogenes was also examined. Overexpression of GAP in NIH 3T3 cells inhibited and suppressed transformation by c-*ras*, c-*src* and an activated c-*src* mutant, but not by v-*ras* or v-*mos*. We conclude GAP as a negative regulator is limiting in cells and overexpression of GAP can inhibit transformation by *ras*-dependent oncogenes. Our results make it unlikely that GAP by itself is the ras target, but they are compatible with the possibility that GAP may be part of a complex that forms the ras target.

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.

Major findings:

1. *ras* oncogenes. Normal *ras* proto-oncogene function is required for growth factor mediated growth of certain cells, 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.

The K*rev*-1 gene has been shown previously to suppress cells that are morphologically transformed by a highly oncogenic version of *ras*. K*rev*-1 encodes a ras-like protein whose amino acids share about 50% identity with those of ras. To genetically localize the segments encoded by *ras* and K*rev*-1 that mediate their opposite function, we have made chimeras between the two genes and studied the ability of each chimera to transform NIH 3T3 cells and to suppress a *ras*-transformed cell line. The results indicated that the *ras*-specific and K*rev*-1-specific amino acids immediately surrounding residues 32-44, which are identical between the two proteins, determined whether the protein induced cellular transformation or suppressed *ras* transformation. Since earlier observations by us and others have implicated this region in ras effector function, we suggest that K*rev*-1 may suppress *ras*-induced transformation by interfering with the interaction between ras protein and its effector.

GAP (for GTPase activating protein) 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 is thus far the only protein known to interact with this region of ras, GAP is a candidate for being the ras target in higher eukaryotes for mediating the ras signal. We have examined the sensitivity to the catalytic activity of GAP (GTP hydrolysis) of several mutant ras proteins with lesions in the effector domain and correlated these results with the biological activity of these mutants. This analysis has identified some mutants which are sensitive to GAP's catalytic activity. However, even when these mutants carry mutations that activate the biological activity of normal ras, they are defective for ras biological activity. These results suggest that a functional interaction between GAP and ras is not sufficient for transmission of the ras signal.

We have also examined the biological effects on ras induced transformation when NIH 3T3 cells overexpress GAP. We have found that co-transfection of GAP with c-ras prevented the ability of c-ras to induce cellular transformation; GAP did not alter the ability of v-ras (which is resistant to GAP catalytic activity) to induce cellular transformation. Overexpression of GAP in cells already transformed by c-ras was associated with morphological reversion to a flat, untransformed phenotype, but GAP overexpression was without effect on cells morphologically transformed by v-ras. The morphological reversion of the c-ras transformed cells appeared to be the consequence of GAP overexpression, since reverted cells and expressed much higher levels of GAP protein than non-revertants, while they continued to express high levels of c-ras protein. These effects of GAP coincided with its catalytic activity in that a truncated version of GAP that encoded the C-terminal one-third of the protein (which contains its catalytic activity) gave results that were qualitatively similar to those obtained with full length GAP. The effect of GAP on two other transforming genes, src and mos, was also examined. Src was chosen because its transforming activity is believed to be dependent upon endogenous ras activity, while mos transformation is ras independent. Both the wild type c-src and an activated c-src gene with a phenylalanine mutation at residue 527 behave similarly to c-ras; either full-length GAP or the GAP C-terminus were able to prevent transformation induced by the src genes, as well as to induce morphological reversion of cells transformed by either src gene. By contrast, GAP did not inhibit the ability of mos to induce cell transformation. 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.

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:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 05550-21 LCO

PERIOD COVERED
October 1, 1989 through May 31, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Retrovirus replication and cellular oncogene expression

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

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COOPERATING UNITS *(if any)*
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National Taiwan University Hospital, Dr. C. Y. Chuang
Yang Ming Medical College, Taiwan, Dr. W. T. Liu

LAB/BRANCH
Laboratory of Cellular Oncology

SECTION

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL: | OTHER |
|---|---|---|
| 1.4 | 1.4 | 0.00 |

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects
☐ (a1) Minors
☐ (a2) Interviews

☒ (b) Human tissues
X 100% AIDS related

☐ (c) Neither

B

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


Using SK37 and SK39 primers in polymerase chain reactions (PCR), we amplified and cloned, from an HIV-1 seropositive mother and her child, a portion of HIV-related *gag* sequence (500 bp). The sequences cloned were very similar to each other, suggesting the possibility of vertical transmission. The few subtle differences between the two sequences suggests they may be considered a quasispecies. These clones showed a homology score of 82.7-87.2% with *gag* sequence of prototype HIV-1. The divergence was particularly prominent in the first 5' proximal 200 bp which gave only 79-80% homology with the corresponding domain of prototype virus sequences. Thus the clones represent variants that are markedly divergent from the prototype HIV-1 stains. There was an early appearance of stop codons in the cloned sequences, suggesting the presence of defective viral genomes.

The ability of cellular factors to transactivate the HIV-1 LTR was investigated in two promonocytic cell lines, U937 and HL-CZ, which represent cells whose differentiation has been arrested at different stages. The two lines differed in their constitutive transactivating abilities and the extent with which they responded to enhancement by TPA and TNF. They also showed differences in their response to deletion of a negative regulatory element from the LTR. These differences may be related to variations in the efficiency with which the latent HIV genome is activated.

Major Findings:

1. Variability and quasispecies of virus sequence cloned from mother and child seropositive for HIV-1:

The AIDS viruses HIV-1 and HIV-2 are highly variable genetically . This variability is in part due to the frequent misincorporation of nucleotides by the viral reverse transcriptase. To study the extent of genetic variability of HIV strains in Taiwan, we collected DNA from peripheral blood samples of seropositive Taiwanese subjects and, by using SK37 and SK39 primers in polymerase chain reactions (PCR), amplified, cloned, and sequenced a portion of *gag* sequence (ca. 500 bp) which is less variable than the *env* region. From among a number of PCR-positive subjects, it was found that sequences cloned from a seropositive mother and her seropositive child were very similar, with a few subtle differences in several nucleotides. These may be better considered as quasispecies. The cloned sequences from the mother and child were compared with the known sequences of the same *gag* region of HIV-1 prototypes, HXB2 (US isolate) and Z2Z6 (Zaire isolate). While these latter isolates exhibit 93.7% homology with each other, the homology score for HXB2 *vs.* TM-1 (a clone from the mother) was 82.7%, and that for HXB2 *vs.* TC-1 (a clone from the child) was 85.8%. Similarly, the score for Z2Z6 vs. TM-1 was 84.0% and that for Z2Z6 *vs.* TC-1 was 87.2%. The divergence was particularly prominent in the first 200 base pairs proximal to the 5' end, giving only 79-80% homology with the corresponding domain of prototype virus sequences. The changes involved transitions more frequently than transversions. There was an early appearance of stop codons in the cloned sequences suggesting the presence of defective viral genomes among these quasispecies. The presence of closely related quasispecies in the paired samples of mother and child suggests possible vertical transmission from the mother to her child. These results demonstrate the potential epidemiological significance of the application of PCR method for tracing the source of infections. They also show the clones from these two Taiwan strains of HIV-1 represent variants that are markedly divergent from the prototype strains tested.

2. Cellular factors activating the transcriptional regulatory genes of HIV-1 in monocytic cell lines:

The latent period of AIDS is influenced by various factors which activate HIV replication in different cell types. Monocytic cells are important as a reservoir for HIV production *in vivo*, but the intracellular factors that regulate HIV transcription in these cells have not been defined.

Recent studies on the recognition of HIV-1 promoters by cellular transactivation factors have revealed that each core promoter consists of an enhancer element for NF-kB, a tandem array of Sp1 binding sites, a TATA motif, and binding sites for LBP-1 and CTF. Since binding to these sites was mapped mainly by studies carried out in cells other than monocytes, we investigated the transactivating ability of cellular factors present in two promonocytic leukemia cell lines, U937 and HL-CZ, and also tried to map the HIV LTR promoter regions functioning in these cells. The U937 cell line was obtained from the American Type Culture Collection, and the HL-CZ cell line was established from a leukemia patient in Taiwan. Although both U937 and HL-CZ express CD15 antigen, only the former expresses both CD4 and CD10 antigens, suggesting that differentiation is arrested at different stages in these two lines.

The full length LTR of HIV-1 and a nested series of 5' deletion mutants of the LTR were linked to the chloramphenicol acetyltransferase (CAT) gene, and transfected into these two monocytic cell lines by means of electroporation. The CAT activity was measured as the indicator of promoter transactivation by cellular factors. It was found that post-transfection treatment of these cells with a tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), or tumor necrosis factor (TNF) markedly enhanced the level of transactivation. The two cell lines showed differences in their constitutive transactivating activities, and the extent with which they responded to TPA and TNF. Deletion of a negative regulatory element (NRE) from the LTR enhanced the transactivation

by cellular factors. The two cell lines also showed differences in their response to NRE. Deletions affecting NF-kB or Sp1 binding sites resulted in a marked reduction in transactivation in these two cell lines.

Thus, monocytes at different stages of differentiation may respond differently to environmental and physiological factors, such as cytokines, mitogens, and tumor promoters, involved in transactivation not only of HIV LTR promoter sites, but also of cellular promoters which share consensus sequences of HIV LTR promoter sites. These differences may contribute to variation among individuals in the efficiency with which the latent HIV genome is activated in cells of the monocytic lineage.

Publications:

Guo H, Chuang CY, Shen MC, Lin HC, Ko YC, Chien CH, Chang KSS. Detection by polymerase chain reaction of HIV-1 DNA in homosexuals, hemophiliacs, and AIDS patients in Taiwan. In: Chuang CY, eds. Human Retroviruses and AIDS. 1989;117-27.

Chang KSS, Wang L, Gao C, Alexander S, Bodner A, Ting R, Kuo A F, Log T, Strickland P. HTLV-I and HIV-1 dual infections in drug abusers: High prevalence of IgG and IgM antibodies. In: Chuang CY, ed. Human Retroviruses and AIDS. 1989;135-47.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 08905-09 LCO

PERIOD COVERED
October 1, 1989 through September 30, 1990

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)*

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| OTHER: | Z. Kiss | Visiting Associate | LCO NCI |
| | Z. Olah | Visiting Fellow | LCO NCI |
| | C. Liapi | Visiting Fellow | LCO NCI |
| | S. A. Lee | Biotechnology Fellow | LCO NCI |
| | J. W. Karaszkiewitz | Biotechnology Fellow | LCO NCI |

COOPERATING UNITS *(if any)*
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INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 5.2 | 5.2 | 0.00 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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


The focus of this project is to better elucidate the possible involvement of transmembrane signal transmission systems in the regulation of cell growth and as possible targets in the process of tumor initiation and tumor promotion. Phospholipase D-mediated hydrolysis of phospholipids may represent an important element in transmembrane signaling. It was determined that activators of protein kinase C (PKC), adenine and guanine nucleotides, and sphingosine all act to stimulate phospholipase D-catalyzed hydrolysis of phosphatidylethanolamine (PtdEtn). Studies performed with both intact cells and isolated membranes established that the prominent lipid peroxidation product 4-hydroxynonenal (HNE) significantly inhibited phospholipase D activity. In contrast, HNE stimulated a phospholipase C activity to enhance PtdEtn hydrolysis and increase DAG levels in NIH 3T3 cells.

Selective oxidative modification of the regulatory domain of PKC by the oxidant tumor promoter m-periodate was found to generate a constitutively active kinase no longer regulated by $Ca^{2+}$ or phospholipids. Oxidation of the catalytic domain resulted in the formation of an inactive PKC, and this inactivation may play a role in the process of down regulation. Activation of PKC, in turn, leads to the activation and subsequent redistribution of Raf-1 protein kinase (Raf-1PK) to the nucleus. Cerebral ischemia in Mongolian gerbils also was found to cause a redistribution of B-raf PK to the nucleus of hippocampal neurons. Together, the above results indicate a possible interrelationship between oxygen free radicals, lipid peroxidation products, and transmembrane signaling systems to modulate cell growth regulation, carcinogenesis, and tumor promotion.

Immunohistochemical evidence, kinase activity measurements, and Western blot analysis all indicate that PKC is significantly elevated in multiple drug-resistant MCF-7/ADR cells. High levels of PKC activity are found in the nucleus of MCF-7/ADR cells, and immunoblot analysis with PKC isotype-specific antisera indicate that nuclei isolated from MCF-7/ADR cells contain markedly elevated levels of a modified form of PKCα. These results suggest that an increase in a form of PKCα may play a role in modulating nuclear events to influence the development of multidrug resistance in MCF-7 cells.

Cooperating Units:

LVC, NCI, FCRF, Dr. U. Rapp
LVC, NCI, NIH, Dr. P. Blumburg
Dept. Pharmacol., USC School of Medicine, Los Angeles, CA, Dr. R. Gopalakrishna
Lab.of Physiopath. Development, Paris, France, Drs. D.Evain-Brion, F. Raynaud
Lab of Molecular Neurobiology, Institute of Biophysics, Szeged, Hungary, Dr. F. Joo

Major Findings:

1. Protein Kinase C. Protein kinase C (PKC), plays a crucial role in relaying a variety of extracellular signals from the cell membrane to other intracellular sites. Thus, it is of importance to better characterize the biochemical events involved in modulating PKC activity to regulate cellular processes, including cell proliferation, tumor promotion, and resistance to drugs of the natural products class. Studies in collaboration with Dr. R. Gopolakrishna have indicated that oxidative modification may be an important regulatory parameter of PKC. Terpenoid tumor promoters (phorbol esters, mezerein) directly bind to PKC to activate this kinase. Other tumor promoters such as benzoyl peroxide and m-periodate are oxidants, and their mechanism of action is not well defined. Results now indicate that low concentrations of the oxidant tumor promoter, m-periodate, can directly modify the regulatory domain of PKC to generate an independent kinase activity no longer regulated by $Ca^{2+}$ or phospholipids. Higher concentrations of periodate modified the catalytic domain, resulting in complete inactivation. The oxidative modification induced by low periodate concentrations (<0.5mM) was completely reversed by brief treatment with 2mM dithiothreitol, suggesting that modification of vicinal thiols are involved. Both oxidative activation and subsequent inactivation of PKC also were observed in intact cells treated with periodate. Thus, oxidant mitogen/tumor promoters such as periodate may be able to by-pass normal transmembrane signaling systems to directly activate pathways involved in cellular regulation.

Several lines of evidence indicate the possible involvement of PKC in modulating cellular resistance to antitumor drugs of the natural products class. PKC activities are increased in multidrug-resistant human breast cancer MCF-7/ADR cells compared to control, drug-sensitive MCF-7/WT cells. Immunohistochemical localization studies indicated that immunoreactivity is enhanced in MCF-7/ADR cells, with pronounced staining noted in the nuclear region. Western blot analysis of protein extracted from purified nuclear preparations further established an increase in PKC associated with the nuclear fraction of MCF-7/ADR cells. MCF-7/ADR cells also showed elevated cytosolic PKC activity, while PKC activity associated with the plasma membrane fraction was similar for both cell types. Examination of these extracts with PKC isotype-specific antisera revealed that nuclei prepared from MCF-7/ADR cells contained markedly elevated amounts of a slightly altered form of PKCα. These data suggest that an increase in a specific form of PKC may play a role in the development and modulation of multidrug resistance.

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. Oncogenic activation of Raf-1 can occur by amino terminal truncation which constitutively activates its kinase activity. Immunocytochemical studies indicated that the Raf-1 kinase is diffusely distributed throughout the cytoplasm of quiescent NIH 3T3 cells. Treatment of serum-deprived NIH 3T3 cells with the mitogen PDGF or with TPA caused the rapid redistribution (along with activation) of Raf-1 PK to the nucleus. This response was maximal at 45-60 minutes of treatment and thereafter the Raf-1 PK began to dissipate from the nuclear fraction. Cells transformed by the v-raf oncogene were found to have high levels of the truncated, active v-raf present in the nuclear fraction without requirement for treatment with mitogen or tumor promoter. Studies also were carried out to determine changes in the intracellular distribution of raf in

hippocampal neurons following cerebral ischemia in Mongolian gerbils. Ischemic insult also was observed to cause a redistribution of B-raf PK to the nucleus. Nuclear Raf-1 PK was in a catalytically active form and was found to be tightly associated to the nuclear scaffold. Raf-1 protein was not released from the nucleus with high salt extraction, but was released by DNase digestion of the nuclear scaffold. Results suggest that activation of PKC may in turn lead to the activation and redistribution of Raf-1 PK to the nucleus. This translocation of Raf-1 PK to the nucleus may play a role as part of the pathway for the transduction of signal to the nucleus.

3. Phospholipid metabolism. Increased phospholipid metabolism to generate second messengers such as diacylglycerol and phosphatidic acid in response to ligand-receptor interaction at the plasma membrane plays a key role in the transmission of extracellular signals across the membrane. Numerous studies have dealt with growth factor and oncogene product modulation of phospholipase C-catalyzed degradation of phosphatidylinositol 1,4,5-bisphosphate. Evidence suggests that the stimulation of phosphatidylcholine and phosphatidylethanolamine (PtdEtn) hydrolysis also might be important for the generation of biologically active compounds. Thus, studies have been carried out to gain a better understanding of the regulation of PtdEtn hydrolysis and its possible role in transmembrane signaling. Activators of PKC, including TPA, were found to stimulate the phospholipase D-catalyzed hydrolysis of PtdEtn. Further, ATP stimulated PtdEtn hydrolysis in isolated membrane preparations, and guanine triphosphates and sphingosine, while having no effect alone, greatly potentiated the stimulatory effect of ATP on PtdEtn hydrolysis. Sphingosine was observed to significantly enhance PtdEtn hydrolysis when added to intact NIH 3T3 cells. The regulatory effects of adenine and guanine nucleotides suggest that PtdEtn hydrolysis also may play an important role in transmembrane signaling.

Studies were also carried out to better elucidate a possible interrelationship between oxygen free radicals, lipid peroxidation products, and transmembrane signaling systems. Peroxidation of membrane lipids leads to the formation of several end products with biological activity, including aldehydes. It was observed that 4-hydroxynonenol (HNE), an aldehyde product of membrane lipid peroxidation, inhibits the hydrolysis of phosphatidylcholine (PtdCho) both in the absence and presence of TPA. With isolated membranes, HNE also inhibited phospholipase D-mediated hydrolysis of PtdCho and PtdEtn determined in the absence or presence of ATP and/or GTPγ-S In contrast, HNE specifically stimulated the degradation of PtdEtn in [$^{14}$C] ethanolamine-prelabeled cells. Results suggest that HNE stimulates a C type phospholipase activity specifically involved in the hydrolysis of PtdEtn in NIH 3T3 cells.

Publications:

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Mihaly A, Olah Z, Krug M, Kuhnt U, Matthies H, Rapp UR, Joo F. Transient increase in Raf protein kinase like immunoreactivity in the dentate gyrus during long-term potentiation. J Neurosci 1990, in press.

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Anderson WB, Liapi C, Strasburger J, Gopalakrishna R, Plet A, Raynaud F, Evain-Brion D. Counteractions of retinoic acid and phorbol ester tumor promotor related to changes in protein kinase activities. In: Meyskens FL, Prasad KN, eds. Nutrients and Cancer Prevention. 1990, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 09051-05 LCO

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biology of fps/fes oncogenes

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

PI: R. A. Feldman Senior Staff Fellow LCO NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Cellular Oncology

SECTION

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National Cancer Institute, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.00 | 1.00 | 0.00 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

This project aims to elucidate the biological function(s) of the c-*fps/fes* proto-oncogene and to understand the molecular basis of its oncogenic potential. We have previously shown that the c-*fps/fes* proto-oncogene, when expressed at sufficiently high levels, can cause tumorigenic transformation of cells that do not express c-*fps/fes*, such as murine fibroblasts. We have now shown that introduction of c-*fps/fes* into a differentiated factor-dependent macrophage cell line where c-*fps/fes* is normally expressed, can relieve their dependence of CSF-1 for their growth. The release of these cells from CSF-1 dependence did not result from a direct action of the c-*fps/fes* product (NCP92) on targets of the CSF-1 receptor. Biochemical analysis showed that the phosphotyrosine substrates of NCP92 were different from those that are phosphorylated by CSF-1 receptor upon CSF-1 treatment of the cells. Neither was endogenous or ectopically expressed c-*fps/fes* a substrate of CSF-1 receptor kinase during ligand-activated signal transduction in these cells. Further analysis showed that in cells that overexpressed NCP92 there was no change in the rate of synthesis, the number of CSF-1 receptors at the cell surface, or in the ability of receptors to bind CSF-1. We conclude that 1) overexpression of c-*fps/fes* can subvert the mechanisms of normal growth control in mature macrophages by acting on targets that are different from those of the CSF-1 receptor pathway, 2) the c-*fps/fes* product does not mediate CSF-1-activated signal transduction, and 3) overexpressed c-*fps/fes* does not up- or down-regulate CSF-1 receptor expression, nor does it affect the binding of ligand to its receptor.



PHS 6040 (Rev. 1/84) GPO 904-917

Major findings:

The gene product of human c-*fps/fes* is a 92 Kda cellular tyrosine kinase (NCP92) that is specifically expressed in myeloid cells. The pattern of expression of NCP92 in certain leukemic cell lines suggests that this protein may be involved in the cellular response to factors that promote growth and/or differentiation in myeloid cells [Feldman et al., Proc. Natl. Acad. Sci. U.S.A. 82: 2379-2383 (1985)]. To further our understanding of the biology of c-*fps/fes*, we have cloned a cDNA copy of this gene and have begun the characterization of its biological and biochemical properties.

Using efficient retroviral vectors, we have recently shown that when the normal c-*fps/fes* gene is expressed at sufficiently high levels, this gene can cause tumorigenic transformation in established murine fibroblasts. The transforming activity of c-*fps/fes* could be regulated at two different levels: a) by controlling its level of expression (1), and by stabilizing phosphotyrosine in the critical substrates of transformation of NCP92 (2). This analysis also yielded basic information about the mode of regulation of the c-*fps/fes* kinase by tyrosine phosphorylation and its mode of action, and provided biological evidence that even closely related tyrosine kinases have different mechanisms of action and may use different pathways to transform cells.

The transforming activity of c-*fps/fes* in fibroblasts suggests that this gene may be involved in the control of cell proliferation and differentiation in myeloid cells, where c-*fps/fes* is normally expressed. To explore this possibility, we have begun to characterize the biochemical and biological consequences of introducing normal and oncogenically activated c-*fps/fes* genes into myeloid cells. As a model system we chose an SV40-immortalized mature macrophage cell line (Bac-1) that expresses moderate levels of endogenous c-*fps/fes* and depends on the presence of CSF-1 in the culture medium for growth. These cells grow in monolayer. As CSF-1 is the major growth factor responsible for inducing cell proliferation and differentiation along the macrophage lineage, we wanted to determine if the c-*fps/fes* product plays any role in the CSF-1 receptor pathway. These studies were done in collaboration with E. Richard Stanley (A. Einstein College of Medicine, New York).

Bac-1 cells were infected with retroviruses carrying normal c-*fps/fes* and wild type oncogenic gag-v-*fps/fes* genes and 3 days after infection, the growth factors were removed from the culture medium. After several days in culture, factor-independent lines were derived at low frequency from c-*fps/fes*-infected cultures, and at much higher frequency from v-*fps/fes*-infected cells, but not from cultures that had been infected with helper virus alone. The surviving factor-independent cells formed focus-like clusters on the background of dying cells. Analysis of individual colonies isolated by limiting dilution showed that all the factor-independent colonies examined expressed very high levels of the corresponding *fps/fes* gene products (up to 50 times above background). Unlike control cells, factor-independent clones were able to grow in soft agar in the absence of CSF-1, suggesting that the cells were transformed. Tumorigenicity studies in nude mice will be carried out to confirm this conclusion with an in vivo assay.

Since Bac-1 cells are dependent on CSF-1 for their proliferation and c-*fps/fes* was able to relieve this dependence, we wanted to determine if this was due to the interaction of c-*fps/fes* kinase with CSF-1 receptor substrates or if NCP92 was a substrate of CSF-1 receptor during signal transduction initiated by CSF-1 binding. To answer these questions, normal or c-*fps/fes*-infected Bac-1 cells were metabolically labeled with $^{32}P$, and cell extracts from untreated or CSF-1-treated cells were immunoprecipitated with antiphosphotyrosine antibodies. SDS-PAGE analysis showed that several proteins were phosphorylated on tyrosine in response to CSF-1 treatment, and that there were no differences in these bands between uninfected and *fps/fes* infected cells. None of these proteins comigrated with the c-*fps/fes* product NCP92. Furthermore,

phosphoamino acid analysis of NCP92 isolated from uninfected or c-*fps/fes*-infected cells showed that the level of phosphotyrosine was undetectable in untreated as well as in CSF-1 treated cells. These results suggest that the c-*fps/fes* protein is not a direct substrate of CSF-1 receptor during CSF-1-mediated signal transduction. In cells overexpressing *fps/fes* proteins, in addition to the CSF-1-dependent substrates, two additional phosphotyrosine-containing proteins were detected. These were two proteins of 62 Kda and 80 Kda that we had previously identified in murine fibroblasts as substrates of NCP92. The level of phosphorylation of these proteins did not change in response to CSF-1 treatment, suggesting that these proteins are not CSF-1 receptor substrates in vivo. In conclusion, the kinase activities of the CSF-1 receptor and NCP92 seem to act on different targets, and under our experimental conditions, they do not phosphorylate each other in vivo. This finding suggests that the major CSF receptor of macrophages and the product of c-*fps/fes* belong to different metabolic pathways, although they can substitute functionally for each other to some extent.

We also wanted to determine if the presence of high levels of NCP92 in these macrophages would have any effects on the numbers of CSF-1 receptors and on their rate of internalization in response to CSF-1 treatment. Binding studies with $^{125}$I-CSF-1 in *fps/fes*-infected cells showed no change in the number of CSF-1 receptors, or in their rate of internalization. Thus, the presence of very high levels of NCP92 in these macrophages caused no up- or- down-regulation of CSF-1 receptors, suggesting that in mature macrophages, c-*fps/fes* plays no role in the regulation of CSF-1 receptor expression. Further analysis will be required to determine if c-*fps/fes* may influence the regulation of CSF-1 receptor expression at other stages of macrophage differentiation.

In conclusion, our first probe of the biological properties of c-*fps/fes* in myeloid cells has shown that this gene is capable of transforming these cells, possibly by a mechanism, possibly involving the direct phosphorylation of critical substrates of transformation. The second major conclusion is that the CSF-1R pathway does not involve NCP92 as an intermediate tyrosine kinase for its action. Conversely, we have found no evidence that c-*fps/fes* can exert any action on the CSF pathway, either directly by phosphorylating major CSF-1 receptor targets, or indirectly, by affecting receptor copy number and rate of receptor internalization. As CSF-1 receptor is the major receptor involved in the control of cell proliferation and differentiation into macrophages, our data casts some doubt on the role of c-*fps/fes* as a major player in macrophage differentiation. Rather, the accumulation of c-*fps/fes* during macrophage differentiation may be the consequence of this differentiation program, and the biological role of NCP92 kinase may be found in some function of the differentiated cell. Our next step will be to test this hypothesis.

Publications:

Feldman RA, Lowy DR, Vass WC, Velu TJ. A highly efficient retroviral vector allows detection of the transforming activity of the human c-*fps/fes* proto-oncogene. J Virol 1989;63:5459-64.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 09052-02 LCO

PERIOD COVERED
October 1, 1989 through September 30, 1990

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

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

| | | | |
|---|---|---|---|
| PI: | J. T. Schiller | Senior Staff Fellow | LCO NCI |
| OTHER: | D. R. Lowy | Chief, LCO | LCO NCI |
| | M. S. Barbosa | IRTA Fellow | LCO NCI |
| | S. A. Sedman | IRTA Fellow | LCO NCI |
| | N. L. Hubbert | Microbiologist | LCO NCI |
| | W. C. Vass | Biologist | LCO NCI |

COOPERATING UNITS *(if any)*
Ludwig Institute for Cancer Research, St. Mary's Hospital Medical School, London, UK, Dr. K. H. Vousden.

LAB/BRANCH
Laboratory of Cellular Oncology

SECTION

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☐ (a1) Minors
☐ (a2) Interviews

B

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

This project is studying transformation by human and animal papillomaviruses. Certain genital human papillomaviruses (HPV), types 6 and 11, are frequently associated with benign condylomas but rarely with carcinomas, while other types, HPV16 and 18, are less frequently detected in condylomas but are the predominate types detected in genital carcinomas. Since the viral E6 and E7 genes are selectively retained and expressed in the carcinomas, we have undertaken a comparative analysis of these genes from the presumed low risk and high risk types. We have determined that the in vitro transforming activities of E6 and E7 from HPV6 are lower than those of E6 and E7 from HPV16 and 18; these results have also been correlated with lower efficiency of Rb binding and casein kinase II phosphorylation by the E7 encoded protein of HPV6, compared with E7 protein of HPV16 and 18. These results provide strong experimental support for the hypothesis that the high risk types, in particular their E6 and E7 genes, play an important role in the genesis of human genital cancer and offer insight into the possible mechanisms of E6 and E7 induced carcinogenisis.

The E5 protein of bovine papillomavirus type-1, although only 44 amino acids in length, can independently transform rodent fibroblasts. Since it seemed unlikely that E5 has intrinsic enzymatic activity, we investigated the possibility that E5, which is a transmembrane protein, transforms by activating growth factor receptors. E5 was shown to potentiate the activity of co-transfected receptors in a ligand independent manner. Analysis of co-transfected EGF receptors suggested that E5 may transform by interfering with receptor processing.

Major Findings:

1. Immortalization of normal human foreskin keratinocytes by E6 and E7. There is a striking correlation between the apparent oncogenic potential of the genital human papillomviruses (HPV) and their ability to immortalize normal targets of genital HPV infection, human foreskin and cervical keratinocytes. HPV16 and 18, which are most frequently detected in human genital carcinomas, can induce immortalization, while HPV6 and 11, which are rarely detected in carcinomas, can not. We were therefore interested in determining the HPV genes responsible for inducing immortalization and the nature of the defect in the low risk types. An LTR activated E6E7 segment of HPV16, previously shown to transform rodent fibroblasts, was able to induce immortalization. Mutational analysis indicated that, in contrast to fibroblast transformation, both E6 and E7 are required for immortalization of human keratinocytes. Co-transfection of HPV16 E6 and E7 on separate plasmids also induced immortalization, indicating that trans-acting products of both genes are required. E7 alone often induced a transient increase in proliferation but these cells grew more slowly than the cells co-transfected with E6 and eventually senesced. All of the immortalized lines were aneuploid, suggesting that, in addition to E6 and E7, changes in cellular genes are also required to induce immortalization. LTR activated E6 and E7 genes of HPV18 were able to complement each other to induce immortalization, but a similar pair of HPV6 constructs were not. When the E6s and E7s of HPV16 and 18 were mixed in co-transfections, the heterologous pairs induced immortalization as efficiently as the homologous pairs. In contrast, neither the E6 nor the E7 of HPV6 was able to functionally substitute for the corresponding HPV16 or 18 gene. These results indicate that there is no type specific interaction between E6 and E7 and strongly imply that both the E6 and E7 proteins of HPV6 are defective in immortalizing activity.

2. Transformation of NIH3T3 cells by E7. Previous studies had established that an LTR activated E6E7 segment from the high risk HPV16 and 18 could induce anchorage independent growth of NIH3T3 cells and that the E7 gene was responsible for the activity. A similar construct containing the E6E7 segment of the low risk HPV6 was inactive. To determine if the defectiveness of the HPV6 clone was due to differences in expression of E7 and/or differences in the intrinsic activity of the E7 protein, we constructed isogenic clones in which the ATG of the three E7 ORFs was the first translation initiation signal and linked to a common upstream sequence. In this construction the HPV6 E7 was expressed at levels comparable to that of HPV16 and 18. In contrast, the clone containing the E6 gene upstream of HPV6 E7 failed to produce E7 protein despite abundant E6E7 mRNA. These results indicate that HPV6 E7, unlike the E7s of HPV16 and 18, is not translated from a polycistronic message. The HPV6 E7 isogenic clone was able to transform NIH3T3 cells, although to a lesser extent than the other clones. Since the levels of E7 protein in the cells were similar, we conclude that the HPV6 E7 has intrinsic transforming activity but that it is less active than HPV16 and 18 E7.

3. Biochemical activities of E7. The E7 proteins of HPV6, 16 and 18 each have a 17 amino acid peptide that shares homology with adenovirus E1a and SV40 T. For E1a, this region has previously been shown to be essential for binding of the tumor suppressor gene product Rb. We have now determined that for HPV16 E7 the N-terminal portion of the homologous peptide is also required for the in vitro binding of E7 to Rb. We have also demonstrated that two serines in the C-terminal portion of the peptide are phosphorylated by casein kinase II (CKII). Phosphorylation by CKII and Rb binding appear to be independent activities, since mutations that eliminate one activity had no effect on the other. Both activities may be involved in E7 mediated transformation of NIH3T3 cells; mutants that fail to bind Rb were transformation defective, while those that were not phosphorylated by CKII exhibited a 5 fold reduction in transforming activity. Both biochemical activities correlate with the apparent oncogenic potential of the viruses. HPV16 and 18 E7 bound similar amounts of Rb, but HPV6 E7 consistently bound less. The rate of HPV18 E7

phosphorylation by CKII was two fold faster than that of HPV16 E7 which in turn was two fold faster than that of HPV6 E7. We conclude that multiple quantitative rather than major qualitative differences in biochemical activities account for the differences in the biological activities of the E7 proteins of the genital HPVs.

4. Analysis of HPV16 E6 proteins. Cells infected with the low risk genital HPVs express a single E6 protein, while those infected with the high risk types have the potential to express both full-length and truncated E6* proteins, translated from spliced mRNAs. To address the question of whether the E6* proteins contribute to the apparent oncogenic potential of the HPVs, we have begun to characterize the individual activities of the HPV16 E6 proteins. We have found that the full-length E6 can complement E7 to immortalize human keratinocytes, but that the E6* proteins cannot. We have also shown that the full-length E6, but not the E6*s, can trans-activate the adenovirus E2 promoter in NIH3T3 cells. The results support the hypothesis that the primary function of the E6 splices is to facilitate translation of E7, and raise the possibility that E6 may exert its effects on cells through the trans-activation of cellular genes.

5. The mechanism of BPV E5 transformation. The bovine papillomavirus (BPV) E5 transforming gene encodes a 44 amino acid protein product that is localized to external plasma and golgi membranes. We have determined that E5 can potentiate the transforming activity of co-transfected human EGF and CSF-1 receptors in NIH3T3 cells. This synergism occurred in the absence of ligand but could be further augmented by the addition of ligand. No synergy was seen with c-fes or c-src, which are also tyrosine kinases but do not span the plasma membrane. Cooperation between E5 and co-transfected EGF receptors was associated with inhibition of receptor degradation and persistence of activated receptors on the cell surface. These results suggest that E5 may potentiate the activity of several receptors, perhaps via inhibition of receptor down-modulation.

Publications:

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SUMMARY REPORT
LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY, DIAGNOSIS, AND CENTERS
NATIONAL CANCER INSTITUTE
October 1, 1989 to September 30, 1990

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 Pathology Section (Dr. William D. Travis, Chief)
Cytopathology Section (Dr. Diane Solomon, Chief)
Ultrastructural Pathology Section (Dr. Maria Tsokos, Chief)
Biochemical Pathology Section (Dr. David D. Roberts, Chief)
Tumor Invasion and Metastases Section (Dr. Lance A. Liotta, Chief)
Hematopathology Section (Dr. Elaine S. Jaffe, Chief)
Gene Regulation Section (Dr. David L. Levens, Chief)
Office of the Chief (Dr. Lance A. Liotta, Chief)

All sections conduct investigative work and provide research opportunities for the residents. Investigative work completed or in progress is listed by section as follows.

Surgical Pathology Section

Approximately 6,000 surgical specimens or biopsies (approximately 60,000 slides) were accessioned in the past year. Approximately 1,000 specimens of fresh human tissues, including the eyes, which are regularly removed during a complete autopsy, were furnished to NIH scientists in various laboratories. A diagnostic immunohistology service is provided for clinical diagnosis and research. A tissue procurement nurse works closely with the staff and helps in the distribution of specimens to scientists. Clinicopathological studies are provided supporting clinical protocols conducted by the Surgery Branch, the Medicine Branch, and the Clinical Center. Dr. David Katz is the staff neuropathologist and participates in teaching and applied research.

Dr. Maria Merino is investigating the use of immunohistochemical markers to study a) the effect of immunotherapy on immune cell infiltration of human renal carcinomas, b) the pathologic distinction between pre-invasive and invasive breast carcinoma, and c) the role of p glycoprotein in endometrium. In addition, she is studying the utility of collagenase, c-erb B2 and GCDFP-15 as cancer markers.

Laboratory of Pathology, DCBDC, NCI

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 30 patients with pulmonary histiocytosis X are also being studied. The light microscopic findings observed in these lung biopsies are being correlated with clinical features, bronchoalveolar lavage data and ultrastructural findings.

The pulmonary and autopsy pathological material is being actively utilized by the staff and residents for research projects involving clinicopathological correlation and pathological characterization of diseases studied at the Clinical Center. Electron microscopic and immunocytochemical techniques are being applied to the study of these diseases. A comprehensive study of the pulmonary pathology of the Acquired Immune Deficiency Syndrome (AIDS) is currently being conducted based on surgical and autopsy lung pathology material from AIDS patients seen at the NIH.

The neuropathology service is integrated with the Surgical Pathology Section, Pulmonary and Postmortem Section, and the Ultrastructural Pathology Section. Neuropathology diagnostic (patient care) service and teaching (of pathology residents) are provided. Approximately 250 neurosurgical specimens were examined last year. The service also functions in a collaborative manner to provide neuropathological support for a wide range of clinicopathologic investigations, including dementia, pituitary adenomas, PML and gliomas.

Cytopathology Section

This section provides diagnostic services in cytology (both exfoliative and fine needle aspiration). During the year, 3800 cytology specimens were accessioned. In addition, there has been a 200% increase in outside consult cases submitted to this section. Special expertise is provided in aspiration cytology. Fine needle aspirations have increased 100%. The section provides a) a pathologist to perform the aspiration; b) a pathologist to instruct the clinician in aspiration technique; c) a cytotechnologist to assist the clinician; d) assistance to the radiologist during the aspiration of a deep tissue specimen.

The section organized three national meetings to address quality assurance in cytology and to develop a new system, named "The Bethesda System", for reporting cervical/vaginal cytopathology diagnoses.

In addition to the diagnostic reports, the staff of this section collaborates closely with the clinical and surgical staff in various clinicopathologic research projects. Dr. Diane Solomon has applied immunocytochemistry to the diagnosis of lymphomas in cytological materials. Over 200 tumor suspensions have been evaluated for tumor-associated antigens and for phenotypic analysis of tumor infiltrating lymphocytes. L6, a murine monoclonal antibody, is being studied for utility as a carcinoma marker. Bone marrow effects of interleukin-1 alpha and B-cell activation in lymph node germinal centers are also being studied.

Laboratory of Pathology, DCBDC, NCI

Ultrastructural Pathology Section

This section provides diagnostic electron microscopy services for a diverse group of Clinical Center physicians, including NCI, NIAMDD, NHLBI, NIAID, and NINCDS, as well as submitted cases from outside physicians. This past year approximately 200 cases were accessioned; over 150 were processed and diagnosed. This facility provides diagnostic training and clinical research opportunities for residents and fellows. Dr. Maria Tsokos has been doing diagnostic EM while investigating the factors such as expression of MyoD1 gene products which correlate with the biologic aggressiveness of alveolar rhabdomyosarcoma compared to embryonal rhabdomyosarcoma.

P-glycoprotein has been associated with a multidrog-resistant (MDR) phenotype in a wide variety of animal and human tumor cell lines and clinical tumor specimens. Pediatric tumors, however, have not been included in the studies reported so far. Tissues from Ewing's sarcomas, primitive neuroectodermal tumors and rhabdomyosarcomas will be evaluated for the presence of P-glycoprotein using a commercially available antibody against P-glycoprotein and employing a modified immunoperoxidase "sandwich" staining method. Hyperdiploidy has been associated with an unfavorable histology (anaplastic) in Wilms' tumor and with a better response to chemotherapy in unresectable neuroblastoma. DNA-aneuploidy was found in rhabdomyosarcoma (RMS) in contrast to Ewing's sarcoma and primitive neuroectodermal tumor (PNET).

The stage of differentiation of the 6 rhabdomyosarcoma (RMS) cell lines will be altered by agents and/or conditions previously used to induce or inhibit differentiation in myoblastic cell cultures and RMS cell lines. The expression of TGF-ß in neural tumors will also be investigated.

Biochemical Pathology Section

The Biochemical Pathology Section, under Dr. David Roberts, is carrying on research on immunochemistry of complex carbohydrates and cell surface receptors. Current approaches include 1) determination of carbohydrate structures of glycoproteins and analysis of oligosaccharide mixtures by gas chromatography/ mass spectrometry (GC/MS), 2) development of hybridoma antibodies against oligosaccharide haptens, and 3) immunochemical studies of cell surface glycoproteins.

The functions of thrombospondin in tumor cell adhesion and migration are being investigated. The carboxyl-terminal domain mediates attachment and haptotaxis, while the amino-terminal domain mediates cell spreading and chemotaxis. One class of receptors are sulfated glycoconjugates which bind to the amino-terminal domain of thrombospondin. A minor heparan sulfate proteoglycan that binds thrombospondin with high affinity was identified in two melanoma cell lines and purified by affinity chromatography on thrombospondin-Sepharose. The intracellular responses of cells to surface receptor binding of thrombospondin to the two types of receptors are also being investigated.

Sulfated glycolipids and α2-3-linked sialyl glycoproteins are two independent receptors for cytoadhesion of *M. pneumoniae*. The former is present at high

concentrations in human trachea which is the primary site of infection. Monoclonal antibodies to the pathogen will be used to define receptors for these, and the respective carbohydrates on affinity matrices will be used to purify their receptors.

Several serotypes of *Chlamydia* were examined for binding to glycolipids and glycoproteins. The elementary bodies bind specifically to glycolipids containing GalNAcß1-4Galß1-4-Glc sequences, heparin or fucoidin conjugated to albumin, and to the adhesive proteins fibronectin and laminin.

Dr. Roberts has determined that sulfated glycolipids participate in tumor cell adhesion by directly promoting adhesion and as receptors for thrombospondin on melanoma cells.

Glycolipids isolated from esophageal tissues obtained from cancer patients, normal individual controls, and a cultured esophageal carcinoma line were examined using several monoclonal antibodies. Both neutral and sialylated glycolipids containing 3-fucosyllactosamine sequences differ in expression between the tumor and control tissues and a heptaglycosylceramide reacting with antibodies to this sequence is found only in the tumor tissues. These glycolipid antigens may be useful as markers of esophageal carcinoma.

Tumor Invasion and Metastases Section

The TIM section members have identified a set of gene products which may regulate tumor invasion and metastasis: a) laminin receptor; b) autocrine factor; c) NM23 metastasis suppressor protein, d) type IV collagenase, and e) TIMP-2 metalloproteinase inhibitor. These metastasis-associated gene products may be different from those which regulate tumor growth, and this is in keeping with the concept that benign tumors may grow in an unrestrained fashion, but fail to invade.

The laminin receptor is a cell surface protein which binds laminin, a glycoprotein of basement membranes, and is augmented in certain aggressive human cancers compared to non-malignant counterpart cells. Dr. Mark Sobel has cloned and sequenced the full-length cDNA for the laminin receptor. He is now studying the structure and function of the receptor.

The autocrine motility factor (AMF) is a new cytokine which is secreted by tumor cells, binds with high affinity to a cell surface receptor, and profoundly stimulates locomotion. Dr. Elliott Schiffmann's group has found that a pertussis toxin sensitive G protein is required for the transduction of the AMF signal through the phosphoinositol pathway. Screening of inhibitors blocking this pathway has led to the identification of a Merck compound which may be of therapeutic value in cancer treatment. Preclinical toxicology and the design of clinical protocols are now complete for this compound. An oral formulation has been developed. We hope that suitable clinical support will be available to immediately initiate clinical trials.

NM23 is a gene expressed to a higher level in low metastatic tumor cells compared to high metastatic cells. It encodes a putative metastases suppressor

protein. Dr. Pat Steeg, a fellow in our section, has cloned and sequenced a full-length NM23 cDNA clone. The encoded protein sequence has properties suggesting a leucine-zipper DNA binding protein. The NM23 protein has a size of 17 kDa by Western blotting of nuclear extracts using anti-peptide antibodies. The NM23 protein is virtually identical to a Drosophila gene (awd) product which plays a critical role in morphogenesis and tissue pattern formation. The discovery of NM23 therefore provides a link between development and metastasis. The negative correlation of NM23 expression with metastases was confirmed in four different animal model systems using multiple clones. Immunogenicity does not appear to play a role in the mechanism of action of NM23. A strong negative correlation was found between human breast carcinoma aggressiveness, in terms of lymph node metastases, and NM23 mRNA expression. The NM23 gene location is chromosome 17, and is associated with allelic loss in a variety of carcinomas. Additional data has demonstrated a strong suppression of experimental metastases in a series of tumor cell clones transfected with NM23 compared to a series of control transfections. NM23 may provide a number of significant prognostic and therapeutic strategies.

The structural backbone of the basement membrane is type IV collagen, a collagen genetically distinct from the other types of collagen. Type IV collagenase is a metalloproteinase which cleaves type IV collagen in a pepsin resistant site. The enzyme is secreted in a latent 70 kDa form which fails to degrade type IV collagen. When the enzyme is activated, it becomes reduced to a molecular size of 62 kDa and can specifically cleave type IV collagen. Both latent and active forms of this enzyme are gelatinolytic. This enzyme is secreted in large amounts by tumor cells and its rate of elaboration is correlated with the metastatic phenotype in several systems. It is a unique member of the metalloproteinase family. Antibodies which block the activity of this enzyme and synthetic inhibitors of this enzyme block tumor cell invasion *in vitro*. This enzyme may play a crucial role during invasion and metastasis.

Analysis of the full-length cDNA clone encoding the human latent type IV collagenase has revealed three separate functional domains: 1) an amino terminal domain of 80 residues which triggers a conformational change in the enzyme resulting in activation from a latent state. Organomercurial activation of the latent type IV collagenase results in autocatalytic removal of this amino terminal domain resulting in the conversion to a 62 kDa activated form of the enzyme. The amino terminal sequence of the purified enzyme before and after activation reveals cleavage at a single locus generating a new amino terminus starting at residue 81. This site is adjacent to the carboxyl end of a conserved region PRCGVPDV which contains an unpaired cysteine residue. An identical sequence is present at the activation site of prostromelysin and interstitial procollagenase. Further studies have demonstrated that this sequence comprises an intrinsic inhibitor which blocks the active site of the enzyme. 2) a cysteine-rich repeat domain which binds to the substrate independent of metal ions and is responsible for the substrate specificity, and 3) a histidine containing domain VAAHEFGHAMGLEHSQ which is the active site of the enzyme, and requires metal ions. Affinity purified antibodies made against synthetic peptides corresponding to the latent versus active enzyme were used to study the expression of the enzyme antigen during progression from human *in situ* to invasive breast carcinoma. The latent type IV collagenase was found

associated to a low degree with benign duct myoepithelial cells. In contrast, the active enzyme form was not present in normal ducts or benign ductal proliferation, was present variably in comedo carcinoma *in situ*, and was markedly augmented in primary invasive carcinoma and metastases. This indicates that enzyme activation may be an important correlate of tumor progression. Type IV collagenase production also shows a strong correlation with invasive grade in human colorectal and gastric cancer, and is markedly augmented following *ras* oncogene transfection in a variety of murine and human cell types.

A very strong correlation was observed between the levels of type IV collagenase and AMF in the urine and bladder carcinoma grade, stage of invasion, and recurrence. These tests have received great interest by urologists, and we are now accepting hundreds of urine samples for a large screening study. The test may be a potential adjunct in the early detection of bladder cancer recurrence.

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

Dr. William Stetler-Stevenson has discovered a novel proteinase inhibitor which binds with a 1:1 stoichiometry to type IV collagenase. The inhibitor has been purified to homogeneity and the complete primary structure has been determined and the full-length human gene has been cloned. The novel inhibitor, termed TIMP-2, is the first new member of the TIMP (tissue inhibitor metallo-proteinases) family with conservation of all 12 cysteine positions. This new inhibitor can be considered in the class of tumor suppressor gene products.

Hematopathology Section

The Hematopathology Section conducts a major program in diagnostic and experimental hematopathology. Dr. Elaine Jaffe supervises an internationally recognized consultation service for diagnostic hematopathology. While most of this material pertains to patients with malignant lymphoma admitted to the clinical services of the National Cancer Institute, collaborations are also conducted with NIAID and NIAMDD for patients with reactive lymphoproliferative lesions. Dr. Jaffe also receives several hundred cases submitted for diagnostic consultation from pathologists in the regional medical community as well as throughout the United States and other parts of the world.

The Hematopathology Section continues its active research program on the immunological characterization of malignant lymphomas. All patients with newly diagnosed lymphomas or recurrences are studied for phenotypic and functional markers. This information is utilized to study the relationship of malignant lymphomas to the normal immune system, to develop improved classifications of disease, and to distinguish new clinicopathologic entities. This information is also being used as a basis for immunotherapy in collaboration with the Medicine Branch, DCT, and the Biological Response Modifier Program in Frederick, Maryland, NCI.

Laboratory of Pathology, DCBDC, NCI

Dr. Jaffe has continued to study the pathologic features of HTLV-I associated lymphomas. In selected populations where HTLV-I is endemic, such as Jamaica, prospective studies of all newly diagnosed lymphoma patients are conducted. Such studies are useful in identifying the clinicopathologic spectrum of HTLV-I associated diseases. Prospective studies of all lymphomas in similar geographic regions with differing incidences of adult T cell leukemia/lymphomas are studies to discern factors which make an impact on the incidence of HTLV-I and HTLV-I associated diseases.

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

Dr. Maryalice Stetler-Stevenson has demonstrated that frequent relapse of follicular lymphoma, the major obstacle to cure, is a consequence of clonal expansion of daughter cells derived from a common stem cell. Thus, despite the "clinical" remission achieved by therapy in most patients, residual lymphoma cells must persist. To detect occult lymphoma, she has specifically amplified the joined bcl-2/JH DNA sequences created by the t(14;18) translocation seen in nearly all follicular lymphomas. Using multiple rounds of primer-directed DNA polymerization (polymerase chain reaction, PCR), 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. She has demonstrated the t(14;18) translocation to be present in 32% of Hodgkin's lymphomas. However, Hodgkin's disease and follicular lymphoma are two distinct clinical-pathological entities. Future studies will examine the expression of the bcl-2/JH transcript and of the bcl-2 protein product in Hodgkin's disease patients and again correlate this information with the clinical course. This data will provide further information concerning the role of bcl-2 in hematopoietic malignancies.

Gene Regulation Section

Dr. David Levens has developed a novel method for identifying sequence-specific DNA binding proteins.

The gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with cellular proteins. These same proteins also bind to a negative cis-element approximately 330 bp upstream of c-myc promoter P1. The gibbon T-cell lymphoma cell line MLA 144 strongly transactivates the GALV-Seato enhancer. Fractionation of MLA 144 extracts by conventional and affinity chromatography allows the separation of the GALV enhancer binding protein complex into 2 components. One of these

components interacts strongly with DNA but does not in itself possess full specificity. The second component does not in itself bind DNA, but upon forming a complex with the first component, confers greatly enhancer power to discriminate between different sequences.

Application of an exonuclease assay developed in his laboratory to identify and map the sites of tight protein-DNA interactions on large pieces of DNA, to the c-myc has revealed multiple cis- and trans-elements both upstream and downstream of the c-myc promoter P1. The group has focused on 3 regions, each of which interacts with one or more sequence-specific binding proteins. A negative cis-element present in intron one of the c-myc gene is frequently mutated in Burkitt's lymphoma.

Office of the Chief

Dr. Susan Mackem is investigating genes which regulate morphogenesis. The elucidation and characterization of genes necessary for establishing pattern formation during morphogenesis and the study of their regulation are problems which are central to many aspects of vertebrate biology. It may be expected that processes including responses to trophic stimuli, cell-cell interactions, migration, differential cell multiplication, programmed cell death, etc. are all involved. Many of these same processes are recapitulated in a pathologic manner during oncogenesis.

It is the aim of this long-term project to isolate genes that regulate morphogenesis in the chick embryo limb bud. Two general types of approaches are being developed to achieve this aim: 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 4 to 5 fold 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.

Dr. Kathleen Kelly is 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. She has isolated over 60 novel cDNA clones that represent mRNA species induced by PHA and PMA in human peripheral blood T cells. A subset of inducible genes (including two putative transcription factors) are constitutively expressed at high levels in T cells infected with HTLV I, and also following transfection of T cells with HTLV I-derived DNA encoding the overlapping reading frames of $p42^{tax}$ and $p27^{rex}$. Such abnormally expressed genes may play a role in the deregulated growth of adult T cell leukemia, a cancer that shows a 100% infection rate with HTLV I. The mechanism of $p42^{tax}$ and $p27^{rex}$ action that leads to over-expression is being examined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00853-37 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.5 | 0.5 | X |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

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.



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

Other Professional Personnel:

| | | |
|---|---|---|
| P. Kragel | Expert | LP NCI |
| W. Travis | Chief, Pulmonary & Postmortem Pathology Section | LP NCI |
| M. Raffeld | Senior Staff Fellow | 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 |
| +K. Schmidt | Medical Staff Fellow | LP NCI |
| +A. Ginsberg | Medical Staff Fellow | LP NCI |
| +J. Taubenberger | Medical Staff Fellow | LP NCI |
| +D. Kleiner | Medical Staff Fellow | LP NCI |
| +D. Roth | Clinical Associate | 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 suramin in advanced stage carcinoma of prostate.

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+These physicians are full-time Residents in the Laboratory of Pathology.
*This Associate Pathologist spends part time in the activities of the Surgical Pathology Section.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09145-06 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Neuropathology

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

PI: D.A. Katz Neuropathologist OCD NINDS

COOPERATING UNITS *(if any)*

Surgical Pathology Section, Pulmonary and Postmortem Section, and Ultrastructural Pathology Section, LP, NCI

LAB/BRANCH

Office of the Clinical Director, NINDS

SECTION

OCD

INSTITUTE AND LOCATION

NINDS, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS. | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1 | 1 | 0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

As described previously, subspecialty expertise in diagnostic neuropathology is provided to the Laboratory of Pathology, NCI, and to all other institutes, via the Office of the Clinical Director, NINDS. The neuropathology service is integrated with the Surgical Pathology, Postmortem, and the Ultrastructural Pathology Sections. Within the Laboratory of Pathology, both diagnostic (patient care) service and teaching (of pathology residents) are provided. The service also functions in a collaborative manner to provide neuropathological support for a wide range of clinicopathologic investigations.



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

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 50 intra-operative frozen section consultations are provided yearly. Current case material includes malignant gliomas, pituitary adenomas, stereotactic biopsies (AIDS), electrocorticographically guided resections for temporal lobe seizures, and PML (progressive multifocal leukoencephalopathy).

Conferences: The case material described above is also utilized for resident teaching conferenes 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).

2. Pituitary adenomas: study of adenomas in Cushing's disease; TSH-producing adenomas (NICHD, NIDDK, NINDS).

3. Progressive multifocal leukoencephalopathy (PML): rapid diagnosis/correlation of immunoperoxidase, in-situ hybridization, and EM findings; pathogenesis (NINDS).

4. Malignant gliomas: diagnosis and grading of tumors prior to experimental therapy; complications of therapy (NINDS, NCI).

Publications:

Houff, SA, Katz D, Kufta CV, Major EO. A rapid method for in situ hybridization for viral DNA in brain biopsies from patients with AIDS. AIDS 1989;3:843-5.

McCutcheon I, Baranco RA, Katz D, Saris S. Adoptive immunotherapy of intracerebral metastasis in mice. J Neurosurg 1990;72:102-9.

Kinsella TJ, Collins J, Rowland J, Klecker R, Wright D, Katz D, Steinberg S, Glatstein E. Pharmacology and phase I/II study of continuous intravenous infusions of iododeoxyuridine (IdUrd) and hyperfractionated radiotherapy in patients with glioblastoma multiforme. J Clin Oncol (in press)

Theodore WH, Katz DA, Kufta CV, Sato S, Patronas N, Bromfield E. Pathology of temporal lobe foci. Correlation with CT, MRI, and PET. Neurology (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09192-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2 | 2 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

The resected specimens of patients with renal cell carcinoma that have received immunotherapy will be evaluated histologically and immunohistochemically and compared with the renal cell cancers of patients which did not receive the same modality of treatment.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09193-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

Sclerosing adenosis is a proliferative breast lesion with pseudoinvasive features frequently misdiagnosed as infiltrating carcinoma. The purpose of this study will be: 1) evalute 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.





GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09194-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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, NICHHD

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3 | 3 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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.



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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09195-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Determination of c-erb B-2 Oncogene Expression in Early Stage Carcinoma of Breast

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

| | | | |
|---|---|---|---|
| PI: | M. Merino | Chief, Surgical Pathology Section | LP NCI |
| OTHER: | E. Glatstein | Chief, Radiation Oncology | NCI |
| | K. Strauss | Senior Staff Fellow | NCI |
| | E. Barker | Molecular Oncology, Inc. | |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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|---|---|---|
| 2 | 2 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

Over-expression of c-erb B-2 oncogene has been detected in human mammary carcinomas, and its presence correlated with poor prognosis and early recurrences.

We will evaluate the presence of this oncogene utilizing monoclonal and polyclonal antibodies in a group of 200 patients treated at NCI in the early stage of breast cancer protocol. The information obtained by immunohistologic evaluation will be correlated with patients' outcome and other parameters such as recurrences and lymph node metastasis, in an attempt to demonstrate whether this marker can be used as a prognostic factor especially in node negative patients.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09196-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Determination of Collagenase Production in Aggressive Variants of Thyroid Cancer

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

| | | | |
|---|---|---|---|
| PI: | M. Merino | Chief, Surgical Pathology Section | LP NCI |
| OTHER: | E. Campo | Guest Researcher | LP NCI |
| | W. Stetler-Stevenson | Senior Staff Fellow | LP NCI |
| | J. Robbins | Chief, Endocrinology | CC |
| | R. Neumann | Chief, Nuclear Medicine | CC |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

Papillary carcinoma of thyroid is associated with indolent biologic behavior, low incidence of distant metastases and good prognosis. Certain special variants, however, such as the TCV, insular and columnar types, have been associated with poor prognosis, early metastases and death.

We will study this unusual forms of cancer to evaluate the presence or absence of enzymes that play an important role in tumor invasion and metastases and compare them with normal thyroid tissues.

Our aim is to demonstrate the possibility to use these enzymes as tumor markers.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09197-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Value of GCDFP-15 to Recognize Breast Cancer Among Tumors of Unknown Origin

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: | C. Monteagudo | Visiting Fellow | LP NCI |
| | R. Neumann | Chief, Nuclear Medicine Department | CC |

COOPERATING UNITS (if any)

Clinical Center

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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| 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.)

Gross cystic disease fluid protein-15 is one of the major components of cystic fluid and a well known marker of apocrine differentiation. Monoclonal antibodies raised against this protein are highly specific and sensitive as breast carcinoma markers.

We will evaluate the usefulness of this monoclonal antibody to recognize breast cancers among metastasis and tumors of unknown origin; for this, a number of primary breast cancer tumors, other primaries, metastases and tumors of unknown origin will be studied in a blind fashion by immunoperoxidase in an attempt to identify those that represent breast cancers. These antibodies may be of tremendous assistance in female patients that present with carcinomas of unknown origin.



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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09198-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Mixed Forms of "Tall Cell Variant" of Papillary Carcinoma

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: | C. Monteagudo | Visiting Fellow | LP NCI |
| | K. Ain | Senior Staff Fellow | CC |
| | J. Robbins | Chief, Endocrinology | CC |
| | R. Neumann | Chief, Nuclear Medicine | CC |

COOPERATING UNITS *(if any)*

Endocrine Branch, Clinical Center

LAB/BRANCH

Laboratory of Pathology

SECTION

Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

The tall cell variant (TCV) of papillary carcinoma has been associated with an aggressive biological behavior. Histologically, these tumors are characterized by the presence of tall oxyphilic cells with the height twice the size of the width. The immunohistochemical profile of these neoplasms, however, has not been reported. We intend to study 18 cases of TCV and to evaluate several tumor markers by the immunohistochemistry technique to investigate if the antigenic profile of TCV differs from usual papillary cancer and if it causes any prognostic significance.





GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09199-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Trophoblastic (Choriocarcinomatous) Differentiation in Endometrial Cancer

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

| | | | |
|---|---|---|---|
| PI: | M. Merino | Chief, Surgical Pathology Section | LP NCI |
| OTHER: | C. Pesce | Visiting Associate | LP NCI |
| | F. Nogales | (Spain) | |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

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Surgical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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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.)*

Production of human chorionic gonadotropins has been reported not only in trophoblastic neoplasms but rarely in carcinomas arising in extragenital sites such as lung and urinary bladder.

HCG production has not been known to occur in endometrial carcinoma. We are in the process of studying HCG production by this type of cancer by immunohistologic techniques, and correlate it with determinations of HCG in serum. The finding of this choriocarcinomatous element in endometrial cancer has significant therapeutic and prognostic significance.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09165-03 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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. Larner, S. Vande Pol, A. Ginsberg, G. Jaffe, K. Schmidt, J. Taubenberger, D. Roth, D. Kleiner, H. Hollingsworth, K. Gardner, R. Doms, 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: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

The Section of Pulmonary and Postmortem Pathology, together with the Cytopathology Section, Hematopathology Section, Surgical Pathology Section, and Ultrastructural Pathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all institutes in those investigations which involve the use and study of human pathological material. The Autopsy Suite was recently renovated and new equipment specially designed for safety was developed in conjunction with the Occupational Safety and Health Branch, Division of Safety, Office of the Director. A set of special autopsy safety policies, many of which were developed in our department, is utilized to protect our staff and residents from exposure to tissues contaminated with high risk infectious agents and radiation.

The pulmonary and autopsy pathological material is being actively utilized by the staff and residents for research projects involving clinicopathological correlation and pathological characterization of diseases studied at the Clinical Center. Electron microscopic and immunocytochemical techniques are being applied to the study of these diseases. A comprehensive study of the pulmonary pathology of the Acquired Immune Deficiency Syndrome (AIDS) is currently being conducted based on surgical and autopsy lung pathology material from AIDS patients seen at the NIH. The autopsies from cancer patients treated with interleukin-2 and other forms of immunotherapy have been reviewed and a paper is almost completed on this subject.

Objectives:

The objectives of the Pulmonary and Postmortem Section are: (a) to provide diagnostic services in pulmonary and autopsy pathology for the clinical research projects conducted at NIH. This includes generating pathology reports for current cases and presenting the pathologic findings of autopsies and lung biopsies at clinical conferences with clinicians and radiologists; (b) to carry out independent research; (c) to provide teaching to the NIH residency program in anatomic pathology; and (d) to collaborate with investigators in research involving the use and study of human materials.

The proposed research program includes (a) continuing to provide the services described; (b) increasing interaction with clinical branches in the design and evaluation of protocols; (c) providing opportunities for residents to participate in teaching, and in research projects; (d) developing data retrieval systems for the autopsy and pulmonary pathology material.

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 are currently being written up as a paper focusing on the contribution of pulmonary pathology to the cause of death in AIDS patients.

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 reviewed and are being written up. These cases include examples of lymphocytic interstitial pneumonitis and nonspecific interstitial pneumonitis.

Publications:

Kettelhut BV, Parker RI, Travis WD, Metcalfe DD. Hematopathology of the bone marrow in pediatric cutaneous mastocytosis: a study of seventeen patients. Am J Clin Pathol 1989;91:558-2.

Stovroff MC, Fraker DL, Travis WD, Norton JA. Altered macrophage activity and tumor necrosis factor: tumor necrosis and host cachexia. J Surg Res 1989;46:462-9.

Doppman JL, Travis WD, Nieman L, Miller DL, Chrousos GP, Cutler GB, Loriaux DL, Norton JA. CT-MR findings in Cushing's syndrome due to primary pigmented nodular adrenocortical disease. Radiology 1989;172:415-0.

Dunbar C, Travis WD, Kan YW, Nienhuis AW. 5-Azacytidine treatment in a nontransfusible beta°-thalassemic. Br J Haematol 1989;72:467-4.

Perry RR, Nieman LK, Cutler GB, Chrousos GP, Loriaux DL, Doppman JL, Travis WD, Norton JA. Primary adrenal causes of Cushing's syndrome: Diagnosis and surgical management. Ann Surg 1989;210:59-8.

Atkinson JC, Fox PC, Travis WD, Popek E, Katz RW, Pillemer SR. IgA rheumatoid factor and IgG-IgA RF complexes in Sjögren's syndrome are related to tissue pathology. J Rheumatol 1989;16:1205-0.

Travis WD, Tsokos M, Doppman JL, Nieman L, Chrousos GP, Cutler GB, Loriaux DL, Norton JA. Primary pigmented nodular adrenocortical disease: a light and electron microscopic study of eight cases. Am J Surg Pathol 1989;13:921-0.

Haas GP, Pittaluga S, Gomella L, Travis WD, Sherina RJ, Doppman JL, Linehan WM, Robertson C. Clinically occult Leydig cell tumor presenting with gynecomastia. J Urol 1989;142:1325-7.

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;17:318-2.

Choyke PL, Filling-Katz MR, Shawker TH, Gorin MB, Travis WD, Chang R, Seizinger B, Dwyer AJ, Linehan WM. Radiologic screening for von Hippel-Lindau disease: the value of CT and ultrasound in visceral manifestations. Radiology 1990;174:697-2.

McIntosh JK, Mule JJ, Travis WD, Rosenberg SA. Studies of the effects of rhTNF-α on autochthonous tumor and transplanted tissue in mice. Cancer Res 1990;50:2463-9.

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;14:555-4.

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;21:493-2.

Travis WD, Lack EE, Oertel JE. Miscellaneous adrenal tumors and tumor-like lesions. Chapter 10. In: Lack EE, ed. Pathology of the adrenal glands. Churchill Livingstone, 1989;351-78.

Lack EE, Travis WD, Oertel JE. Adrenal cortical nodules, hyperplasia and hyperfunction. Chapter 2. In: Lack EE, ed. Pathology of the adrenal glands. Churchill Livingstone, 1989;75-113.

Lack EE, Travis WD, Oertel JE. Pathology of adrenal cortical neoplasms. Chapter 3. In: Lack EE, ed. Pathology of the adrenal glands. Churchill Livingstone, 1989;115-71.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09166-03 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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: |
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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

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 45 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.



PHS 6040 (Rev. 1/84)

GPO 914-916

Publications:

Masur H, Ognibene FP, Yarchoan R, Shelhamer JH, Baird BF, Travis WD, Suffredini AF, Deyton L, Kovacs JA, Falloon J, Davey R, Polis M, Metcalf J, Baseler M, Wesley R, Gill VJ, Fauci AS, Lane HC: CD4 counts as predictors of opportunistic pneumonias in human immunodeficiency virus (HIV) infection. Ann Intern Med 1989;111:223-1.

Curiel DT, Holmes MD, Okayama H, Brantly ML, Vogelmeier C, Travis WD, Stier L, Perks WH, Crystal RG. Molecular basis of the lung and liver disease associated with the alpha$_1$-antitrypsin deficiency allele $M_{malton}$. J Biol Chem 1989;264:13938-5.

Smith WS, Lesar MS, Travis WD, Lubbers P, Sen RP, Ginsberg AM, DeLaney T, Lack EE, Gomes MN. MR and CT findings in pulmonary artery sarcomas. J Comput Assist Tomog 1989;13:906-9.

Davey RT, Margolis D, Deyton L, Kleiner DE, Travis WD. Disseminated *Pneumocystis carinii* infection during aerosolized pentamidine prophylaxis. Ann Intern Med 1989;111:681-2.

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Feinberg L, Travis WD, Ferrans V, Sato N, Bernton HF. Pulmonary fibrosis associated with tocainide: report of a case with literature review. Am Rev Respir Dis 1990;141:505-8.

Feuerstein IM, Archer A, Pluda J, Francis PS, Falloon J, Masur H, Pass HI, Travis WD. Thin-walled cavities and pneumothorax in patients with *Pneumocystis carinii* pneumonia: CT demonstration. Radiology 1990;174:697-2.

Travis WD, Schmidt K, Masur H, MacLowry J. Respiratory cryptosporidiosis. Case report and review of the literature. Arch Pathol Lab Med 1990;114:519-2.

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 1990;14:615-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00852-37 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 | Senior Staff Fellow | LP NCI |
| | C. Copeland | Cytotechnologist | LP NCI |
| | L. Galito | Biologist | LP NCI |
| | A. Wilder | Cytotechnologist | LP NCI |
| | E. Sanders | Bio. Lab. Technologist | LP NCI |
| | C. King | Medical Technologist | LP NCI |
| | J. Colandrea | Biotechnology Fellow | 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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 7 | 2.5 | 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 pathologist instruct the clinician in aspiration technique; 3) a cytotechnologist assist the clinician in handling the specimen; 4) aspirations of deep lesions be performed by the radiologist with the assistance of a cytotechnologist to evalute adequacy of the specimen.

An example of one collaborative clinical research project involves clinical trials currently being conducted to study the use of the monoclonal antibody 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. We are also performing in situ hybridization studies on cytopathologic material, probing for CMV viral DNA.

Major Findings:

Approximately 3800 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 increased in number by 100% compared to 2 years ago. This modality has been embraced by clinicians as a minimally invasive technique which provides diagnoses rapidly and cost effectively, with minimal discomfort to the patient often obviating more invasive biopsy procedures.

Cases submitted by outside pathologists for consultation by the Cytopathology Section have increased in number by approximately 200% over previous years.

Cytological techniques are utilized in collaborative work with other sections and branches of NIH. For example, single cell tumor suspensions, tumor cell lines, and stimulated lymphocyte cultures are evaluated microscopically and by immunocytochemical techniques.

Publications:

Manyak MJ, Nelson LM, Solomon D, Thomas GF, Stillman RJ, and Russo A. Photodynamic therapy of rabbit endometrial transplants: a model for treatment of endometriosis. Fertility and Sterility 1989;52:140-5.

Rosengart TK, Pass H, Cannon R, Miller DL, Solomon D, Clark RE. Ruptured gastroepiploic artery aneurysm and vascular collapse in a patient with thoracid aneurysm. J Cardiovasc Surg 1989;30:514-6.

Hamburger JI, Husain M, Nishiyama R, Nunez C, Solomon D. Increasing the accuracy of fine needle biopsy for thyroid nodules. Arch Pathol Lab Med 1989;113:1035-1.

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. JB Lippincott (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00897-07 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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.S. Jaffe | Chief, Hematopathology Section | LP NCI |
| | L. Elwood | Senior Staff Fellow | 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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.0 | .5 | .5 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

The cytological diagnosis of malignant lymphoma can be extremely difficult because the cytological features of the malignant cells in small cell and mixed small and large cell lymphomas may be indistinguishable from those of reactive lymphoid cells. We have examined the usefulness of the avidin biotin immunoperoxidase technique and a battery of antibodies to T and B cell markers to the diagnosis of lymphoma in cytological specimens. We conclude that immunocytochemistry is very useful in the cytological diagnosis of non-Hodgkin's lymphoma. Further, it is possible to diagnose the vast majority of lymphomas using only the immunoglobulin light chain markers κand 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.



PHS 6040 (Rev. 1/84)

GPO 914-916

Major Findings:

Immunocytochemistry in the evaluation of lymphoid cell populations:

We have investigated 395 specimens, including 208 pleural and peritoneal effusions, 80 cerebrospinal fluids, and 99 fine needle aspiration specimens. We have found 181 cases to be positive for lymphoma and 114 to be reactive in nature. Of the 181 positive cases, 142 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 34 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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09153-04 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 |
| | P. Aebersold | 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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.50 | .25 | .25 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

D

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

Clinical trials employing the adoptive transfer of expanded tumor infiltrating lymphocytes to patients with metastatic disease are currently underway under the direction of the Surgery Branch, NCI. Our collaborative effort in this project involves immunocytochemical analysis of tumor cell suspensions to identify (1) the percentage and phenotypic expression of subsets of tumor infiltrating lymphocytes present in the tumor and (2) tumor markers, if any, which are expressed by the tumor cells. Once the tumor infiltrating lymphocyte cultures have been expanded and are to be harvested for patient therapy, we analyze the material using routine cytologic preparations and immunocytochemistry to ensure the cultures are free of tumor cells.

Major Findings:

Over 200 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 200 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:

Topalian SL, Solomon D, Rosenberg SA. Tumor-specific cytolysis by lymphocytes infiltrating human melanomas. J Immunol 1989;142:3714-25.

Haas GP, Solomon D, Rosenberg SA. Tumor infiltrating lymphocytes from non-renal urologic malignancies. Cancer Immunol Immunother 1990;30:342-0.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 09176-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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. Smart | Chief, Early Detection Branch | DCPC NCI |

COOPERATING UNITS *(if any)*

DCPC: CDC

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .25 | .25 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

The cervical/vaginal Papanicolaou smear is arguably the most effective cancer screening technique in general use today. However, the accuracy of cervical/vaginal cytopathologic diagnosis has been subject to intense scrutiny and criticism recently.

A workshop organized and sponsored by DCPC and DCBDC, NCI to develop a uniform system of reporting cervical/vaginal cytopathology resulted in The Bethesda System for Reporting Cervical/Vaginal Cytopathology Diagnoses. Widespread implementation of The Bethesda System will provide for effective communication between laboratories and clinicians as well as provide a meaningful data base for research into the biology and epidemiology of cervical neoplasia.

The Bethesda System (TBS) has received widespread support nationally and internationally. Virtually every professional society involved in cervical cancer cytology screening has endorsed TBS. I continue to be involved in the dissemination of TBS through presentations at national and international meetings and as an information resource for laboratories and physicians. 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; the Laboratory Initiatives for the year 2000 Health Objectives for the nation; and an NCI Working Group to assess the current role of cytopathology in cervical cancer screening.



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

Publications:

Solomon D. Introduction to the proceedings of the conference on the state of the art in quality control measures for diagnostic cytology laboratories. Acta Cytol 1989;33:427-0.

National Cancer Institute Workshop: Solomon D, Chairman, Terminology Working Group. The 1988 Bethesda System for reporting cervical/vaginal cytologic diagnoses. JAMA 1989;262:931-4; Acta Cytol 1989;33:567-4; Diagn Cytopathol 1989;5:331-4.

Solomon D, Wied GL. Cervicography: An assessment. J Reprod Med 1989;34:321-3.

Erozan Y, Greening S, Gupta P, Kraemer B, Kurman R, Luff R, Rosenthal D, Saigo P, Solomon D, Wied G. Cervical cytology and reimbursement issues (Letter to the Editor). JAMA 1990;263:515.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09177-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Immunocytochemistry of Metastatic Colorectal Carcinoma

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

| | | | |
|---|---|---|---|
| PI: | L. Elwood | Deputy Chief, Cytopathology Section | LP NCI |
| OTHER: | M. Lotze | Senior Staff | SB NCI |
| | R. Chang | Staff Radiologist | DR CC |
| | A. Wilder | Cytotechnologist | LP NCI |
| | C. King | Medical Technologist | LP NCI |

COOPERATING UNITS *(if any)*

Surgery Branch, DCT, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.25 | 0.10 | 0.15 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

Studies to assess the effectiveness of combined recombinant IL-2 and anti-tumor antibodies with and without lymphokine activated killer cells (LAK) in treating metastatic cancer are currently underway. We are involved in protocols assessing these therapies in patients with liver metastases from primary colorectal carcinoma. Computed tomography-guided aspiration biopsy specimens are evaluated by a trained cytotechnologist for adequacy in the radiology suite. the procured specimen is then stained in our laboratory by immunocytochemistry with the antibodies L6 and 17-1A. L6 is a murine monoclonal antibody raised against human lung cancer that stains a carbohydrate antigen present on a variety of adenocarcinomas, including most colon cancers. Only scattered cells in normal tissues appear to react with L6. 17-1A is a murine monoclonal antibody generated against a colorectal cell line. Most colorectal cancers react with 17-1A. Some reactivity for 17-1A has been demonstrated in normal colon. Patients are assigned to a protocol according to their immunocytochemical staining pattern.

Major Findings:

Since the first patient was evaluated on 10/26/88, 36 specimens in 35 patients have been evaluated for this study. With L6 antibody 6 cases yielded unsatisfactory specimens. Of 30 evaluable cases, 22 (73%) showed positivity. For 17-1A, 6 cases were unsatisfactory. Of the 29 evaluable cases, 28 (97%) were positive. A significant problem is obtaining sufficient viable material from metastatic colon cancer. Immediate evaluation of material for adequacy in the radiology suite has been helpful; however, unsatisfactory specimens are still obtained in some cases. The biopsies are done as outpatient procedures and have been associated with no clinical morbidity. It appears that fine needle aspiration biopsy of metastatic liver lesions yields satisfactory specimens for immunocytochemical characterization in many cases, minimizing morbidity to the patient and cost of tissue procurement.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09178-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Immunophenotypes of T Cells and Stromal Cells in Mouse Peyer's Patches

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

| | | | |
|---|---|---|---|
| PI: | L. Elwood | Deputy Chief, Cytopathology Section | LP NCI |
| OTHER: | G. 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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.20 | 0.10 | 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 in the germinal center are poorly understood. In an attempt to better define some of the factors involved, studies in mice have been underway to characterize the B-cell-associated T-cells in mouse Peyer's patches by flow cytometry and to isolate B-cell associated stromal cells. In our laboratory, we are using frozen section immunohistochemistry to localize the various T-cell subsets found by flow cytometry. For this purpose, dual staining techniques have been utilized. Query stromal cells obtained from tissue culture are being characterized by immunocytochemistry.





GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09186-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 | Deputy Chief, Cytopathology Section | 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

SECTION

Cytopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.25 | .10 | .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 pleiotropic biological effects and is involved in inflammation, immunologic challenges, and the response to infections. The genes for two different forms of IL-1, IL-1 alpha and IL-1 beta, have been cloned allowing production of large quantities of these cytokines by genetic engineering.

IL-1 alpha is membrane bound in vivo and is biologically active. Specifically, in animal studies, IL-1 alpha has been shown to have immunoenhancing, bone marrow restorative and direct anti-tumor effects. Because of these effects, it is likely that IL-1 alpha will have significant clinical utility in the treatment of malignant disease and a phase I study of its toxicity and hematological and immunologic effects in humans in currently underway. Specifically, I will be looking at pre- and post-treatment bone marrow aspirates and peripheral blood smears in patients with metastatic malignant disease given increasing doses of IL-1 alpha.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09184-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

P-Glycoprotein Expression in Pediatric Cell Tumors

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

| | | | |
|---|---|---|---|
| PI: | C. Axiotis | Cancer Expert | LP NCI |
| OTHER: | M. Tsokos | Chief, Ultrastructural Pathology Section | LP NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3/4 | 3/4 | |

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.)*

P-glycoprotein has been associated with a multidrug-resistant (MDR) phenotype in a wide variety of animal and human tumor cell lines and clinical tumor specimens. Pediatric tumors, however, have not been included in the studies reported so far. Given the known chemosensitivity of certain solid pediatric tumors, such as Ewing's sarcoma, primitive neuroectodermal tumor and rhabdomyosarcoma, to the point that all clinical trials use chemotherapy and radiotherapy as primary methods of treatment, it is important to detect levels of P-glycoprotein in these tumors before and after treatment.

Tissues from Ewing's sarcomas, primitive neuroectodermal tumors and rhabdomyosarcomas are available from patients included in the clinical therapeutic protocols in the Pediatric Oncology Branch of NIH. All these tumors are well characterized and classified histologically by light and electron microscopy. Moreover, tissues from primary sites before treatment and from metastatic sites or sites of recurrence after treatment are available in many cases. All these tissues will be evaluated for the presence of P-glycoprotein using a commercially available antibody against P-glycoprotein and employing a modified immunoperoxidase "sandwich" staining method (Chan et al., Lab. Invest. 59: 870, 1988). The results of immunostaining for P-glycoprotein will be correlated with clinical data of patient outcome and response to treatment.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09187-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Expression of Transforming Growth Factor-b in Human Neural Cell Lines

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 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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1 | 1 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

D

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

Although little is known about the physiologic role of Transforming Growth Factor (TGF)-b in vivo, immunohistochemical studies in the developing mouse embryo have implicated TGF-b in proliferation and differentiation of tissues in general, especially the mesenchymally (bone, muscle, and blood vessels) and neural crest derived ones. Interestingly enough, intense staining is found in the craniofacial mesenchyme of neural crest origin, destined to become mandible, maxilla, palate, nose, and nasal sinuses.

We have recently obtained cDNA probes for all 3 TGF-b subtypes (1,2 and 3) from Dr. Anita Roberts (NIH), and antibodies against the TGF-b1 and 2 are available commercially. We also have in our laboratory 12 human tumor cell lines originated from various neural tumors, ranging from conventional neuroblastoma, to peripheral primitive neuroectodermal tumors, to olfactory neuroblastoma.

We will study the expression of TGF-b1, 2, and 3 in all these neural cell lines at the mRNA and protein level by Northern and Western blotting, and immunocytochemical staining by immunofluorescence. Various patterns of expression of these 3 TGF-b subtypes may be observed implicating a role of this growth factor in the neural childhood tumors. In addition, several possible emerging patterns of expression may further confirm our suspicion of different pathogenetic pathways in the development of these tumors, and finally may serve as differential markers in their diagnosis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09188-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Flow Cytometric Analysis of Childhood Sarcomas

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

| | | | |
|---|---|---|---|
| PI: | C. McLeod | Biotechnology Fellow | LP NCI |
| OTHER: | M. Tsokos | Chief, Ultrastructural Pathology Section | LP NCI |
| | M. Horowitz | Senior Investigator | PB NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 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.)*

Hyperdiploidy has been associated with an unfavorable histology (anaplastic) in Wilms' tumor and with a better response to chemotherapy in unresectable neuroblastoma. Moreover, DNA-aneuploidy was found in rhabdomyosarcoma (RMS), in contrast to Ewing's sarcoma and primitive neuroectodermal tumor (PNET) in a recent report using a limited number of tumors. The latter was suggested to be an additional criterion in the differential diagnosis of childhood sarcomas.

We will analyze the cellular DNA content of 100 tumor samples including RMS, PNET, and Ewing's sarcoma using histologic sections (10 or more per tumor sample) from paraffin blocks. DNA histograms will be used to determine: (1) the number of malignant stem lines in each sample; (2) the DNA index of the stem line(s) (i.e., ratio of the modal Go/G1 channel for tumor cells versus that of normal diploid cells) and (3) the percentages of tumor cells in S phase and G2+M phase. The findings will be compared with the histology of the tumors and the histologic subtypes in the case of RMS (embryonal vs. alveolar, and the newly defined by us histologic subtype with aggressive clinical outcome, the solid variant alveolar RMS). Given the fact that all patients were treated homogeneously according to clinical protocols, meaningful comparisons between DNA-ploidy and clinical outcome or response to treatment will be made as well.

The findings will help us draw conclusions as to a possible prognostic or diagnostic role of flow cytometry in this group of pediatric sarcomas.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09189-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Regulatory Pathways in the Differentiation of Human Rhabdomyosarcoma In Vitro

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

PI: M. Tsokos Chief, Ultrastructural Pathology Section LP NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have found previously that 5-Azacytidine (5-AZA) induces and Transforming Growth Factor (TGF)-b inhibits differentiation of human rhabdomyosarcoma (RMS) cells in vitro. The effect of 5-AZA was associated with increased levels of expression of the muscle determination gene $Myo_{D1}$. However, remarkable changes in the expression of the $Myo_{D1}$ gene after addition of 1 ng/ml ($4 \times 10^{-11}$ M) TGF-b were not observed.

Although in a recent study $Myo_{D1}$ expression has been found repressed by TGF-b1, this was achieved at a 100-fold higher concentration of the agent, and was demonstrated in myoblasts derived from a 5-AZA treated pluripotential cell line and not RMS. In other studies, it was found that TGF-b at a concentration of $4 \times 10^{-10}$ M blocks the appearance of functional slow $Ca^{2+}$ channels in differentiated myocytes by inhibiting the expression of the dihydropyridine receptor (DHPR) gene, which is skeletal muscle specific. The expression of $Myo_{D1}$ gene was not influenced.

We have obtained the DHPR cDNA and we will investigate changes of the expression of this gene, and $Myo_{D1}$ in 5 human rhabdomyosarcoma cell lines treated with various concentrations of TGF-b. Although it is not known if the DHP-sensitive $Ca^{2+}$ channels are necessary events in the myogenic differentiation, extracellular $Ca^{2+}$ is necessary for muscle development. Possible detectable changes of the DHPR gene in relation to the action of TGF-b and the expression of the $Myo_{D1}$ gene will suggest that $Ca^{2+}$ channels may be part of the intracellular mechanisms that control myogenesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09172-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

| | | | |
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| PI: | D.D. Roberts | Chief, Biochemical Pathology Section | LP NCI |
| OTHER: | V. Zabrenetzky | Biotechnology Fellow | LP NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.8 | 1.8 | 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 functions of thrombospondin in cell adhesion and migration are being investigated. We have identified two regions of the thrombospondin molecule that mediate adhesive and migratory responses of cultured human melanoma cells to thrombospondin. The carboxyl-terminal domain mediates attachment and haptotaxis, while the amino-terminal domain mediates cell spreading and chemotaxis. The cell receptors recognizing these two regions of thrombospondin are under investigation. One class of receptors are sulfated glycoconjugates which bind to the amino-terminal domain of thrombospondin. A minor heparan sulfate proteoglycan that binds thrombospondin with high affinity was identified in two melanoma cell lines and purified by affinity chromatography on thrombospondin-Sepharose (Cancer Res. 48: 6875, 1988). An unusual sulfated glycolipid present only in melanoma cell lines that spread on thrombospondin was also found to bind thrombospondin (ibid.). This glycolipid purified from peripheral nerve and a monoclonal antibody to the glycolipid specifically inhibit melanoma cell spreading on thrombospondin but not on fibronectin. To further define the mechanism of thrombospondin interactions with tumor cells, receptors for the carboxyl-terminus of thrombospondin will be characterized. 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 to the two types of receptors are also being investigated.



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

Major Findings:

1. 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 the adhesive glycoproteins thrombospondin and laminin to immobilized sulfatide with ID50 values of 220 µg/ml and 470 µg/ml, respectively. Sulfated glycoconjugates on melanoma cells mediate spreading on thrombospondin by binding to the amino-terminal heparin and sulfatide binding domain. This domain is also required for chemotaxis on thrombospondin. We therefore examined the effect of suramin on human melanoma cell spreading and migration. Suramin at 50 to 400 µg/ml specifically inhibited G361 melanoma cell spreading on thrombospondin without affecting cell attachment. Suramin also inhibited spreading of A2058 melanoma cells on thrombospondin and laminin and partially inhibited cell attachment. However, surramin had no effect on G361 or A2058 cell attachment or spreading on fibronectin. Chemotaxis of A2058 and G361 melanoma cells to thrombospondin and laminin were also specifically inhibited by suramin, as was haptotaxis of A2058 melanoma cells to laminin. However, suramin only weakly inhibited haptotaxis of G361 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. Cellular responses to binding of thrombospondin to the two classes of receptors are being investigated. Changes in intracellular inositol phosphate and cyclic AMP were observed following exposure of melanoma cell monolayers to thrombospondin. Experiments are in progress to determine the role of these changes in mediating the effects of thrombospondin on cell adhesion, growth and motility.

Publications:

Sherwood JA, Roberts DD, Spitalnik SL, Lawler JW, Miller LH, Howard RJ. Falciparum malaria infected erythrocytes bind to a 140 kilodalton thrombospondin fragment and not to the amino treminal heparin-binding region. Mol Biochem Parasitol (in press)

Taraboletti G, Roberts DD, Liotta LA, Giavazzi R. Platelet thrombospondin modulates endothelial cell adhesion, motility and growth: a potential angiogenesis regulatory factor. J Cell Biol (in press)

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 (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09173-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Carbohydrate Receptors for Human Pathogens

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

PI: D.D. Roberts Chief, Biochemical Pathology Section LP NCI

COOPERATING UNITS *(if any)*

T. Hackstadt, Dept. of Pathology, University of Texas Medical Branch, Galveston; T. Walsh, Pediatric Oncology, NCI, NIH

LAB/BRANCH

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INSTITUTE AND LOCATION

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| 0.3 | 0.3 | 0 |

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☐ (a1) Minors

☐ (a2) Interviews

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

Adhesive specificities of some *Enterococcus* species, *Candida albicans*, and elementary bodies of *Chlamydia trachomatis* are being examined. These will be 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:

*M. pneumoniae* was also tested for adhesion to immobilized glycoproteins. Several purified glycoproteins including laminin, thrombospondin, fetuin, and human chorionic gonadotropin (hCG) promote dose-dependent and saturable adhesion of *M. pneumoniae*. Adhesion to all of the proteins requires sialic acid, and only those glycoproteins with α2→3-linked sialic acid are active. Structural analysis of the asparagine-linked oligosaccharides of thrombospondin demonstrated α2→3-linked sialic acid. The α-subunit of hCG contains only mono- and biantennary oligosaccharides and also promotes attachment, suggesting that a biantennary asparagine-linked sialyloligosaccharide is sufficient for binding. Binding to the glycoproteins is inhibited by 3'-sialyllactose and fetuin oligosaccharides, weakly by 6'-sialyllactose, but not by dextran sulfate. Conversely, dextran sulfate but not 3'-sialyllactose inhibits *M. pneumoniae* adhesion to sulfatide. Thus, two distinct receptor specificities mediate binding to the two classes of carbohydrate receptor. Both 3'-sialyllactose and dextran sulfate partially inhibit *M. pneumoniae* adhesion to WiDr cells at concentrations that completely inhibit binding to laminin or sulfatide, respectively, and in combination they inhibit adhesion of *M. pneumoniae* to WiDr cells by 90%. Therefore, both receptor specificities contribute to *M. pneumoniae* adhesion to cultured human cells.

*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 GalNAcß1-4Galß1-4Glc 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, Olson LD, Barile MF, Ginsburg V, Krivan HC. Sialic acid-dependent adhesion of *Mycoplasma pneumoniae* to purified glycoproteins. J Biol Chem 1989;264:9289-3.

Krivan HC, Olson LD, Barile MF, Ginsburg V, Roberts DD. Adhesion of Mycoplasma pneumoniae to sulfated glycolipids and inhibition by dextran sulfate. J Biol Chem 1989;264:9283-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09174-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

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COOPERATING UNITS *(if any)*

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J. Cashel, Biochemical Pathology Section, LP, NCI

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| 1.8 | 0.8 | 1.0 |

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☐ (a1) Minors
☐ (a2) Interviews

B

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

We have found that sulfated glycolipids participate in tumor cell adhesion by directly promoting adhesion (Cancer Res. 48: 3367, 1988) and as receptors for thrombospondin on melanoma cells (Cancer Res. 48: 6785, 1988). Relatively few of the glycolipids belonging to this class have been characterized. We are purifying novel sulfated glycolipids from several sources including human kidney and meconium and breast and small cell lung carcinoma cell lines. The structures of these glycolipids will be examined using chemical and immunological approaches. Monoclonal antibodies to these will be used to examine the potential of these structures as tumor markers. The molecular basis of binding of adhesive glycoproteins to sulfatide is being investigated by identifying sequences in these molecules responsible for binding. To further characterize the effects of sulfated glycolipids on tumor cell behavior, we are also examining the intracellular responses following attachment of melanoma cells on sulfatide-coated substrates.

Major Findings:

Two novel sulfated glycosphingolipids belonging to the globo series were isolated from human kidney. The lipids were isolated from pooled kidney by chloroform/methanol extraction, akali treatment, DEAE-Sephadex and silicic acid column chromatography and preparative thin layer chromatography. Based on infrared and two-dimensional proton magnetic resonance spectroscopy, negative secondary ion mass spectroscopy, methylation analysis, and interaction with monoclonal antibodies and sulfatide-binding proteins, the structures are proposed to be $HSO_3$-3Galß1-3GalNAcß1-3Galα1-4Galß1-4Glcß1-1ceramide and $HSO_3$-GalNAcß1-3Galα1-4Galß1-4Glcß1-1ceramide. These glycolipids react with a monoclonal antibody SSEA-3 on thin layer chromatograms and by radioimmunoassay and bind $^{125}$I-labeled thrombospondin. The yield of glycolipids was 0.19 and 0.034 nmol/g of kidney, respectively.

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 [$^{35}$S]-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.

Publications:

Nagai K, Roberts DD, Toida T, Kushi Y, Handa S, Ishizuka I. Sulfated globopentaosylceramide from human kidney. J Biol Chem 1989;264:16229-7.

Nagai K, Roberts DD, Toida T, Kushi Y, Handa S, Ishizuka I. Monosulfated globotetraosylceramide from human kidney. J Biochem 1989;106:878-6.

Taraboletti G, Rao CN, Krutzsch HC, Liotta LA, Roberts DD. Sulfatide-binding domain of the laminin A chain. J Biol Chem (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09175-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Glycolipid Antigens Expressed in Esophageal Carcinoma

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

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| PI: | D.D. Roberts | Chief, Biochemical Pathology Section | LP NCI |
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L. Zhang and V. Ginsburg, LSB, NIDDK, NIH

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☐ (a1) Minors

☐ (a2) Interviews

B

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

Esophageal carcinoma is endemic in the Ho Nam Province in China. Characterization of antigens unique to these tumors would be useful for diagnosis of this carcinoma. Glycolipids isolated from esophageal tissues obtained from 11 cancer patients, 10 normal individual controls, and a cultured esophageal carcinoma line were examined using several monoclonal antibodies. Several glycolipids differ in amount between the tumor samples and normal controls. Both neutral and sialylated glycolipids containing 3-fucosyllactosamine sequences differ in expression between the tumor and control tissues and a heptaglycosylceramide reacting with antibodies to this sequence is found only in the tumor tissues. The same glycolipids are detected in the cultured cell line, confirming that the tumor antigen is produced by the tumor and is not due to contamination by infiltrating granulocytes.





GPO 914-918

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00891-07 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

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| | M. Beckner | Biotechnology Fellow | LP NCI |
| | D. Savarese | Biotechnology Fellow | LP NCI |
| | H. Krutzsch | Expert | LP NCI |
| | P. Hwang | Visiting Scientist | LP NCI |

COOPERATING UNITS *(if any)*

Cpt. C. Evans, Dept. Surgery, WRAIR; Dr. Lee Helman, Sr. Invest., COP, DCT, NCI

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☐ (a1) Minors

☐ (a2) Interviews

B

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

We have been studying tumor cell motility as a requirement for the metastatic dissemination of malignant cells. The autocrine motility factor (AMF) from human melanoma cells has been purified to homogeneity and its N-terminal sequence determined. The material is highly potent (50 pmol), a labile protein, and has an Mr ~ 60-65 kDa. A protein that is a candidate for a motility receptor on these cells has been identified by a monoclonal antibody. The latter inhibits migration of cells to a variety of stimuli. Type IV collagen, a chemoattractant for the melanoma cells, stimulates a rapid transient mobilization of intracellular calcium; this suggests a role for such $Ca^{++}$ pools in the induction of the motile response by type IV collagen. Type I collagen does not stimulate motility. Insulin-like growth factor-I (IGF-I) previously shown to stimulate motility via a type I receptor, has now been demonstrated to stimulate a receptor-associated tyrosine kinase. This study provides insight into the signal transduction mechanism for IGF-I, an agent that may direct homing of metastatic cells. Preliminary results suggest that granulocyte-macrophage colony stimulating factor may also induce tumor cell migration. In collaborative studies with other investigators, it has been shown that rat prostatic carcinoma cells produce an AMF different from that from melanoma cells, and that IGF-II stimulates motility in rhabdomyosarcoma cells via a receptor distinct from the type I. These results emphasize the heterogeneity and versatility of malignant tumor cells during their metastatic dissemination.



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

Major Findings:

We have been studying the role of cell motility in the metastatic dissemination of malignant tumor cells. The work has focussed upon the agents and the biochemical mechanisms by which they stimulate migration in tumor cells. In these efforts, we have identified an autocrine motility factor (AMF) which stimulates melanoma cell migration.

A) Melanoma Cells - A2058 Strain

I) Purification of the autocrine motility factor (AMF)

The factor, a protein, has been purified to homogeneity. The procedure involves the following sequence of steps. A2058 conditioned media is passed over a gelatin column to separate AMF from type IV collagenase, which has a similar molecular weight to AMF and binds to the column; the AMF-containing material (not binding to column) is concentrated and subjected to 1.2 M $(NH_4)_2SO_4$ precipitation in the cold; the supernatent from this is freed of salts and then chromatographed on a phenyl sepharose column; chemotactically active fractions (identified by procedures detailed in Stracke et al.) were then applied to a phenyl TSK column (hydrophobic interaction) and active fractions again identified. These, in turn, were applied to a weak anion exchange column. Active material from this step was then applied to a concanavalin A affinity column and eluted with α-methylmannoside. The active material was then subjected to 'non-denaturing' gel electrophoresis. Active material from this step was then subjected to SDS gel electrophoresis, transferred by electroblotting to an immobilon matrix which was then visualized with Coomassie dye. A band of material of Mr 60-65 kDa was observed.

II) N-terminal sequence of the AMF

The material from the electroblot (mw 60-65 kDa) was subjected to micro Edman degradation and an N-terminal sequence of 12 amino acids was determined.

III) Plans for the AMF study

It is planned to produce antibodies in a rabbit injected with a conjugate of the peptide to bovine serum albumin. Also, we will attempt to 'renature' the specific band of material from SDS gel electrophoresis to verify the biological activity of the AMF so isolated.

IV) Surface protein on melanoma cells required for motility

A monoclonal antibody has been isolated that inhibits both attachment and motility of melanoma cells; in the latter case, motility to AMF insulin-like growth factors, type IV collagen, laminin and fibronectin are all affected. The antibody reacts on Western blotting with a protein, derived from melanoma cell extracts, which has a molecular weight of 95-107 kDa and differs from the ß1 subunit of the integrin receptor and from the laminin receptor.

Fluorescence studies show it to be a surface protein, and enzymatic treatments as well as behavior on binding columns indicate this protein to be glycosylated. It is planned to obtain sufficient protein to secure an N-terminal sequence. Also, a cDNA library from A2058 cells is being screened for a clone that produces this protein. Isolation of a positive clone would enable us to determine the major sequence of the proteins as well as, it is our hope, to also clone the gene for this protein. The protein itself may be a necessary adherence component for the cell involved in its metastatic progression.

V) The mechanism of action of type IV collagen in stimulating motility

We have previously shown that type IV collagen stimulates motility in melanoma cells through a pertussis-toxin sensitive mechanism. Further studies on this suggest that neither the inositol phosphate nor the adenylate cyclase pathway is involved in signal transduction of the collagen stimulus. Studies with A2058 cells loaded with the dye FURA-2 indicate that the collagen causes a rapid elevation of intracellular $Ca^{++}$. These efforts are being pursued with populations of cells selected for their motility. A variety of $Ca^{++}$ channel blockers did not affect motility. The uptake and release of $^{45}Ca$ is being studied as a function of collagen levels. Another possible mechanism, lipid methylation, was suggested by the finding that inhibitors of biological methylation markedly reduced the motility induced by type IV collagen. We plan to determine whether the attractant stimulates methylation in A2058 cells. This response is <u>not</u> induced by type I collagen indicating specificity for the collagenous ligand. Studies to isolate the domain of type IV collagen responsible for initiating motility are in progress.

VI Other ligands stimulating motility in melanoma cells

a) IGF-I and its mechanism of action

We have previously shown that the insulin-like growth peptide, IGF-I, potently stimulates motility in melanoma cells via a receptor. In signal transduction studies with this agent, it has now been found that there is a good correlation between the ability of IGF-I to stimulate motility and its capacity to activate the tyrosine kinase associated with the IGF-I receptor on melanoma cells. In addition, some agents which are known to inhibit protein kinases reduce this IGF-I stimulated motility and tyrosine kinase activity in a parallel manner. It is planned to identify the substrate for the receptor-associated kinase in succeeding experiments. IGFs may act as homing signals for metastasizing tumor cells.

b) Suramin as an inhibitor of cell motility

Suramin is a polysulfonated drug that inhibits binding of certain growth factors to cells. It has now been found that suramin can inhibit the spreading of melanoma cells on layers of both thrombospondin and laminin. The motility of these cells to these agents was also inhibited in the presence of low (50 to

400 µg/ml) concentrations of the drug. The drug had no effect upon the actions of fibronectin. The anti-tumor effects of this drug are likely to be the result of its interaction with the sulfatide components on the ligands laminin and thrombospondin, preventing their binding to appropriate cell receptors.

c) Granulocyte-macrophage colony stimulating factor (GM-CSF) as a motility stimulant in tumor cells

Highly motile cells such as neutrophils have been shown to be primed for responsiveness to chemoattractants after exposure to GM-CSF. We exposed melanoma cells to low levels (1-10 nM) of this agent and observed a motility response that was principally chemotactic. Pertussis toxin treatment of the cells markedly reduced this response. It is planned to identify the receptor for this agent as well as to search for the corresponding mRNA. The significance of these findings may be that this agent can influence the general motility and homing of tumor cells. This could lead to modification of therapies utilizing GM-CSF, especially in cancer patients.

B) Other Types of Tumor Cells

I) Prostatic cancer cells

In a collaborative study with WRAIR, it has been found that rat prostatic carcinoma cells produce and respond to an AMF, which differs from the melanoma AMF. It is susceptible to a variety of proteases. It is more of a chemotactic agent than melanoma AMF, which is highly chemokinetic; it is unstable to both acid and base, both oxidation and reduction inactivate it; it is stable in 6M urea; it markedly differs from melanoma AMF in not showing pertussis toxin sensitivity and in being sensitive to both choleratoxin and forskolin treatment of the cells. Highly metastatic cells were more responsive than the low metastatic variety. This attractant did not stimulate neutrophil motility. Further studies on the characterization of this protein are planned.

II) Rhabdomyosarcoma (RD)

In a collaboration with the Pediatric and Medicine Branch of the NCI, it has been found that insulin-like growth factor II (IGF-II), in addition to acting as a growth factor for RD cells, also stimulates primarily chemokinetic motility in these cells. This motility is not inhibited by the antibody $\alpha IR_3$, a monoclonal for the type I IGF receptor. Further studies are planned to characterize the receptor that mediates motility. The studies may emphasize the role of the IGFs in directing homing of metastasizing tumor cells.

Publications:

Stracke ML, Engel JD, Wilson LW, Rechler MM, Liotta LA, Schiffmann E. The type I insulin-like growth factor receptor is a motility receptor in human melanoma cells. J Biol Chem 1989;264:21544-9.

Kohn EC, Liotta LA, Schiffmann E. Autocrine motility factor stimulates a three-fold increase in inositol trisphosphate in human melanoma cells. Biochem Biophys Res Commun 1990;166:757-4.

Aznavoorian S, Stracke ML, Krutzsch HC, Schiffmann E, Liotta LA. Signal transduction for chemotaxis and haptotaxis by matrix molecules in tumor cells. J Cell Biol 1990; 110:1427-38.

Liotta LA, Kohn EC. Cancer invasion and metastases. Grand Rounds of the National Institutes of Health. Saul Rosen, Ph.D., M.D., Section Editor. JAMA 1990;263:1123-6.

Kohn EC, Francis EA, Liotta LA, Schiffmann E. Heterogeneity of the motility responses in malignant tumor cells: A biological basis for the diversity and homing of the metastatic process. Int J Cancer (in press)

El-Badry OM, Minniti C, Kohn EC, Houghton PJ, Daughaday, WH, Helman LJ. Insulin-like growth factor II acts as an autocrine growth and motility factor in human rhabdomyosarcoma tumors. Cell Growth Differen (in press)

Zabrenetzky V, Kohn EC, Roberts DD. Suramin inhibits laminin- and thrombospondin-mediated cell adhesion and migration and binding of these adhesive proteins to sulfatide. Cancer Res (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00892-07 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)

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| | A. Rosengard | Biotechnology Fellow | LP NCI |
| | A. Leone | Guest Researcher | LP NCI |
| | J. Stahl | Howard Hughes Research Scholar | LP NCI |
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COOPERATING UNITS (if any)

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| 4.7 | 3.0 | 1.7 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The genetic regulation of tumor metastasis was investigated. A novel gene, *nm23*, was identified on the basis of its consistent reduced expression in highly metastatic rodent and human tumor cell lines, as well as human breast carcinomas from patients with lymph node metastases. A 17 kDa human *nm23-H1* protein was identified, which is highly conserved in evolution and may regulate normal development. The *nm23* gene is of potential prognostic and therapeutic importance to the cancer field.

*Nm23* has been demonstrated to have two characteristics of a suppressor gene for cancer progression, allelic deletion in tumors and suppression of the metastatic phenotype upon transfection. Human lung, colon, renal and breast tumor tissues exhibited allelic deletion of *nm23-H1* sequences, as compared to the patient's normal tissue. *Nm23-H1* mapped to human chromosome 17 near the centromere, identifying a new site for allelic deletion in cancer.

Transfection of murine *nm23-1* cDNA into high metastatic potential murine K-1735 TK melanoma cells resulted in the identification of two stable, high expressing lines. Each of these *nm23-1* expressing lines produced at least 90% fewer metastases upon injection into mice than randomly chosen control transfectants. A reduced incidence of primary tumor formation was also observed in the *nm23-1* transfected lines, as well as alterations in their responsiveness to the cytokine transforming growth factor-beta *in vitro*.

A second human *nm23* gene has been identified and sequenced. Expression of the *nm23-H2* gene is also reduced in high metastatic potential tumor cells, but appears to be under separate regulatory controls than *nm23-H1*.



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

Major Findings:

My laboratory has investigated the genetic regulation of the tumor metastatic process. The *nm23* gene was identified by differential colony hybridization of RNAs from related, low and high metastatic potential murine melanoma cell lines. *Nm23* RNA levels were quantitatively reduced in high metastatic potential tumor cells, as compared to low- or nonmetastatic tumor cells, in four rodent metastasis model systems. In a study of human primary infiltrating ductal breast carcinomas *nm23* RNA levels were reduced in all tumors from lymph node positive patients, as well as 25% of the tumors from lymph node-negative patients. In the latter cases, the low *nm23* tumors were estrogen receptor negative and poorly differentiated, both indicative of aggressive behavior. A 17 kDa nm23 protein was identified in murine and human cells. Anti-nm23 synthetic peptide antibodies, used on Western blots or in immunoperoxidase staining of tumor sections, indicated reduced *nm23* protein levels in high metastatic potential tumor cells. The predicted nm23 amino acid sequence shared a 77% identity, 95% similarity to the predicted amino acid sequence of a *Drosophila* developmental gene, *abnormal wing discs* (*awd*). The high degree of amino acid identity suggests a functional identity, that *nm23* regulates normal development.

Function of the *nm23* gene.

The consistent down-regulation of *nm23* in highly metastatic tumor cells has led to the hypothesis that *nm23* is a metastasis suppressor gene. Suppressor genes such as *Rb* and *p53* have been identified for the tumorigenesis phase of cancer progression; these genes have been characterized by their allelic deletion in tumor tissue, and restoration of a normal phenotype upon transfection into tumor cells.

The human *nm23-H1* gene was mapped in the human to chromosome 17, near the centromere, by Dr. O. Wesley McBride, NCI. A restriction fragment length polymorphism (RFLP), indicative of allelic forms of *nm23-H1*, was observed in a Bgl II digest of genomic DNA. Matched normal and tumor tissue DNAs were analyzed for *nm23-H1* expression in colon, non- small cell lung, renal and breast carcinomas, in collaboration with Drs. Marston Linehan, Robert Callahan and Curt Harris of NCI and Dr. Robert Rees of the University of Sheffield, England. Allelic deletion of *nm23-H1* was observed in all of the tumor types studied, in approximately 50% of informative colon, breast and lung carcinomas, and 10% renal carcinomas. The data identify a new locus for allelic deletion in cancer, and indicate that *nm23-H1* has one characteristic of a cancer suppressor gene.

Transfection studies were performed to directly test the suppressive effect of *nm23*. Murine *nm23-1* cDNA, in SV40 promoter driven expression constructs, was transfected into the highly metastatic murine K-1735 TK melanoma cell line. To date, 88 *nm23-1* transfectants have been analyzed, as well as an equal number of control transfectants. Two experimental designs were used to test the metastatic potential of the *nm23-1* and control lines. In initial experiments *nm23-1* and control transfected lines were randomly chosen.

In these lines expression of the transfected *nm23-1* construct was low and unstable with time in culture, but the *nm23-1* transfected lines exhibited 15-75% reductions in tumor metastatic potential upon injection into mice. In subsequent experiments, all 88 *nm23-1* transfected lines were screened to identify stable high expressing lines. Two lines, 4-6 and A3, were identified, and compared to randomly chosen control transfected lines. Both the 4-6 and A3 lines exhibited greater than 90% reductions in tumor metastatic potential upon injection into mice, confirming *nm23-1* to be a metastasis suppressor gene for TK cells. A reduced incidence of primary tumor formation was also noted, although primary tumor size, size of the metastases and growth rate *in vitro* were not significantly reduced. The latter data suggests that *nm23-1* may regulate only certain aspects of tumorigenesis.

The mechanism(s) of *nm23-1* suppression of metastasis are under investigation. The *nm23-1* cDNA was obtained from a transformed fibroblast library. We have cloned and sequenced *nm23* from normal mouse liver as well as K-1735 TK cells, and mutations which would alter its amino acid sequence were not detected. Suppression of metastasis therefore resulted from either the net increase in *nm23* expression, or *nm23-1* expression outside of the normal cellular regulatory controls. The control transfected lines were stimulated in soft agar colony formation assays by transforming growth factor-beta (TGF-beta), while the *nm23-1* transfected lines 4-6 and A3 were not. The data suggest that *nm23-1* transfection alters the responsiveness of tumor cells to cytokine regulatory signals.

Additional confirmatory transfections into K-1735 TK cells using sense and antisense constructs are ongoing, and transfections into human tumor cells are planned.

Identification of a second human *nm23* gene.

A pNM23-H2 cDNA clone was identified, which encoded only the C-terminus of a nm23-like protein. A full-length pNM23-H2s cDNA clone has been identified from a normal human fibroblast library. Southern blot analysis of *nm23-H1* and *H2* expression indicate that *nm23-H2* is not an allele of *H1*, but is a separate gene. It predicts a 17 kDa protein with 95% identity to the human nm23-H1 protein. Expression of *nm23-H2s* RNA is reduced in the highly metastatic tumor cells studied, but its pattern is not identical to that of *nm23-H1*. The data suggest that the two genes are independently regulated. Transfection studies with *nm23-H2* are also planned.

Expression of *nm23* in human tumor cell lines.

In collaboration with Dr. Curt Harris, NCI, *nm23* expression in *ras* transfected human bronchial tumor cell lines is under investigation. Preliminary results indicate that expression of *k-ras* and *N-ras* in immortalized bronchial epithelial cells reduced *nm23* expression, and more highly metastatic variants of each ras transfected line were further reduced in *nm23* expression.

In collaboration with Dr. Robert Dixon, Georgetown University, a series of oncogene transformed human breast tumor lines are being screened for *nm23* expression. Preliminary data indicate that immortilization of normal human mammary tumor cells with benzopyrene increased *nm23* expression, while subsequent transformation with *ras* or *mos* oncogenes significantly reduced *nm23* expression.

Prognostic significance of *nm23*.

Anti-peptide antibodies to *nm23* are being tested for optimal immunoreactivity in immunoperoxidase staining of tumor sections. Retrospective clinical studies of *nm23* expression are planned in collaboration with Dr. Dennis Slamon, UCLA, in lung and ovarian cancers. In breast cancer, a limited retrospective study is underway in collaboration with Dr. Robert Barnes, University of Florida. Preliminary data indicate that patients with high *nm23* expression have a significantly longer survival time than those with low *nm23* expression. A large scale retrospective breast study is planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

# NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09130-06 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Laminin Receptor in Breast Tissue, Benign and Malignant Tumors

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

| | | | |
|---|---|---|---|
| PI: | M. Sobel | Senior Investigator | LP NCI |
| OTHER: | L. Liotta | Chief, Tumor Invasion & Metastases Section | LP NCI |
| | V. Castronovo | Breast Cancer Research Fellow | LP NCI |
| | V. Cioce | Guest Researcher | LP NCI |
| | A. Claysmith | Biologist | LP NCI |

COOPERATING UNITS *(if any)*

Dr. Barry Shmookler, Washington Hospital Medical Center, Washington, D.C.
Dr. Claus Colin and Dr. Eric Lifrange, University of Liege, Liege, Belgium

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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|---|---|---|
| 1.3 | 1.1 | 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.)*

Both Scatchard analysis of laminin binding studies and immunocytochemical localization of the high affinity laminin receptor using monoclonal and polyclonal antibodies have indicated that the number of laminin receptors on the cell surface may be increased in human carcinomas. Studies on human breast carcinoma-derived cell lines suggested that the increased cell surface expression of the laminin receptor was due to an increased steady state level of laminin receptor mRNA. The latter experiments suggested that assessing the laminin receptor mRNA levels of human carcinoma cells may be of diagnostic and/or prognostic value. We have developed anti-laminin receptor synthetic peptide antibodies as well as cDNA restriction fragments which are being used to assess the cell surface expression of the laminin receptor as well as laminin receptor mRNA levels in different neoplastic cell types. We evaluated the expression of laminin receptor using the immunoperoxidase technique in breast aspirates. Cells obtained from benign samples exhibited a low level of laminin receptor antigen, while 71% of smears obtained from malignant breast lesions contained cells that were strongly stained by the antibody. These data suggest that the immunodetection of laminin receptor in cells obtained by fine needle aspiration of breast lesions could be a valuable adjunct in the evaluation of breast lesions. We have analyzed paired tumor and normal tissues from individual patients with colorectal carcinoma. In each of the 18 patients studied, the neoplastic tissue contained significantly more laminin receptor mRNA than did the normal tissue. Comparable increases in laminin receptor protein were found in the carcinomatous tissue by immunoblot as well as immunohistochemical techniques. These findings suggest that increased expression of laminin receptor may be a useful marker in the evaluation of cancer patients.

Major Findings:

Immunodetection of laminin receptor in human breast cancer cells obtained by fine needle aspiration biopsy

Fine needle aspiration biopsy of the breast is a very useful technique for the evaluation of a suspect lesion prior to surgical removal. We evaluated the expression of laminin receptor using the immunoperoxidase technique in 81 breast aspirates, including 26 benign and 55 neoplastic lesions. Cells obtained from benign samples exhibited a very low level of laminin receptor antigen detected by affinity-purified antibody raised against a cDNA-derived laminin receptor synthetic peptide. In contrast, 71% of smears obtained from malignant breast lesions contained cells that were strongly stained by the antibody. Expression was also increased in fixed malignant tissues obtained at the time of surgery. These data suggest that the immunodetection of laminin receptor in cells obtained by fine needle aspiration of breast lesions could be a valuable adjunct in the evaluation of breast lesions.

Differential expression of laminin receptor in human colorectal carcinomas

We analyzed paired tumor and normal tissues from individual patients with colorectal carcinomas for their steady state levels of laminin receptor mRNA, and determined their expression of laminin receptor by immunoblot and immunohistochemistry techniques. The relative levels of laminin receptor mRNA were significantly greater in 18 colon carcinomas than in matched normal colon samples ($p<0.0001$). Immunologic evaluation of the tumor and normal samples for expression of laminin receptor protein showed a direct correlation with the mRNA levels. These findings suggest that the differential expression of laminin receptor in malignant disease is the result of increased content of laminin receptor mRNA in a variety of human tumors.

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 (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09131-06 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Molecular Cloning of Connective Tissue Matrix Molecules

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

| | | | |
|---|---|---|---|
| PI: | M. Sobel | Senior Investigator | LP NCI |
| OTHER: | A. Claysmith | Biologist | LP NCI |
| | V. Castronovo | Breast Cancer Research Fellow | LP NCI |
| | V. Cioce | Guest Researcher | LP NCI |
| | H. Chen | Stay-in-School | LP NCI |
| | K. Barker | Guest Researcher | LP NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.5 | 1.9 | 1.6 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The interaction of the tumor cell with its extracellular matrix may play an important role in determining its metastatic and invasive properties. To better understand the protein components that make up the extracellular matrix, their regulation, and how they interact with the tumor cell, we have constructed, isolated, and characterized molecular clones of a high affinity 67 kilodalton laminin receptor, and a newly identified 31 kilodalton laminin binding protein. Laminin is a high molecular weight glycoprotein specifically associated with basement membranes. The high affinity 67 kilodalton laminin receptor is a cell surface protein to which laminin binds. We have previously isolated cDNA clones of the human and murine laminin receptor, and have predicted the entire primary structure of the receptor. Antibodies to specific peptides deduced from the cDNA clones have been used to identify a potential transmembrane domain as well as the ligand binding domain of the receptor. Furthermore, a synthetic peptide successfully inhibited tumor cell attachment to endothelium, thus blocking a key step in the metastatic cascade. The laminin receptor is induced by exposure of cancer cells to extracellular attachment proteins such as laminin and fibronectin, suggesting that as tumor cells are exposed to the extracellular environment they synthesize receptors required for migration and invasion through the connective tissue. Multiple genes for the laminin receptor have been detected, including pseudogenes. The variable expression of this multicopy gene family is under investigation in normal and metastatic tissues. Another laminin binding protein with an approximate molecular weight of 31 kilodaltons was identified in human melanoma cells. Partial protein sequence showed homology with a previously identified carbohydrate binding protein. Polymerase chain reaction technology was used to obtain a partial cDNA clone of this alternate laminin binding protein. Its expression in a variety of carcinoma-derived lines is different from that of the previously identified high affinity laminin receptor.





GPO 914-918

Major Findings:

Putative transmembrane domain of the laminin receptor

The cDNA-predicted sequence of the laminin receptor does not include a traditional signal peptide, nor does the sequence contain a classic transmembrane domain. A short, 16 amino acid long, putative transmembrane domain predicted by computer analysis is relatively amino-terminal, suggesting that approximately two-thirds of the receptor may be extracellular. To test the computer-predicted transmembrane domain hypothesis, a series of immunofluorescence studies were performed in which affinity purified antibodies directed against specific 20-residue long domains of the cDNA-predicted receptor were reacted with permeabilized and nonpermeabilized melanoma cells. As expected, all the antibodies reacted with the permeabilized cells. Antibodies directed against domains carboxy-terminal to the predicted transmembrane domain reacted with the nonpermeabilized cells; however, antibodies directed against domains amino-terminal to the predicted transmembrane domain did not react with them. These studies suggest that the computer-predicted transmembrane domain is correct.

Ligand binding domain of the laminin receptor

To define the ligand-binding domain of the laminin receptor, we used a combination of cDNA-derived synthetic peptides and their corresponding antibodies. The possible binding activity of eight different cDNA-derived synthetic peptides was investigated by affinity chromatography assays. The ability of iodinated laminin to bind to synthetic peptide affinity columns was tested; the iodinated laminin bound specifically to peptide "G", containing the sequence IPCNNKGAHSVLMWWMLAR. A synthetic peptide containing a scrambled sequence but the same amino acid composition as peptide "G" did not have binding ability. In contrast, the reverse sequence of peptide "G" did have binding activity. Since this peptide contains the palindromic sequence LMWWML, we tested an octapeptide containing the palindrome. The octapeptide was also active, suggesting that the ligand binding site is contained within this 8 amino acid long region.

Role of laminin receptor in the attachment of cancer cells to endothelium

Attachment of cancer cells to the endothelium is an important step in the metastatic cascade. We proposed a model in which laminin bound to the cancer cell surface may mediate this attachment to endothelium by direct binding of the laminin on the cancer cell surface to apical laminin receptors on endothelial cells. We demonstrated the presence of laminin receptors on the apical surface of endothelium by Scatchard analysis and developed an assay in which the ability of metabolically labelled melanoma cells to attach to confluently grown endothelial cells was measured. Attachment was specifically inhibited by peptide "G". Presumably, the peptide competes with the laminin receptors on the endothelium for binding to the laminin-coated melanoma cells. These studies suggest that synthetic peptides may be developed to block the ability of laminin-coated metastatic cells to attach to endothelium.

Regulated expression of the laminin receptor

During invasion, cancer cells actively influence the surrounding extracellular matrix, and the host extracellular matrix components exert biochemical and mechanical influences on the cancer cell via plasma membrane receptors. We examined the modulation of laminin receptor mRNA levels in cancer cells by laminin and fibronectin, two prevalent attachment proteins in the extracellular matrix. Both laminin and fibronectin increased the total cellular level of laminin receptor mRNA in two human cancer cell lines, T47D breast carcinoma cells and A2058 melanoma cells. Since the steady state level of mRNA levels has been shown to control the number of receptors expressed at the cell surface, these results suggest that contact of the cancer cells with laminin and fibronectin in the host matrix may be an important regulatory mechanism by which cancer cells maintain a high number of laminin receptors at their cell surface as they progress through the several steps of tumoral invasion and metastasis.

Multiple genes for the human laminin receptor

Using genomic DNA hybridization blots, we have previously predicted that there are multiple laminin receptor genes in the human and mouse genomes. We have performed gene copy experiments and have found evidence for 28 copies of the laminin receptor gene per human diploid genome. We have isolated 97 phage clones containing human genomic fragments which hybridize to the laminin receptor cDNA sequence. These clones are being studied to identify regulatory regions and pseudogenes.

Identification of a 31 kilodalton laminin binding protein

Extracts of human melanoma A2058 cells contain a 31 kilodalton protein which specifically binds to laminin affinity columns. The protein can be eluted under stringent conditions such as acetic acid or formic acid. The protein was purified by electroelution, digested with cyanogen bromide and trypsin, and partially sequenced. There was significant homology to a previously identified rodent carbohydrate binding protein. Using the human sequence data, we developed oligonucleotides, and reverse transcribed a specific 270 base long mRNA transcript from human A2058 cellular RNA. We then amplified this specific sequence by PCR, and cloned it. It recognizes a 1000 base long mRNA on Northern blots. The relative level of mRNA for this alternate laminin binding protein in a variety of carcinoma-derived cell lines is different from that of the 67 kilodalton laminin receptor.

Publications:

Rahman Q, Paneerselvam M, Guirguis R, Castronovo V, Sobel ME, Daddona PE, Liotta LA. Anti-laminin receptor antibody targeting of adriamycin encapsulated liposomes to human breast cancer cells in vitro. J Natl Cancer Inst 1989;81:1794-1800.

Rao CN, Castronovo V, Schmitt MC, Wewer UM, Claysmith AP, Liotta LA, Sobel ME. Evidence for a precursor of the high affinity metastasis-associated murine laminin receptor. Biochemistry 1989;28:7476-86.

Castronovo V, Sobel ME. Laminin and fibronectin increase the steady state level of the 67 kD high affinity metastasis-associated laminin receptor mRNA in human cancer cells. Biochem Biophys Res Commun 1990;168:1110-7.

Bason CT, Knowles WJ, Bell L, Albelda SM, Castronovo V, Liotta LA, Madri JA. Spatiotemporal segregation of endothelial integrin and nonintegrin extracellular matrix binding proteins during adhesion events. J Cell Biol 1990;110:789-801.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 09163-03 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Anticancer Effects of a Novel Drug, Merck L651582

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

| | | | |
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| | L. Travers | Guest Researcher | LP, NCI |
| | E. Schiffmann | Research Chemist | LP, NCI |
| | L.A. Liotta | Chief, Tumor Invasion and Metastases Section | LP, NCI |

COOPERATING UNITS *(if any)*

G. Curt, Associate Director, DCT, NCI; M.A. Sandeen, Research Technician, FCRF/ DCBDC Animal Holding Unit; C. Felder, Fellow, Lab. of Cell Biology, NIMH

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Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS. | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 0.6 | 0.9 |

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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.)*

We have continued our investigation on the effects of MSD L651582 and recently have developed chemical modifications of L651582. In addition, we have initiated preclinical development of L651582 with the projection of submitting an IND application and Phase I clinical trial protocol to the FDA within this annual report year. We have shown previously that L651582 inhibits proliferation of a variety of cancer cell lines and prevents several steps in the process of tumor cell invasion and metastasis including adhesion and migration. We demonstrated the anti-proliferative and anti-metastatic effects of L651582 in animal models of spontaneous and experimental metastasis and tumorigenesis. We have expanded our in vitro observations to include several new cancer cell lines, effects on laminin-stimulated adhesion and migration, direct effects of phospholipases A2 and C and calcium modulation in noncancer models, and have developed analogs of the parent compound. Animal studies are in progress to evaluate the clinical efficacy of the analogs. A continuation-in-part has been submitted for the use patent application and a new patent application has been made describing the analog work. In in vivo studies, we have shown that L651582 inhibits experimental pulmonary metastases due to HT-29 human colon carcinoma.



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

Major Findings:

We have been studying a novel anti-cancer agent, the carboxyamide-imidazole L651582 (heretoforth called CaI) developed by Merck Laboratory for Therapeutic Research (Rahway, NJ). This compound inhibits several steps in nucleoside biosynthesis and signal transduction utilizing the effector activities of phospholipases A2 and C and calcium fluxes.

We have previously shown that this agent 1) decreases autocrine motility factor (AMF) and laminin-stimulated tumor cell motility; 2) inhibits tumor cell adhesion to tissue culture plastic, laminin, and fibronectin substrata; 3) decreases AMF-stimulated phosphotidyl inositol metabolism; 4) inhibits tumor cell proliferation *in vitro*; and, 5) prolongs survival of human ovarian cancer OVCAR3-bearing mice.

Over the course of the last year, we have approached our study of this drug in several ways: 1) further evaluation of its effects on signal transduction; 2) development of a HPLC separation method for the extraction of drug and potential analogs and quantitation of drug in experimental and patient specimens; 3) synthesis, purification, and analytical validation of analogs developed in our laboratory; 4) bioassay and animal experiments with our analogs; 5) collaborative preclinical development and drafting of the Investigational New Drug Application and Phase I clinical trial protocol.

1. In collaboration with Dr. Christian Felder of the NIMH, we have pursued evaluation of the mechanism of action of L651582. The model system in Dr. Felder's laboratory uses Chinese hamster ovary cells which have been transfected with specific muscarinic receptors and thus respond to carbachol stimulation. Carbachol causes activation of phospholipases A2 and C and calcium fluxes in these M1 and M5 muscarinic receptor-transfected cells. L651582, 1 - 10 μM, inhibited the carbachol stimulation of both enzymes; the time course of action was rapid, within 5 - 10 minutes. In addition, in one system, L651582 inhibited carbachol-mediated 45 $Ca^{++}$ uptake in a non-voltage dependent calcium channel. Recent unpublished work by Dr. Felder has linked that calcium channel to an undefined G protein. These data independently confirm the effect of L651582 on signal transduction pathways, but do not yet define a specific mode of action. Work is ongoing to address the effect of the analogs developed in this laboratory and to better localize the most proximal site of inhibition of L651582 on signal transduction.

2. Our laboratory has developed a very sensitive method of separation of L651582 using reverse phase high pressure liquid chromatography (HPLC). Using this HPLC assay with the internal standard trazodone, chosen for its structural homology to the triazole moiety of L651582, we have demonstrated a linear relationship between the ratio of peak area of L651582/peak area of trazodone and the concentration of L651582. This standard curve relationship will allow us to quantitate amounts of drug from experimental specimens and for patient samples. We have used it to demonstrate that the uptake of L651582 by A2058 human melanoma cells is extremely rapid, and are presently extracting mouse organs to evaluate organ distribution of drug after oral administration.

3,4. We have postulated that the triazole moiety of the L651582 molecule may be the important site for its effects on signal transduction due to the structural similarity to guanine. To evaluate this postulate, we have engineered an analog with a adipic acid residue linked to the amine of the triazole. This analog, confirmed by mass spectrometry and nuclear magnetic resonance spectrometry, appears to be different in its solubility, stability, and efficacy when compared to the parent. Preliminary laboratory studies with this purified analog have shown that it is more rapid in its inhibition of tumor cell adhesion, more active in inhibition of $^{3}H$-thymidine incorporation, and that these effects cannot be accounted for simply by the addition of adipic acid and the parent compound simultaneously in these assays. This compound has a more stable cyclized form with marked ring homology to 8-aza-guanine. Further studies are ongoing to increase the yield of the cyclized form and to evaluate its biologic function. In addition, we are evaluating the effect of this analog in the experimental metastasis model using 5R Ha-ras transfected rat embryo fibroblasts. A patent application describing this novel compound is being filed.

5. We have taken our laboratory and animal studies on the effects of L651582 on cancer cell lines and cancer-bearing animals to the Decision Network of the Division of Cancer Treatment. The Decision Network is the organization which determines the appropriateness of NCI support for development of new anticancer agents. L651582 received a unanimous vote of confidence for support for continued preclinical development. In collaboration with Drs. Bruce Chabner, Director, DCT, Greg Curt, Associate Director, DCT, and Charles Grieshaber, Chief, Toxicology Branch, DTP, we have advanced our understanding of the pharmacology of L651582. While an intravenous formulation for animal and patient Phase I clinical studies is preferred, the hydrophobic nature of L651582 has made intravenous use difficult. Due to the excitement and urgency to get L651582 into trial quickly, an oral formulation using capsules has been devised. Large animal toxicology studies and animal efficacy and schedule dependence studies are in progress using this oral formulation. Pending this toxicology and pharmacology data, an Investigational New Drug Application (INDA) and Phase I clinical trial protocol will be completed and sent to the FDA and Institutional Review Board for approval of use in adult and later pediatric oncology patients.

Publications:

Kohn EC, Liotta LA. L651582: A novel antiproliferative and antimetastasis agent. J Natl Cancer Inst 1990;82:54-0.

Kohn EC, Liotta LA. Therapeutic application of an anti-invasion compound. Application for letters patent of the U.S., serial #07/355,744, filed May 15, 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09164-03 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

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| OTHER: | A. Levy | Microbiologist | LP NCI |
| | P. Brown | BCSG Fellow | LP NCI |
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| | M. Wacher | Visiting Scientist | LP NCI |
| | M. Onisto | Visiting Fellow | LP NCI |
| | L. Liotta | Chief, Laboratory of Pathology | LP NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

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Tumor Invasion and Metastases Section

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| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.0 | 3.3 | 0.7 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

In order to investigate the role of type IV collagenase in tumor invasion and metastases, we have undertaken the purification, molecular cloning, enzymatic characterization, and the study of structure-function correlation of this 72 kDa metalloproteinase. We have also studied the binding of type IV collagenase to both type IV collagen and gelatin. These studies have demonstrated that enzyme binding to these substrates is independent of metal atoms and can be competed by fibronectin and fibronectin-gelatin binding domain peptides. Recent studies have focused on 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 the 72 kDa collagenase is unique among the members of the matrix metalloproteinase enzymes in the manner of its regulation by various cytokines. Also, the 72 kDa collagenase IV probes shows elevated levels in colorectal tumor tissues when compared with adjacent normal mucosa tissue. In expanding this project, we have also cloned and sequenced the human tumor cell interstitial collagenase. The tumor enzyme was found to be identical to the fibroblast collagenase, thus demonstrating definitively and for the first time that ductal epithelial cells are capable of producing this enzyme.

Major Findings:

1. The 72 kDa collagenase IV enzyme is activated by organomercurial compounds.

2. This activation results in removal of an 80 amino acid profragment peptide.

3. The 80 amino acid profragment contains a highly conserved peptide region which is responsible for maintaining the latency of the proenzyme.

4. The latency of the proenzyme is maintained by a sulfhydryl-metal atom interaction as determined by titration studies of the free sulfhydryls associated with the holoproenzyme and apoproenzyme preparations.

5. Immunohistochemical localization studies have shown that in normal human breast tissues the 72 kDa collagenase IV enzyme is primarily localized to the myoepithelial cells.

6. Both *in situ* and invasive human breast carcinoma tissues demonstrated an intense immunoreactivity for 72 kDa type IV collagenase in the malignant epithelial cells, clearly demonstrating for the first time that it is the tumor cells which are responsible for the augmented levels of this enzyme in primary human tumors.

7. We have developed a novel substrate capture assay which allows quantification of the 72 kDa type IV collagenase enzyme in complex biological substrates.

Publications:

Liotta LA, Stetler-Stevenson WG. Principles of molecular cell biology of cancer: Cancer metastasis. In: DeVita VT Jr, Hellman S, Rosenberg SA, eds. Cancer: Principles and practice of oncology, vol 1, 3rd ed. Philadelphia: JB Lippincott Co, 1989;98-15.

Stetler-Stevenson WG, Krutzsch HC, Wacher MP, Margulies IMK, Liotta LA. The activation of human type IV collagenase proenzyme. Sequence identification of the major conversion product following organomercurial activation. J Biol Chem 1989;264:1353-6.

Liotta LA, Stetler-Stevenson WG. Editorial: Metalloproteinases and malignant conversion: Does correlation imply causality? J Natl Cancer Inst 1989;81:556-7.

Monteagudo C, Merino MJ, San-Juan J, Liotta LA, Stetler-Stevenson WG. Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. Am J Pathol 1990;136:585-2.

Wacher MP, Krutzsch HC, Liotta LA, Stetler-Stevenson WG. Development of a novel substrate capture immunoassay for the detection of a neutral metalloproteinase capable of degrading basement membrane (type IV) collagen. J Immunol Meth 1990;126:239-5.

Templeton NS, Brown PD, Levy AT, Margulies IMK, Liotta LA, Stetler-Stevenson WG. Cloning and characterization of human melanoma interstitial collagenase. Cancer Res (in press)

Liotta LA, Stetler-Stevenson WG. Metalloproteinases and cancer invasion. In: Gottesman M, ed. The role of proteases in cancer. (in press)

Liotta, LA, Stetler-Stevenson WG, Steeg PS. Cancer invasion and metastasis: Positive and negative regulatory elements. Cancer Invest (in press)

Zucker S, Moll UM, Lysik RM, DiMassimo EI, Stetler-Stevenson WG, Liotta LA, Schwedes JW. Extraction of type IV collagenase/gelatinase from plasma membranes of human cancer cells. Int J Cancer (in press)

Stetler-Stevenson WG, Kreck ML, Talano JA. Assaying type IV collagenase activity using [$^{3}$H]-pepsinized human placental type IV collagen, NET-931. NEN/Dupont Biotechnology Update, May 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 09179-02 LP

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Novel Metalloproteinase Inhibitors: Role in Tumor Invasion and Metastasis

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

| | | | |
|---|---|---|---|
| PI: | W.G. Stetler-Stevenson | Senior Staff Fellow | LP NCI |
| OTHER: | M. Onisto | Visiting Fellow | LP NCI |
| | P. Brown | BCSG Fellow | LP NCI |
| | A. Levy | Microbiologist | LP NCI |
| | H. Krutzsch | Expert | LP NCI |
| | L. Liotta | Chief, Laboratory of Pathology | LP NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pathology

SECTION
Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS. | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.5 | 2.2 | 0.3 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have 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. Inhibition of the activated enzyme occurs with 1:1 molar stoichiometry. TIMP-2 contains 12 cysteine residues, the positions of which are highly conserved when compared with the primary structure of TIMP-1. We have obtained a 1.0 kB cDNA clone which encodes for the pro-TIMP-2. This clone encodes for a 194 amino acid mature TIMP-2 molecule and a 26 amino acid signal peptide. Recent studies have focused on the production of recombinant TIMP-2 and transcriptional regulation of the TIMP-2 transcript levels.

Major Findings:

1. There is a novel 21 kDa protein which binds selectively and with 1:1 molar stoichiometry to the latent 9 form of the human 72 kDa type IV collagenase.

2. This protein has been purified, isolated and sequenced.

3. Primary structure information reveals that this 21 kDa protein shows 37.6% identity and 65.6% overall homology with the previously identified TIMP.

4. Based on its ability to inhibit type IV collagenase activity and the homology with TIMP, we refer to this protein as TIMP-2.

5. The identification of TIMP-2 suggests that there is a family of TIMP's which may show selective interactions with members of the matrix metalloproteinase family.

Publications:

Stetler-Stevenson WG, Krutzsch HC, Liotta LA. Tissue inhibitor of metalloproteinase (TIMP-2): A new member of the metalloproteinase inhibitor family. J Biol Chem 1989;264:17374-8.

Liotta LA, Stetler-Stevenson WG. Metalloproteinases and cancer invasion. In: Gottesman M, ed. The role of proteases in cancer. (in press)

Liotta LA, Stetler-Stevenson WG, Steeg PS. Cancer invasion and metastasis: Positive and negative regulatory elements. Cancer Invest (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09180-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Identification of Novel Metastasis Associated Gene Products

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

| | | | |
|---|---|---|---|
| PI: | W.G. Stetler-Stevenson | Senior Staff Fellow | LP NCI |
| OTHER: | E. Kohn | Senior Staff Fellow | LP NCI |
| | N. Templeton | BTP Fellow | LP NCI |
| | L. Liotta | Chief, Laboratory of Pathology | LP NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1 | 1 | 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 used synthetic oligonucleotide probes to screen a human melanoma cDNA library. Probes containing sequences similar to those contained in metastasis associated metalloproteinases were used to screen this library. Screening yielded several cDNA clones. Two of these clones have been fully sequenced.

The first clone, referred to as Cll14.12, contains a 1.7 kB insert. The sequencing of this clone revealed that it contained a novel sequence with a single open reading frame encoding for a unique peptide sequence. Primer extension data are consistent with this clone representing a full-length message. Northern blot analysis suggests that the message size is on the order of 1.9-2.0 kB and that this is a transcript of relatively low abundance. Northern blot analysis also revealed that this message was more abundant in normal mucosal tissue when compared with the transcript levels seen in colon tumors. This suggests that possibly the Cll14.12 transcript levels are down regulated in tumor cells. This possibility is being explored further.

The second clone is referred to as pM5. This clone contained a 4086 bp insert which contained a single open reading frame which encoded a polypeptide of 1144 amino acid residues. Computerized homology searches revealed no homology with any known sequences both at the protein and at the nucleotide level. This suggests that pM5 is a unique gene product. Specific hydrophilic domains from the pM5 protein sequences have been selected for the generation of antipeptide antibodies. These will be used to determine the subcellular localization of the pM5 gene product and to determine the tissue localization using immunohistochemical techniques.





GPO 914-916

Major Findings:

1. Identification and sequencing of the C1114.12 cDNA clone.

2. Identification of a low abundance mRNA which hybridizes to the C1114.12 cDNA probe.

3. Demonstration that C1114.12 transcripts are more abundant in normal vs. tumor tissues.

4. Identification and sequencing of the pM5 cDNA clone.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09185-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

G Proteins and Tumor Cell Motility

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

| | | | |
|---|---|---|---|
| PI: | S. Aznavoorian | Biotechnology Fellow | LP NCI |
| OTHER: | L. Liotta | Chief, Tumor Invasion and Metastases Section | LP NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.1 | 1.1 | |

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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 goal of this project is to identify and isolate a cDNA(s) encoding the pertussis toxin sensitive G protein(s) operating in signal transduction pathways responsible for tumor cell motility. The human melanoma cell line A2058 is known to have one or more pertussis toxin sensitive G proteins which transduce chemotactic signals initiated by laminin, type IV collagen, and "autotaxin". In addition, this cell line has been shown to have at least one 40 kDa G protein labeled by ADP ribosylation catalyzed by pertussis toxin. Previous studies with this cell line (Aznavoorian et al., J Cell Biol 1990;110:1427-37) have shown that transduction of signals initiating motility by extracellular matrix proteins differed depending on the type of matrix molecule and whether the ligand was in solution or bound to a substratum. Following pretreatment with pertussis toxin, chemotactic responses to laminin, fibronectin, and type IV collagen were distinctly different. Chemotaxis to type IV collagen was profoundly sensitive to PT, laminin was intermediate in sensitivity, but fibronectin was completely insensitive. Haptotaxis initiated by all three ligands was unaffected by PT. Modulators of the cAMP pathway had no effect on chemotaxis or haptotaxis. Thus, a PT sensitive, non-cAMP pathway is required to stimulate chemotaxis via laminin or type IV collagen, components of the basement membrane. The specific identity of the G protein involved in A2058 motility is not yet known, although its pertussis toxin sensitivity eliminates $G_s$ as a possibility. Potential candidates include one of the $G_i$-"like" proteins already cloned, or $G_o$, which is abundant in brain tissues (and has also been found in non-neuronal tissues), but whose physiological function is largely unknown; alternatively, an as yet undiscovered G protein could be involved. The present approach to isolating the cDNA(s) encoding the relevant G protein(s) is to screen the A2058 cDNA library with nucleotide probes after making filter lifts of phage plaques. A series of potential G protein encoding human cDNA clones have been isolated.

Major Findings:

The A2058 library was initially screened at low stringency with a probe consisting of a mixture of full-length cDNAs corresponding to the α subunits of $G_{i1}$, $G_{i2}$, and $G_o$, and a 600 bp fragment of the cDNA corresponding to $G_{13}$ (originally isolated from rat olfactory neuroepithelium, and kindly provided by Dr. Randall R. Reed of Johns Hopkins University). To accomplish this, the phage particles (lambda gt11) containing the A2058 cDNA inserts were grown on lawns of the appropriate strain of *E. coli* which allowed phage-induced lysis of the bacterial colonies to occur. Plaques produced by lysis were screened for DNA sequences encoding the above-mentioned pertussis toxin sensitive G proteins by first transferring the DNA in the plaques to nitrocellulose filters using standard blotting procedures. The transferred DNA was then exposed to the labelled probe, and the hybridization sequences were detected by autoradiography. Autoradiography was performed first after a low stringency wash of the filters, then after a high stringency wash. Over 200 hybridization spots remained after the high stringency wash; secondary screenings of these will include the use of probes with increasing specificity for particular subtypes of G proteins. For example, oligonucleotide probes synthesized from the highly conserved pertussis toxin-sensitive carboxyl-terminal region will be useful in separating pertussis toxin-sensitive from - insensitve G protein genes, which may have been detected in the low-stringency screen. Probes specific for $G_o$ will be utilized to search for $G_o$ (or a close variant) in the library. $G_o$, a major component of neuronal growth cone membranes, has been shown to be a potential signal transducer in the pertussis toxin-sensitive phosphatidylinositol pathway of *Xenopus* oocytes (Nature 1990;343:79-2). It has also been postulated to regulate calcium and potassium channels in some systems, and to regulate cellular proliferation, morphology, and phospholipid metabolism when transfected into murine adrenal cells (Mol Cell Biol 1990;9:5434-9). These studies raise the possibility that a variant of $G_o$ could play a role in one or more of the signal pathways leading to tumor cell motility.

In a separate approach to designing a probe with which to screen the A2058 cDNA library, PCR was performed on the library. There are several guanine nucleotide binding domains in the G protein α subunits. These domains are highly conserved in amino acid sequence among all known G proteins. Oligonucleotide probes were designed to flank one of these domains, specifically that between the predicted amino acids 152 and 218 of the cDNA clones corresponding to $G_{i1-3}$ and $G_o$ from rat olfactory neuroepithelium (J Biol Chem 1987;262:14241-9). (This domain includes the arginine residue which is ADP-ribosylated by cholera toxin). Degenerate oligonucleotides (26- and 27-mer) with Spe 1 linkers to facilitate subcloning were synthesized, deblocked, and used in the PCR reaction to amplify the A2058 cDNA in lambda gt11. A low stringency annealing temperature of 37°C was required for successful amplification of the human cDNA. A PCR product of approximately 230 base pairs was formed from this reaction, and has been purified from 6% acrylamide gels. The purified product is ready for subcloning, sequencing, and use as a specific human probe in higher stringency secondary screening of the A2058 cDNA library. Also, sequence information obtained from the PCR

product may reveal as yet undiscovered variants of G protein α subunits which are present in the human melanoma cDNA library.

The possibility exists that other types of tumor cells, unlike the A2058 cells, are constitutively "turned on" in their motility responses, and therefore no longer require extracellular signals in order to migrate. Point mutations which constitutively activate the α subunit of $G_s$ by inhibiting GTPase activity have been described in a subset of growth hormone-secreting human pituitary tumors (Nature 1989;340:692-6). It is conceivable that mutations in the corresponding regions of genes encoding the α chains of other types of G proteins would have a similar effect. The result would be a constitutive activation of other second messenger systems such as the phosphoinositide/$Ca^{2+}$ cascade, which may in turn permanently activate tumor cell motility. Therefore, this project will include the screening of human tumor tissue DNA (along with corresponding normal DNA from the patients' lymphocytes) for point mutations in conserved regions of the Gα genes, which may constitutively activate the proteins. This will be accomplished using a combination of PCR amplification and "chemical mismatch analysis" (Nucl Acids Res 1989;17:3347-58).

Publications:

Aznavoorian S, Stracke ML, Krutzsch HC, Schiffmann E, Liotta LA. Signal transduction for chemotaxis and haptotaxis by matrix molecules in tumor cells. J Cell Biol 1990;110:1427-38.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00550-10 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Immunologic Characterization of Malignant Lymphomas

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

| | | | |
|---|---|---|---|
| PI: | E.S. Jaffe | Chief, Hematopathology Section | LP NCI |
| OTHER: | L.J. Medeiros | Senior Staff Fellow | LP NCI |
| | D.L. Longo | Senior Investigator | BRMP NCI |
| | M. Raffeld | Senior Staff Fellow | LP NCI |
| | M.A. Stetler-Stevenson | Expert | LP NCI |
| | C.L. Gonzalez | Biotechnology Fellow | LP NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.0 | 3.0 | 1.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

In order to assess the clinical and pathologic significance of the immunologic characterization of human malignant lymphomas, fresh biopsy tissues are obtained from patients referred to the Clinical Center for treatment. Biopsies are obtained with patient permission prior to therapy and processed in the Hematopathology Section. The neoplastic cells are characterized as to their origin from T cells, B cells, or histiocytes, and in addition can be identified as belonging to specific developmental and functional subpopulations. This data is then correlated with clinical and pathologic data. Morphologic features are analyzed to achieve improved classification of lymphoproliferative lesions.

Selected cases of hematologic malignancies are also referred for detailed immunophenotypic, genotypic, and morphologic analysis. Such cases are selected for unusual clinical and/or histologic features.

This information is utilized to develop improved classifications of disease and to distinguish new clinicopathologic entities. It also will be used as a basis for potential immunotherapy or adjunctive immunotherapy in a program of autologous bone marrow transplantation.



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

Major Findings:

All cases of lymphocytic lymphoma of intermediate differentiation (IDL) referred to the NCI were reviewed in order to define the histopathologic spectrum of disease and to investigate morphologic and immunophenotypic features with potential prognostic relevance. Although morphologically such cases are classified as diffuse small cleaved (intermediate grade in the working formulation), the clinical features are characteristic of a low-grade non-Hodgkin's lymphoma. All patients presented with stage III or IV disease. Median survival was 56.3 months with only three patients having a prolonged relapse-free survival greater than two years. A histologically distinctive variant with blastic cytologic features was identified in seven cases. This blastic variant was associated with a higher mitotic index and shortened survival (24.9 months). All cases had a mature B-cell phenotype; lambda was more often present than kappa. Seventy-eight percent of cases were CD5 positive with the majority of CD5 negative cases presenting in extranodal mucosal associated sites. The proliferative rate measured by K1-67 positivity correlated with the mitotic index and showed a trend towards shortened survival when elevated (greater than 200 cells per 20 grids examined).

A phenotypic and genotypic study of diffuse mixed lymphoma was completed. Immunophenotypic studies demonstrated that all lesions were composed predominantly of T cells. However, in nine cases evidence of B-cell lymphoma was identified with a concomitant reactive T-cell proliferation. In 12 cases with uncertain phenotypic results, the genotypic analysis provided evidence of clonality in eight. Thus, molecular genetic analysis is a complementary tool to immunophenotypic studies. Evidence of the t(14;18) translocation involving bcl-2 was identified in seven cases, five of which were not considered to be of follicular center cell origin based on morphologic features.

A study of surface light chain phenotype in low-grade B-cell lymphomas demonstrated a lack of prognostic significance for light chain expression.

Publications:

Rosolen A, Nakanishi M, Poplack DG, Cole D, Quinones R, Reaman G, Cotelingam JD, Trepel JB, Sausville E, Marti GE, Jaffe ES, Neckers LM, Colamonici OR. Expression of interleukin-2 receptor ß subunit in hematopoietic malignancies. Blood 1989;73:1968-2.

Elias JM, Gown AM, Nakamura RM, Wilbur DC, Herman GE, Jaffe ES, Battifora H, Brigati DJ. Quality control in immunohistochemistry - Report of a workshop sponsored by the Biological Stain Commission. Am J Clin Pathol 1989;92:836-3.

Jaffe ES. The role of immunophenotypic markers in the classification of non-Hodgkin's lymphomas. Sem Oncol 1990;17:11-9.

Longo DL, Glatstein E, Duffey PL, Ihde, DC, Hubbard SM, Fisher RI, Jaffe ES, Gilliom M, Young RC, DeVita VT, Jr. Treatment of localized aggressive lymphomas with combination chemotherapy followed by involved-field radiation therapy. J Clin Oncol 1989;7:1295-2.

Kamel AM, Asem MM, Jaffe ES, Magrath I, Aboul Enein MI, Hindawy DS. Immunological phenotypic pattern of acute lymphoblastic leukaemia in Egypt. Leuk Res 1989;13:519-5.

Jaffe ES, Raffeld M. The avidin-biotin complex immunoperoxidase method for the identification of cells in tissue sections. In Shevach EM, ed. Current protocols in immunology. (in press)

Pombo de Oliveira MS, Jaffe ES, Catovsky D. Leukaemic phase of mantle zone (intermediate) lymphoma: its characterisation in 11 cases. J Clin Pathol 1989;42:962-72.

Abbondanzo SL, Sato N, Straus SE, Jaffe ES. Acute infectious mononucleosis: CD30 (Ki-1) antigen expression and histologic correlations. Am J Clin Pathol 1990;93:698-2.

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 Livingston. (in press)

Boumpas DT, Wheby MS, Jaffe ES, Steinberg AD, Klippel JH, Balow JE. Synovitis in angioimmunoblastic lymphadenopathy with dysproteinemia stimulating rheumatoid arthritis. Arth Rheum (in press)

VanderMolen LA, Steis RG, Duffey PL, Urba WJ, Foon KA, Smith JW II, Clark JW, Conlon K, Stevenson HC, Hartmann LC, Watson T, Jaffe ES, Longo DL. Low vs. high dose interferon alfa-2a in relapsed low-grade non-Hodgkin's lymphoma. J Natl Cancer Inst (in press)

Medeiros LJ, Rizzi R, Lardelli P, Jaffe ES. Malignant lymphoma involving a Warthin's tumor of the parotid gland. A case with immunophenotypic and gene rearrangement analysis. Hum Pathol (in press)

VanderMolen LA, Duffey PL, Cossman J, Jaffe ES, Longo DL. Surface light chain phenotype in indolent lymphomas: Lack of prognostic significance. Am J Hematol (in press)

Lardelli P, Steinberg SM, Jaffe ES, Garcia del Moral R. Usefulness of histological criteria as prognostic indicators for Hodgkin's disease in southern Spain. Cancer J 1989;2:454-0.

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 (in press)

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 (in press)

Bookman MA, Lardelli P, Jaffe ES, Duffey PL, Longo DL. Lymphocytic lymphoma of intermediate differentiation: Morphologic, immunophenotypic, and prognostic factors. J Natl Cancer Inst (in press)

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)

Jaffe ES, Andrade R, Elwood LJ, Medeiros LJ, Cossman J, Raffeld M. Peripheral T-cell lymphomas and the spectrum of their clinicopathologic presentation. In Dammacco F, ed. Immunological aspects of malignant lymphomas and cryoglobulinemia. Milan: Edi Ermes, 1990. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 00855-08 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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.S. Jaffe | Chief, Hematopathology Section | LP NCI |
| OTHER: | W.A. Blattner | Senior Investigator | EEB NCI |
| | R.C. Gallo | Senior Investigator | LTCB NCI |
| | P. Levine | Senior Investigator | EEB NCI |
| | M. Raffeld | Senior Investigator | LP NCI |
| | H.C. Hsieh | Guest Researcher | LP NCI |
| | M.A. Stetler-Stevenson | Expert | LP NCI |
| | L. Al-Gwaiz | Special Volunteer | LP NCI |

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.20 | 0.15 | 0.05 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

Pathologic material from patients identified to be seropositive for HTLV-I is reviewed and correlated with clinical and epidemiologic features of disease. Material is derived from patients in the United States as well as other parts of the world. Where possible, immunologic phenotyping of the lymphomas is performed and tumor DNA is directly analyzed for viral genome.

For cases in which fresh material is not available, DNA will be extracted from paraffin sections and examined for HTLV-I sequences using the PCR amplification technique. This information will be correlated with serologic, clinical and pathologic data to determine the validity of the PCR technique in establishing the diagnosis of adult T-cell lymphoma/leukemia (ATL).

In selected populations where HTLV-I is endemic, such as Jamaica or Trinidad, prospective studies of all newly diagnosed lymphoma patients are conducted. Such studies are useful in identifying the clinicopathologic spectrum of HTLV-I associated diseases. Prospective studies of all lymphomas in similar geographic regions with differing incidences of adult T cell leukemia/lymphomas are included to discern factors which may have an impact on the incidence of HTLV-I and HTLV-I associated diseases.

Other diseases are being investigated with respect to a possible viral association: angiocentric immunoproliferative disorders (lymphomatoid granulomatosis), sinus histiocytosis with massive lymphadenopathy, systemic Castleman's and Kikuchi's disease. Viruses under investigation include EBV, HHV-6, HTLV-I, and HTLV-II.

Major Findings:

A registry for ATL diagnosed in the United States was established. Approximately 100 cases have been entered in the registry thus far; epidemiologic, clinical, and pathologic data are being analyzed.

A prospective study of all malignant lymphomas diagnosed in Trinidad-Tobago over a three-year period has been completed. Preliminary studies indicate a high incidence of ATL. It has been possible to demonstrate a T-cell phenotype in paraffin-section immunoperoxidase studies, and a T-cell phenotype has correlated with positive serology for HTLV-I.

Studies in progress include the use of the PCR technique to identify HTLV-I viral sequences in paraffin sections. This technique will allow confirmation of the diagnosis of ATL in cases with or without positive serologies.

In a study of leukemia and lymphoma in Egypt, only one case of HTLV I-associated lymphoma was identified.

An association of HHV-6 with sinus histiocytosis with massive lymphadenopathy has been demonstrated.

An association of EBV with the angiocentric immunoproliferative lesions has been suggested, both in the Western and Far Eastern populations.

Publications:

Levine PH, Cuevas M, Arosemena JR, Jaffe ES, Saxinger WC, Altafulla M, De Bernal J, Espino H, Rios B, Xatruck H, Barnett M, Drummond J, Alexander S, Blattner W, Reeves W. Human T-cell leukemia virus (HTLV)-I and hematologic malignancies in Panama. Cancer 1989;63:2186-1.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09181-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Expression of Oncogenes in Lymphoproliferative Disorders

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

PI: M. Stetler-Stevenson Senior Staff Fellow LP NCI

COOPERATING UNITS *(if any)*

Medicine Branch, NCI

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.2 | 0.2 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

We have continued in our studies of the expression of bcl-2 in follicular lymphoma and Hodgkin's disease. We developed a procedure for isolation of RNA followed by reverse transcription and PCR amplification. Using this procedure, we are able to measure the levels of expression of bcl-2 in biopsy specimens. In a small series of follicular lymphoma cases, we have found the levels of expression to have a strong correlation with prognosis; however, a greater number of cases must be studied before final conclusions can be drawn. 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 thus are undertaking a study of levels of expression of myc m-RNA in these same follicular lymphoma specimens using a technique we have developed. As point mutations leading to a protein of increased activity may be as important as levels of RNA, we are also looking for mutations in the myc gene in tumor specimens. This will provide important information as to the interrelationship of various oncogenes in the development of follicular lymphoma.

We have demonstrated the t(14;18) translocation to be present in 32% of Hodgkin's lymphomas. However, Hodgkin's disease and follicular lymphoma are two distinct clinical-pathological entities. We shall also examine the expression of the bcl-2/JH transcript and of the bcl-2 protein product in Hodgkin's disease patients and again correlate this information with the clinical course. This data will provide further information concerning the role of bcl-2 in hematopoietic malignancies.



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

Major Findings:

1. The level of expression of bcl-2 differs among follicular lymphoma specimens and may relate to clinical prognosis.

2. The t(14;18) translocation is found in 32% of Hodgkin's lymphomas.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09182-02 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Molecular Biology of Human Lymphoproliferative Diseases

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

| | | | |
|---|---|---|---|
| PI: | M. Raffeld | Senior Staff Fellow | LP NCI |
| OTHER: | T. Yano | Visiting Fellow | LP NCI |
| | J. Medeiros | Senior Staff Fellow | LP NCI |
| | H. Van Krieken | Special Volunteer | LP NCI |
| | R. Swaby | Special Volunteer | LP NCI |
| | E. Jaffe | Chief, Hematopathology Section | LP NCI |
| | M. Stetler-Stevenson | Senior Staff Fellow | LP NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.3 | 1.8 | 0.5 |

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.

We are initiating a study utilizing monoclonal antibodies to the bcl-2 protein (obtained from D. Mason) designed to give us a better understanding of the biological role of this protein in lymphomagenesis and, in particular, lymphoma progression.

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 studying the feasibility of using PCR to follow response to therapy and predict relapse using peripheral blood samples and in other tissue samples. We are also designing retrospective studies using paraffin embedded tissues.

We have shown the bcl-1 breakpoint region is frequently rearranged in malignant lymphoma of intermediate differentiation. We are pursuing this relationship to study the bcl-1 locus and to search for the postulated proto-oncogene which is thought to become activated by the rearrangement event.

We have identified and sequenced a delta locus gene rearrangement occurring in over a third or precursor B ALL. We are developing a PCR based blood assay to detect this common rearrangement as we have done for bcl-2 related lymphomas.

To determine whether or not there are biologic differences to justify the continued separation of the undifferentiated lymphoma group into Burkitt-like and non-Burkitt subcategories, we analyzed both groups for abnormalities of oncogene loci commonly associated with lymphoma. We have found molecular genetic differences which support the distinction of these two entities.

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.

3. Precursor B cell ALL shows a high frequency of rearrangement of its T cell receptor delta gene locus involving V delta 2, D delta 2 and D delta 3. There is no coordinate J region joining.

4. Malignant lymphoma of intermediate differentiation shows a high frequency of rearrangement of the bcl-1 locus.

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 type occasionally show bcl-2 rearrangement, but rarely involvement of c-myc suggesting that the subtle morphologic and clinical differences between the two groups are indeed meaningful.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09191-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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: | A. Ginsberg | Medical Staff Fellow | LP NCI |
| | H. Van Krieken | Special Volunteer | LP NCI |
| | J. Cossman | Senior Investigator | LP NCI |

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Hematopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.5 | 0.5 | |

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. We have found that immunoglobulin gene restriction pattern changes occur frequently (30% of the cases studied). Changes in the bcl-2 gene restriction fragments were not observed. We believe the changes in the immunoglobulin genes may be due to the normal physiologic "mutator" function which gives rise to somatic mutation in B cell development. If a cell containing a mutation at a restriction enzyme site undergoes a second genetic event that imparts a growth advantage, the first mutation will become detectable as a restriction pattern change. In this way, changes in the immunoglobulin genes may serve as a marker for subsequent growth promoting mutations.

We have begun to investigate potential second events which could impart a selective growth advantage to a lymphoma cell or result in aggressive transformation of a lymphoma. To this end, we are currently analyzing the potential role of several oncogenes and anti-oncogenes. We have found retinoblastoma gene abnormalities at the DNA and RNA levels in several different types of lymphomas and are currently attempting to extend these findings by studying expression at the protein level in a collaborative effort with Dr. William Benedict. 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 other loci.



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

Major Findings:

1. Immunoglobulin restriction patterns show frequent changes over time in follicular lymphomas.

2. Rb abnormalities occur occasionally in lymphoproliferative diseases, and seem to be associated with a more aggressive clinical course.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09144-06 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.25 | 2.75 | 0.5 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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

Application of an exonuclease assay developed in this laboratory to identify and map the sites of tight protein-DNA interactions on large pieces of DNA, to the c-myc gene has revealed multiple cis- and trans-elements both upstream and downstream of the c-myc promoter P1. We have focused on 4 regions, each of which interacts with one or more sequence-specific binding proteins.

1. 300 bp upstream of P1, fos/jun (AP1) and octamer binding proteins bind to a common negative element.

2. Approximately 1 kb downstream, a 140 kD phosphoprotein binds to a negative element.

3. 1.5 kb upstream of P1, a cell-type and differentiation specific 75 kD protein containing peptides, binds to a positive element.

4. 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.

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 promonomyelocytic 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. The purification from HL60 cells of a 75 kD polypeptide binding FUSE is complete, and further characterization of the protein as well as cloning of its gene are in progress. 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.

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. Functional analysis of the cis-element to which this protein binds, as well as studies on the pathways of signal transduction responsible for modulating the protein's phosphorylation are in progress.

A region from - 100 to -140 bp upstream of c-myc promotor 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

two of the proteins, one of which has novel properties. Further purification of these proteins as well as cloning of their structural genes is in progress.

Major Findings:

1. A 138 kd protein binds in a phosphorylation dependent manner to a cis-element frequently mutated in Burkitt lymphoma.

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. One protein in particular with Mr of 59,000 has novel regulatory properties.

Publications:

Takimoto M, Quinn JP, Farina AR, Staudt LM, Levens DL. fos/jun and octamer-binding protein interact with a common site in a negative element of the human c-myc gene. J Biol Chem 1989;264:8992-9.

Zajac-Kaye, M., Levens DL. Phosphorylation-dependent binding of a 138 kDa myc intron factor to a regulatory element in the first intron of the c-myc gene. J Biol Chem 1990;265:4547-1.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09168-03 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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: | J. Quinn | Visiting Associate | LP NCI |
| | A. Farina | Visiting Fellow | LP NCI |
| | K. Gardner | Biotechnology Fellow | LP NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pathology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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|---|---|---|
| 3.5 | 3.0 | 0.5 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The gibbon ape leukemia virus is a family of type C retroviruses associated with several distinct hematopoietic malignancies. The Seato strain is associated with granulocytic leukemia. The principal determinant of enhancer activity has been shown to reside in a 22 bp element which interacts with cellular proteins. The gibbon T-cell lymphoma cell line MLA 144 strongly transactivates the GALV-Seato enhancer. Fractionation of MLA 144 extracts by conventional and affinity chromatography allows the separation of the GALV enhancer binding protein complex into 2 components. One of these components interacts strongly with DNA but does not in itself possess full specificity. The second component does not in itself bind DNA, but upon forming a complex with the first component confers greatly enhancer power to discriminate between different sequences. These proteins are distinct and separable from fos/jun (AP1). However, a minor complex is present in MLA 144 which contains a fos-related antigen (FRA). The two components of the major complex can be independently activated in a cell-line specific manner.

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 low molecular weight, proteinaceous component 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 dynamics govering 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. A variety of transfection studies has confirmed the existence of a moderately inducible cycloheximide resistant activator of transcription from the GALV enhancer in T cells.

Major Findings:

1. A core complex composed of a functionally equivalent 38 and 42 kD polypeptides (approximately 35 kD) interacts with a second factor to produce a specific GALV/AP1 site binding complex. This complex is distinct from fos/jun.

2. A minor complex contains fos-related antigens.

3. The components of the major complex can be activated and regulated in a cell line-specific manner.

Publications:

Quinn JP, Farina AR, Gardner K, Krutzsch HC, Levens DL. Multiple components are required for sequence recognition of the AP1 site in the gibbon ape leukemia virus enhancer. Mol Cell Biol 1989;9:4713-1.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09170-03 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Identification of Genes Differentially Expressed in Developing Embryonic Limb Buds

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

| | | | |
|---|---|---|---|
| PI: | S. Mackem | Senior Staff Fellow | LP NCI |
| OTHER: | M. Ranson | Visiting Fellow | LP NCI |

COOPERATING UNITS *(if any)*

K. Mahon, Senior Staff Fellow, Lab. of Mammalian Genes and Development, NICHD (research collaborator)

LAB/BRANCH

Laboratory of Pathology

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 1.5 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The elucidation and characterization of genes necessary for establishing pattern formation during morphogenesis and the study of their regulation are problems which are central to many aspects of vertebrate biology. It may be expected that processes including responses to trophic stimuli, cell-cell interactions, migration, differential cell multiplication, programmed cell death, etc. are all involved. Many of these same processes are recapitulated in a pathologic manner during oncogenesis. Chick limb development is an attractive system for studying the molecular basis for pattern formation during morphogenesis. Critical events at the level of tissue/cellular interactions involved in pattern formation have been well characterized in this organism, the events regulating pattern formation appear to be overall very similar to those in mammalian systems, and this system is readily amenable to biochemical and molecular analysis as well as studies involving 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 types of approaches are being developed to achieve this aim: 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 4 to 5 fold 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.

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 fo 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 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 (< 1 mm length each), and considering that regulatory signals may be expressed in only a small proportion of the total limb bud cell population at a given stage, techniques were sought to amplify limiting amounts of mRNA so that hybridization subtraction probes could be easily generated and to increase the likelihood of isolating low abundance sequences. The different mRNA populations were stabily 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. Several screening approaches are being employed, including the use of homologous DNA probes in low stringency hybridizations, anti-peptide antibodies recognizing highly conserved protein domains for immunoscreening, and degenerate oligonucleotide primers based on amino acid sequence for selective pcr amplification of gene fragments from pooled library cDNA. 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 16 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 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. This homeobox gene may also play a role in determining pattern and/or establishing limb-type identity. A second new homeobox gene 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 two 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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09171-07 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

The Regulation of Lymphocyte Proliferation

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

PI: K. Kelly Expert LP NCI

COOPERATING UNITS (if any)

U. Siebenlist, Laboratory of Immune Regulation, NIAID, NIH

LAB/BRANCH

Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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| 3.5 | 1.5 | 2.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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. We initially have selected clones for complete sequencing analyses whose expression was shown to be restricted in tissue specificity or constitutively high in adult T cell leukemia (ATL) cells. Primary sequence analysis on seven clones has been completed.

Three clones restricted in expression to hematopoietic cells encode two related but nonidentical lymphokines (464 and 744) and a cell surface protein (237). Four clones responsive to signals in a number of cell types appear to encode 1) a $C_2H_2$-type zinc finger DNA binding protein (225), 2) a protein related to the steroid receptor family of transcription factors (416), 3) a secreted protein (154) and 4) a protein whose structure is not defined by a structure/function category (120). Those clones that have been sequenced entirely are being examined with regard to 1) their possible required role in cell cycle progression and/or "downstream" gene expression and 2) predicted function as assessed from conserved structural motifs in the translated protein sequence. In addition, we have begun defining upstream DNA elements in the 744 gene that regulate mitogen inducibility in T cells.

Major Findings:

Primary Structure Determination

Previously, we have determined that two induced genes (pAT 464 and pAT 744) encode hematopoietic-specific cytokines that most likely are involved in inflammatory responses. Two additional clones (pAT 225 and pAT 416) encode putative transcription factors. We have extended completed sequence analyses to include the following additional clones. 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. pAT 154 appears to encode a secreted factor expressed by both activated lymphocytes and fibroblasts in addition to bone marrow. An extensive tissue distribution for 154 expression is being determined as is the production of recombinant protein to be utilized in investigating the biological activity of the 154 protein. pAT 120 appears to encode a cysteine-rich, approximately 32 kd protein that is expressed in growth-activated lymphocytes and fibroblasts. A comparison to sequences in GenBank shows that pAT 120 does not appear to contain any obvious nucleic acid or amino acid homologies with known genes. pAT 120 does contain a consensus sequence for myristoylation which often serves to localize proteins to the inner side of the plasma membrane. We anticipate that the use of anti-peptide antibodies to study the subcellular localization of pAT 120 and potentially associated proteins will contribute significantly to our understanding. Also, sequenced determinations for additional full-length, mitogen-induced clones (including pAT 140, pAT 243, pAT 270, pAT 276, pAT 281, pAT 402, and pAT 563) are in progress.

Gene Regulation Studies

We have chosen pAT 464 and 744 to initiate gene regulation studies. We anticipate that these genes will encode functionally interesting proteins. Importantly, these two genes share common regulatory properties that are similar but distinguishable from IL-2 and are clearly different from the seven other novel genes that have been assayed. In order to begin characterizing the upstream regulatory regions for 464 and 744, we have isolated multiple genomic clones. 464 and 744 are closely linked in the human genome, separated by 14 kb, and are organized in a head to head fashion. Each of the genes is present in an additional nonallelic copy (referred to as 464.2 and 744.2) as part of an apparent amplification unit in the genome of many individuals. The 464.2 gene is expressed and potentially encodes a protein highly related to 464.1, varying in 5 of 92 amino acids. As expected, 464.2 and 744.2 are also closely linked to each other as determined by population linkage disequilibrium studies. Individuals bearing a chromosome with a third amplification event, involving a 464-related gene but not a 744-related gene, are also infrequently observed. These genes are all located on chromosome 17 in bands q11-q21, the region implicated in von Recklinghausen neurofibromatosis (NF1) and in acute promyelocytic leukemia (AMF-M3).

Approximately 1.2 kilobases of sequence has been determined 5' to the first exons of 744, 464.1, and 464.2. These sequences feature canonical TATA and

CAT boxes. In addition, similarly positioned consensus cAMP responsive elements and regulatory elements commonly found in lymphokine genes (Pu Pu Pu Pu TT Py CA Py) exist within the first 100 base pairs of the upstream sequences for the three genes. Not unexpectedly, the overall upstream sequence homology for 464.1 and 464.2 is greater than 90 percent. By contrast, there is no overall homology between 464 and 744, although there is conservation of sequence and position for multiple short stretches of less than 20 nucleotides within the first 500 nucleotides of upstream sequence. DNA footprinting studies will be required to determine whether these short stretches of conserved sequences represent sites for DNA binding proteins. In an effort to begin defining DNA regulatory sequences utilizing a functional assay, obvious amounts of the pAT 744 upstream sequence has been connected to a CAT reporter gene. Such hybrid genes are transfected into Jurkat cell followed by treatment with activating agents. Deletional analysis have shown that sequences between -112 and -143 relative to the mRNA CAP site are necessary and sufficient for mitogen induction. Further fine structure determinations are in progress.

Publications:

Irving SI, 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 (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09190-01 LP

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Abnormal Expression of a Set of Mitogen-Inducible Genes in HTLV-I-Infected Cells

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

PI: K. Kelly Expert LP NCI

COOPERATING UNITS (if any)

U. Siebenlist, Laboratory of Immune Regulation, NIAID, NIH

LAB/BRANCH

Laboratory of Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 0.5 | 1.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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. A subset of inducible genes (including two putative transcription factors) are constitutively expressed at high levels in T cells infected with HTLV I, and also following transfection of T cells with HTLV I-derived DNA encoding the overlapping reading frames of $p42^{tax}$ and $p27^{rex}$. Such abnormally expressed genes may play a role in the deregulated growth of adult T cell leukemia, a cancer that shows a 100% infection rate with HTLV I. The mechanism of $p42^{tax}$ and $p27^{rex}$ action that leads to over-expression is being examined.





GPO 914-918

Major Findings:

By analogy to known activation-induced growth regulatory genes such as c-fos, c-myc, and IL-2 receptor that are thought to play a causative role in various lymphoid tumors, we have begun investigating the possible abnormal expression in transformed T cells of the mitogen-induced genes described here. The model we have used is the HTLV I transformed adult T-cell leukemia (ATL) for several reasons. 1) The normal progenitor of ATL is the mature $T3^+T4^+$ T helper cell which is a PHA responsive cell within the population from which the induced clones were isolated. 2) The etiological agent of ATL, HTLV I, is structurally well characterized and can be manipulated in culture. 3) HTLV I encodes a transactivating protein, $p42^{tax}$, that is thought to play a role in transformation via affecting expression of cellular genes. One such mitogen-induced gene that is transcriptionally upregulated by $p42^{tax}$ encodes the IL-2 receptor. An additional viral protein, $p27^{rex}$, appears to affect viral RNA accumulation by post-transcriptional mechanisms which include increasing the efficiency of RNA transport from the nucleus. The effect of $p27^{rex}$ on cellular mRNA expression has not been well investigated as yet. $p42^{tax}$ and $p27^{rex}$ represent alternative reading frames of overlapping DNA segments within the viral genome.

In order to address the effects of HTLV I on mitogen-induced genes, we have compared levels of expression in an HTLV I-infected human T helper clone (TM11-H) relative to the matched, uninfected T helper parental clone (TM11), provided by Hirokai Mitsuya, NCI. TM11 demonstrates tetanus toxoid-specific growth stimulation *in vitro* followed by a quiescent period requiring further antigenic stimulation to reinitiate growth. By contrast, TM11-H grows constitutively and its growth is unaffected by antigen.

Differential screening against greater than 60 unique phage clones of induced genes utilizing $^{32}P$ labelled cDNA derived from TM11 and TM11-H demonstrated that several induced genes are overexpressed in TM11-H relative to TM11. Such overexpression has been confirmed for 15 genes by Northern blot analyses with TM11-H, other HTLV I-infected T cell clones, and ATL lines. Although the quiescent TM11 cells did not express those 15 genes which are constitutively expressed in the HTLV I transformed (TM11-H) cells, autogenic stimulation of TM11 cells did lead to a transient induction of expression, as expected. Many other novel induced genes are not expressed in HTLV I-infected cells or are expressed equivalently in quiescent TM11 and TM11-H. Thus, one consequence of HTLV I infection is the selective overexpression of some early mitogen-induced genes. These results demonstrate specific changes in cellular gene expression mediated by HTLV I infection.

As the $p42^{tax}$ is a known transactivator for cellular gene transcription, we have initiated experiments that directly test the affect of $p42^{tax}/p27^{rex}$ in the absence of other viral gene products. To this end, we have obtained from Warner Greene Jurkat cells transfected with an expression vector for the TAX/REX gene products (114.9J). Following treatment with PMA and forskolin, $p42^{tax}$ and $p27^{rex}$ can be induced in 114.9J cells after approximately 8 hours. To date, ten genes known to be overexpressed in HTLV I infected T cell clones have been assayed for mRNA levels in 114.9J cells. None of the eleven

genes is expressed in unactivated nontransfected Jurkat cells. However, six of these genes (pAT 154, 225, 276, 591, 416 and 120) demonstrate constitutive expression in the TAX/REX expressing Jurkat cells, implying potentially a direct effect of one or both gene products in regulating mRNA levels for the three genes. The results obtained with 114.9J cells have been confirmed in another cell line (KATI 40) derived from a continuously-growing primary T lymphocyte line infected with a herpesvirus vector containing the pX region of HTLV I.

In order to investigate the mechanisms of abnormal gene expression in cells producing pX region products, we have carried out both run-on transcription studies and mRNA half-life determinations for a number of effected genes. For those genes that have been tested, transcriptional activation clearly is observed in the presence of $p42^{tax}$ and $p27^{rex}$ while mRNA half-lives remain relatively constant in the absence or presence of pX region gene products.

Publications:

Wright JJ, Gunter KC, Mitsuya H, Irving SG, Kelly K, Siebenlist U. Expression of a zinc finger gene in HTLV I- and HTLV II-transformed cells. Science 1990;248:588-1.

SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY BRANCH
DCBD, NCI

October 1, 1989 through September 30, 1990

The Dermatology Branch conducts both clinical and basic research studying the etiology, diagnosis and treatment of inflammatory and malignant diseases involving the skin and the host's response to these diseases. The basic research involves biochemical as well as biological studies of skin and is subdivided into six separate, though frequently interacting, areas. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center (approximately 1500 patients are seen in consultation each year). The main research achievements of the Dermatology Branch for the past year are as follows:

Immunopathologic Mechanisms Involved in Inflammatory and Neoplastic Skin Diseases:

We have continued our studies of the immunological functions of cells of the epidermis. During this past year, we have sought to resolve two very important, though controversial, pieces of information with regard to Langerhans cells. The first is the question of whether freshly-prepared Langerhans cells express class I MHC molecules and the second is whether when cultured, Langerhans cells can actually process as well as present soluble protein antigens for the activation of T cells. With regard to the former, in collaborative studies with Ms. S. Sharrow and Dr. A. Singer, EIB, DCBD, we have found that Langerhans cells express markedly diminished amounts of H-2K whereas they express normal, if not increased, amounts of H-2D compared to keratinocytes. The potential significance of this finding may relate to the ability of Langerhans cells to function as targets and/or stimulators of CTL responses. With regard to the ability of cultured Langerhans cells to process antigen, we have found that although there appears to be a strain specific difference in this function, freshly prepared cells from all strains can process antigen and present the relevant peptides after culture.

We have also continued 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 then attempt to generate primary in vitro responses to viral-or tumor-associated antigens. As well, we are assessing the role of class II-bearing human keratinocytes in the activation or suppression of specific T cell responses.

We have also investigated the very earliest events which occur after skin is exposed to haptens and other chemicals, and have found that within 24 hours there is "activation" of Langerhans cells as demonstrated by their expressing greatly increased amounts of class II MHC on their surfaces, as well as their becoming much more potent antigen presenting cells than are "unstimulated" Langerhans cells. We are currently studying the early activating signals.

Regulation of Cutaneous Accessory Cell Activity in Health and Disease:

The major focus of this laboratory, which was established this year, has been to determine how ultraviolet B (UV-B) radiation modulates the epidermal immune system. We have found that low doses of UV-B radiation administered to epidermal cells (EC) in vitro completely inhibit the ability of freshly isolated Langerhans cells (fLC) to support the mitogenic response of T cells to anti CD3 antibodies. This inhibitory effect appears to result from the direct action of UV-B on LC and is not due to cytotoxicity. LC that have been cultured for 24-72 hours (cLC) become progressively more resistant to UV-B radiation, an observation which correlates with the progressive increase in the expression of the intercellular adhesion molecule ICAM-1 by cLC. We have found that anti-murine ICAM-1 antibodies inhibit the proliferative response of T cells to anti CD3 antibodies supported by fLC or cLC, confirming that the ICAM-1/LFA-1 interaction is critical in this system. We have also found that exposure of fLC to low doses of UV-B radiation in vitro prevents them from expressing increased levels of ICAM-1 after a 24 hours culture period. Finally, irradiated fLC do not form clusters with T cells in response to anti CD3 suggesting that ICAM-1 is functionally deficient as well. We are also studying the effect of UV-B radiation on protein antigen processing in a model system where expression of costimulatory molecules (eg. ICAM-1) is unimportant.

In collaborative studies, we are examining the accessory cell activity of EC from essential fatty acid (EFA) deficient mice. EC from EFA deficient mice are better stimulators of T cell responses to protein antigens and alloantigens than EC from normal mice. LC number and class II antigen expression by LC is unaltered in EFA deficient mice, as is cytokine production. The most striking difference between EC from EFA deficient and normal mice is that some EFA deficient keratinocytes (KC) express high levels of class II antigens. We have suggested that EFA deficient KC serve as "costimulators" of LC dependent T cell responses.

Molecular Basis of Autoimmune Skin Diseases:

This laboratory studies autoantibody-mediated skin diseases in order to further our understanding not only of the pathophysiology of these diseases but also of the structure and function of normal epidermis and epidermal basement membrane zone. 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. With antibody screening of a λgtll expression cDNA library derived from cultured human keratinocytes, we have isolated a partial cDNA clone encoding the C-terminal end of this antigen. In order to find a cDNA with the full length coding sequence, we have used portions of this partial cDNA as primers and probes to construct and screen new cDNA libraries. We have thus cloned overlapping cDNAs encoding almost the total molecule. Antibodies raised against fusion proteins and synthetic peptides from sequences coding the carboxy-domain bind the hemidesmosome plaque, inside basal cells, as determined by immunoelectron microscopy. Analysis of protein structure and amino acid sequence indicates marked homology with desmoplakin 1, a desmosome plaque protein. These studies indicate that BP antigen and desmoplakin I form a gene family of adhesion plaque proteins.

We have also characterized, by immunochemical methods, the pemphigus vularis (PV) and pemphigus folaceus antigen complexes extracted from normal human epidermis, and shown that they contain the adhering junction molecules desmoglein (PF), a core desmosomal glycoprotein, and plakoglobin (both antigens), a protein found in the dense plaques of both desmosomes and adherens juntions. Thus, in diseases of defective cell adhesion, autoantibodies are directed against adhering junction components.

Molecular and cell biology of immunoreactive molecules in the Skin:

ICAM-1 is a cell surface glycoprotein that serves as the major ligand for LFA-1, an anchoring receptor on all T cells whose interaction with ligand is critical for proper T-cell function. The regulation of ICAM-1 expression is critically important in both normal inflammatory and pathologic processes. We have established the kinetics and molecular regulatory level for cytokine-induced de novo ICAM-1 expression by human keratinocytes (HK) in culture. We have established an in vitro leukocyte adhesion assay for resting and blast T cells to cultured HK and have demonstrated that the marked increase in adhesion of T cells to IFN-$\gamma$-treated HK is primarily due to the induction of HK ICAM-1 expression. We have further explored the regulatory mechanisms of ICAM-1 gene expression and shown that ICAM-1 expression in HK is induced by short-term exposure to cycloheximide, implicating the existence of a trans-acting protein with a short half-life that is involved in the constitutive repression of ICAM-1 in HK. We have shown that the kinetics for IFN-$\gamma$-induced expression of HLA-DR$\beta$ by HK (DR) is different from those for ICAM-1. Additionally, TGF-$\beta$ enhances the induced expression of ICAM-1 and suppresses the induced expression of DR.

The transcriptional regulatory region of the human ICAM-1 gene has been cloned and characterized: A 17.4 kb genomic lambda phage clone has been isolated, restriction mapped and restriction fragments subcloned. Multiple subclones have been sequenced and analyzed, including a 2.05 kb subclone containing the first exon and 1350 bp of the 5' flanking region. The transcription start site (cap site) has been established by primer extension and S1 nuclease protection assays and is located 40 bp 5' of the translation start codon of the first exon.

Therapy of Skin Cancer and Disorders of Keratinization:

We are continuing to evaluate safety and effectiveness of new oral and topical agents particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne as well as in the prevention of cancer.

The efficacy of isotretinoin at high dose as a chemopreventive agent is being studied further in a series of 7 patients with xeroderma pigmentosum and nevoid basal cell carcinoma syndrome. Preliminary results indicate significant partial, but not complete, efficacy in inhibiting new tumor formation. Furthermore, impressive increases in the rate of new tumor formation have been observed after stopping therapy. Low-dose isotretinoin is now being used in these patients in order to minimize toxicity.

Seven patients developed symtoms of severe depression during treatment with oral isotretinoin. All depressive symtoms resolved completely within 2 to 7

days after discontinuation of the drug. One patient who was rechallenged with a second course of isotretinoin at a lower dose, again eveloped depressive symtoms that remitted on discontinuation. There was no predictable relationship between dose or duration of drug administration and the onset of depressive symptoms. None of the patients had signs of benign intracranial hypertension during their depressive episodes, and none had a pretreatment history of a major depressive episode indicating that depression is a rare, idiosyncratic side effect of isotretinoin.

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

All XP patients have abnormally increased frequences of UV-induced skin cancers. It is known that nucleotide excision-repair-deficient XP patients have abnormally high levels of UV-induced chromosomal aberrations. We are continuing our studies to determine the levels of such aberrations in cells from XP variant patients (who have normal levels of nucleotide excision repair). These studies may indicate whether or not abnormal levels of UV-induced chromosomal aberrations are causally related to the development of sunlight-induced skin cancer in XP patients. Our results indicate that XP variant cells have only a minimal, if any, abnormally high level of UV-induced chromosomal aberrations. We are now attempting to determine whether caffeine will increase the number of UV-induced chromosomal aberrations in the XP variant cells.

In collaboration with Dr. Kenneth H. Kraemer, we transiently transfect human lymphoblastoid cell lines with a plasmid (pRSVcat) which contains the bacterial cat gene coding for chloramphenicol acetyltransferase. The plasmid is irradiated with different doses of 254-nm UV. Then, in order to evaluate the repair of dimer and nondimer photoproducts, an aliquot containing the irradiated plasmid is 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 have 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.

Chemistry, Structure, and Biosynthesis of Mammalian Epidermal Keratin Filaments:

The availability of complete amino acid sequences of several keratin chains has now led us to propose more advanced models of intermediate filament structure. Even though filaments are polymorphic, a major structural form consists of 8 4-chain molecules, arranged in an antiparallel mode. The structure of the 4-chain molecule is being examined by scanning transmission electron microscopy (with Dr. Wall) using platinum-clathrates complexed onto cystine residues. In collaboration with Drs. A.C. Steven and David Parry, we have constructed "surface-lattice" models of filament structure in which the rod domains of the protein chains form the basic filament core, and the variable end domains occupy peripheral positions. Limited proteolytic cleavage experiments have provided direct evidence in support of these structural models and of the precise alignment of the neighboring rod domains. In

addition, scanning transmission electron microscopy is being used to explore the mass, structure and association of 4-chain units in an attempt to describe intermediate levels of filament substructure. However, the structural organization of the end domains is still uncertain. In the case of the keratins, we propose that their glycine-serine-rich sequences form omega-loop structures and interact with other cellular components including filaggrin and the cell envelop by H-bonding and disulfide cross-links. In collaboration with Dr. James Mack, these ideas are being tested by use of solid-state NMR of keratin filaments isotopically labeled with glycine (to study end domains) and leucine (to study the rod domain). Our data suggests the glycine-rich domains have little or no structural order, whereas the rod-domains are considerably more ordered.

A major project to determine the structure, complexity, linkage and evolution of keratin genes has been initiated. In collaboration with Dr. Stuart Lessin, we have found that type II keratins seem to be linked in one cluster in the general region of 12q11-12 and type I keratins are clustered on 17q12-21. Using keratin probes generated by PCR reaction to known sequence, we are employing pulse field electrophoresis to further map the linkage of the keratin genes. In related experiments in collaboration with Drs. Compton and Roop, the human keratin 1 and 10 genes have been used to generate transgenic mice with the aim to study their regulation. Also mutant versions of these genes will be used to explore the possibility of generating disorders of keratinization due to the expression of the mutant phenotype. We have now found that the loricrin gene is also located near the 1g21 region, in the same region where filaggrin and the involucrin gene are located. Accordingly, we will do RFLP studies on DNA from patients with keratinizing disorders to determine whether any disorders are linked at or near these three genes expressed in the epidermis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 CB 03630-20 D

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Effects of Retinoids and Other Differentiating Agents on Skin

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

P.I.: G.L.Peck, Senior Investigator, Dermatology Branch, DCBD, NCI

OTHER: J.J. DiGiovanna, Expert Scientist, Dermatology Branch, DCBD, NCI
P.M. Steinert, Senior Investigator, Dermatology Branch, DCBD, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 0.2 | 0.2 | |

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [X] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

B

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

This project proposed to morphologically and biochemically define the mechanism of action of vitamin A and its derivatives (retinoids) in altering epidermal differentiation in normal skin and in benign and malignant lesions of skin. Topical alltrans retinoic acid, but not systemic 13-cis-retinoic acid, increased gap junction density and decreased desmosome density in treated basal cell carcinomas. This indicates that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms. A specific cytosol retinol binding protein (CRBP) has been identified in normal human skin, newborn mouse skin and human skin from patients with Darier's disease, psoriasis and basal cell carcinomas. A specific cytosol retinoic acid binding protein (CRABP) has also been identified in newborn mouse and normal human adult skin and newborn foreskin. The qualitative and quantitative distribution between the epidermis and dermis of both CRBP and CRABP has been determined in adult human lower limb skin. Using molecular hybridization probes specific for transcripts of individual keratin genes, keratin gene expression in skin cancer and cutaneous disorders of keratinization indicate the following. In contrast to normal epidermis where the expression of proliferation-associated keratin genes is suppressed after cells migrate from the basement membrane, hyperproliferative disorders of the epidermis exhibit inappropriate expression of proliferation-associated keratin genes in the more superficial layers of the epidermis. In addition, skin cancers fail to express differentiation-associated keratins, indicative of their undifferentiated state. Utilizing in situ hybridization techniques and specific probes, filaggrin production is being characterized in skin from normal persons and from a variety of disorders of keratinization.



PHS 6040 Rev. 1 84)

GPO 914-918

Project Description

Major Findings:

A project studying keratin gene expression in skin cancer and cutaneous disorders of keratinization has been initiated. The study employes molecular hybridization probes that are specific for transcripts of individual keratin genes. The transcripts are localized at the cellular level within frozen sections by in situ hybridization with 35-S labeled RNA probes. Diseases studied to date include Darier's disease, psoriasis, lamellar ichthyosis, basal cell carcinoma and squamous cell carcinoma. Preliminary findings suggest that, in contrast to normal epidermis where the expression of proliferation-associated keratin genes is suppressed after cells migrate from the basement membrane, hyperproliferative disorders of the epidermis exhibit inappropriate expression of proliferation-associated keratin genes in the more superficial layers of the epidermis. In addition, skin cancers fail to express differentiation-associated keratins, indicative of their undifferentiated state. Recent experiments on normal skin indicate a sequential expression of differentiation-associated keratins (K1, K10).

Utilizing in situ hybridization techniques and specific probes, filaggrin production is being characterized in skin from normal persons and from a variety of patients with disorders of keratinization.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 03638-21 D

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Studies of DNA Repair in Human Degenerative Diseases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
PI: J. H. Robbins, M.D., Dermatology Branch, DCBD, NCI

OTHER: V. A. Bohr, M.D., Senior Investigator, LMPH, DCT, NCI
A. J. Fornace, Jr., M.D., Senior Investigator, LMPH, DCT, NCI
K. H. Kraemer, M.D., Senior Investigator, LMC, DCE, NCI
L. Seguin, Ph.D., Consultant, D., DCBD, NCI
R. E. Tarone, Ph.D., Mathematical Statistician, BB, DCE, NCI

COOPERATING UNITS (if any)
Laboratory of Molecular Pharmacology, DCT, NCI; Laboratory of Molecular Carcinogenesis, DCE, NCI; Biostatistics Branch, DCCP, NCI

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 3.5 | 1.5 | 2.0 |

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

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

Studies in this laboratory are designed to elucidate the role of DNA repair processes in carcinogenesis and in 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.



PHS 6040 (Rev. 84)

GPO 914-918

Project Description

Major Findings:

In collaboration with Drs. Vilhem 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's disease.

In collaboration with Dr. Kenneth H. Kraemer, we transiently transfect human lymphoblastoid cell lines with a plasmid (pRSVcat) which contains the bacterial cat gene coding for chloramphenicol acetyltransferase. The plasmid is irradiated with different doses of 254-nm UV. Then, in order to evaluate the repair of dimer and nondimer photoproducts, an aliquot containing the irradiated plasmid is 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 have 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.

With Dr. Albert J. Fornace, Jr., we have studied 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.

All XP patients have abnormally increased frequences of UV-induced skin cancers. It is known that nucleotide excision-repair-deficient XP patients have abnormally high levels of UV-induced chromosomal aberrations. We are continuing our studies to determine the levels of such aberrations in cells from XP variant patients (who have normal levels of nucleotide excision repair). These studies may indicate whether or not abnormal levels of UV-induced chromosomal aberrations are causally related to the development of sunlight-induced skin cancer in XP patients. Our results indicate that XP variant cells have only a minimal, if any, abnormally high level of UV-induced chromosomal aberrations. We are now attempting to determine whether caffeine will increase the number of UV-induced chromosomal aberrations in the XP variant cells.

We have previously reported the existence of XP complementation group H. Scientists in the Netherlands and in Italy are unable to obtain complementation between the group-H stain and group-D stains. We and scientists in Japan obtain such complementation. Joint studies are underway to determine the basis for these results which may be due to differences in culture conditions among the laboratories.

The relation between age of symptomatic onset of XP neurologic disease and the patient's DNA-repair defect will be tested in cells from an unusual group-A patient from Egypt (who has no neurological abnormalities by age 35 years) and from a sibling pair from Mexico (characterized by severe neurologic disease and a reportedly mild DNA-repair defect). Colony-forming ability studies of their cells will be conducted.

Publications:

Robbins, JH. Defective DNA repair in xeroderma pigmentosum and other neurologic diseases. Curr Opin Neurol Neurosurg 1989;2:953-958.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 03656-17 D

PERIOD COVERED

October 1, 1989 to December 31, 1989

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

Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments

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

P.I.: P.M. Steinert, Biologist, Dermatology Branch, NCI

OTHER: see next page

COOPERATING UNITS (if any)

Lab of Cellular Carcinogenesis and Tumor Promotion, DCE, NCI; Laboratory of Physical Biology, NIAMS; Laboratory of Biochemistry, NIDR

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 7.5 | 5.5 | 2.0 |

CHECK APPROPRIATE BOX(ES)

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

B

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

The biosynthesis, structure and function of the principal differentiation products of human and mouse epidermis are being studied. Keratin subunits polymerize in vitro into native-type intermediate filaments. Details of their structure are being investigated by use of: solid state NMR on isotopically-labeled filaments; transmission and scanning transmission electron microscopy of intact filaments or subfilamentous forms; optical diffraction and image analysis procedures; and limited proteolysis experiments to ascertain alignment of constituent subunits. Model structures generated by these methods are being computationally tested for compatibility with other physico-chemical data and amino acid sequence information on individual subunits. PCR-generated probes to type I and type II keratins are being used to characterize the number, organization and complexity of their genes. The expression of the human keratin genes 1 and 10 are being studied by the production of transgenic mice produced from various constructs of these genes. Both cDNA and αDNA clones have been used to describe the properties of the human filaggrin gene. It encodes a large polyprotein precursor that displays considerable variation in sequence and size. Clones encoding a major cell envelope protein, loricrin, have also been isolated and are being sequenced. Clones encoding an epidermal transglutaminase have been isolated and will be characterized. Constructs of keratins, filaggrin and the cell envelop protein clones have been assembled with pGEM vectors for use in insitu hybridization experiments in order to study the expression of these proteins in epidermal keratinizing disorders.

Other Professional Personnel:

S.-Q. Gan, M.D., Visiting Fellow, Dermatology Branch, DCBD, NCI
W.W. Idler, B.S., Chemist, Dermatology Branch, DCBD, NCI
J. Mack, Ph.D., Staff Fellow, Dermatology Branch, DCBD, NCI
J. Compton, Ph.D., Jackson Laboratories, Bar Harbor, ME
S. Chung, Ph.D., Senior Investigator, Laboratory of Biochemistry, NIDR
R.D. Goldman, Ph.D., Professor, Dept. Antmy & Cell Biol., Northwestern Univ.
D. Hohl, M.D., Dermatology Clinic, University Hospital, Zurich, Switzerland
D.A.D. Parry, Ph.D., Professor, Dept. of Physics and Biophysics, Massey Univ., Palmerston North, New Zealand
D.R. Roop, Ph.D., Associate Professor, Dept. Cell Biology, Baylor College of Medicine, Houston, TX
A.C. Steven, Ph.D., Visiting Scientist, Lab. of Phys. Biol., NIAMS
J. Wall, Ph.D., Professor, Department Biology, Brookhaven National Laboratory, Upton, NY
S.H. Yuspa, M.D., Branch Chief, Lab. of Cellular Carcinogenesis and Tumor Promotion, DCE, NCI

Project Description

Major Findings:

The availability of complete amino acid sequences of several keratin chains has now led us to propose more advanced models of intermediate filament structure. Even though filaments are polymorphic, a major structural form consists of 8 4-chain molecules, arranged in an antiparallel mode. The structure of the 4-chain molecule is being examined by scanning transmission electron microscopy (with Dr. Wall) using platinum-clathrates complexed onto cystine residues. In collaboration with Drs. A.C. Steven and David Parry, we have constructed "surface-lattice" models of filament structure in which the rod domains of the protein chains form the basic filament core, and the variable end domains occupy peripheral positions. Limited proteolytic cleavage experiments have provided direct evidence in support of these structural models and of the precise alignment of the neighboring rod domains. In addition, scanning transmission electron microscopy is being used to explore the mass, structure and association of 4-chain units in an attempt to describe intermediate levels of filament substructure. However, the structural organization of the end domains is still uncertain. In the case of the keratins, we propose that their glycine-serine-rich sequences form omega-loop structures and interact with other cellular components including filaggrin and the cell envelop by H-bonding and disulfide cross-links. In collaboration with Dr. James Mack, these ideas are being tested by use of solid-state NMR of keratin filaments isotopically labeled with glycine (to study end domains) and leucine (to study the rod domain). Our data suggests the glycine-rich domains have little or no structural order, whereas the rod-domains are considerably more ordered.

We are using NMR techniques to study the nature of the molecular interaction of keratin filaments and filaggrin, which is an important filament associated protein in the epidermis. Keratin filaments isotopically labeled with glycine (for end domains) and glutamic acid or lysine (for rod domains) are complexed with filaggrin. Changes in the molecular motions of the filaments will provide information on their mode of interaction with filaggrin. Preliminary data suggests the molecular motions of the glycine regions are unchanged, Experiments using lysine indicate that the rod domains are considerably more constrained. These preliminary observations will be continued in an attempt to establish whether filaggrin interacts with the rod domains.

Many cDNA clones to human filaggrin have now been isolated and sequenced. The human filaggrin gene encodes a polyprotein containing a large number of filaggrin repeats arranged in tandem. Sequencing by Dr. Gan has shown that these repeats are highly polymorphic but conserved in size. Also, the gene from different individuals contain differing numbers of repeats. In collaboration with Dr. Lessin, we have localized the human gene to chromosome 1q21 region. Attempts are now underway to isolate the human gene in order to define regulatory sequence regions by use of CAT assays and eventually production of transgenic mice bearing the human gene.

Dr. Hohl has isolated, cloned and sequenced the gene for the major human cornified cell envelop proteins, named loricrin. Fragments of this gene are now being subcloned into CAT vectors to define regulatory regions. In collaboration with Dr. Dennis Roop, the clones bearing the complete gene will be used for the production of transgenic mice. In collaboration with Dr. Ron Chung, we have begun to isolate and characterize cDNA clones for epidermal transglutaminase, the enzyme which cross-links the loricrin to the cell envelop. In addition, we have synthesized peptides corresponding to loricrin sequences for in vitro transglutaminase assays, and $^{3}$H-lysine-labeled cross-linked peptides of loricrin from labeled foreskins will be characterized to define the structural organization of loricrin in the cell envelop.

A major project to determine the structure, complexity, linkage and evolution of keratin genes has been initiated. In collaboration with Dr. Stuart Lessin, we have found that type II keratins seem to be linked in one cluster in the general region of 12q11-12 and type I keratins are clustered on 17q12-21. Using keratin probes generated by PCR reaction to known sequence, we are employing pulse field electrophoresis to further map the linkage of the keratin genes. In related experiments in collaboration with Drs. Compton and Roop, the human keratin 1 and 10 genes have been used to generate transgenic mice with the aim to study their regulation. Also mutant versions of these genes will be used to explore the possibility of generating disorders of keratinization due to the expression of the mutant phenotype. We have now found that the loricrin gene is also located near the 1g21 region, in the same region where filaggrin and the involucrin gene are located. Accordingly, we will do RFLP studies on DNA from patients with keratinizing disorders to determine whether any disorders are linked at or near these three genes expressed in the epidermis.

In collaboration with Drs. Hohl and Roop in situ hybridization techniques are being used to study the expression of filaggrin, loricrin and keratins 1 and 10 in disorders of keratinization.

Publications:

Rothnagel JA, Steinert PM. The structure of the gene for mouse filaggrin and a comparison of the repeating units, J Biol Chem 1990;265:1862-1865.

Green KJ, Parry DAD, Steinert PM, Virata MLA, Wagner RM, Angst BD, Niles LA. Structure of the human desmoplakins: implications for function in the desmosomal plaque, J Biol Chem 1990;265:2603-2612.

Steinert PM. The two-chain coiled-coil molecule of native epidermal keratin intermediate filaments is a type I - type II heterodimer, J Biol Chem 1990;265:8766-8774.

Mehrel T, Hohl D, Rothnagel JA, Longley MA, BUndman D, Cheng CK, Lichti U, Steinert PM, Yuspa SH, Roop DR. Identification of a novel gene encoding a major cell envelope protein, loricrin, Cell 1990 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 03657-16 D

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (50 characters or less. Title must fit on one line between the borders.)
Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases

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

P.I.: S.I. Katz, Branch Chief, Dermatology Branch, DCBD, NCI

OTHER: P. Cohen, Medical Staff Fellow, Dermatology Branch, DCBD, NCI
S. Aiba, Visiting Fellow, Dermatology Branch, DCBD, NCI
E. Dugan, Medical Staff Fellow, Dermatology Branch, DCBD, NCI

COOPERATING UNITS (if any)
Dermatology Dept., USUHS, Bethesda
Immunology Branch, DCBD, NCI

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER |
|---|---|---|
| 5 | 4 | 1 |

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [X] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

B

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

The major area of study of this laboratory is the role of the epidermis as an immunological organ. We have found that epidermal Langerhans cells are derived from precursor cells in the bone marrow and play an essential role in many of the immunological reactions affecting the skin. We have also identified an epidermal Interleukin 1-like cytokine which may serve as a second signal in the generation of T cell responses as well as a proinflammatory agent affecting other cells - especially neutrophils. We have demonstrated that when murine epidermal Langerhans cells are cultured for 2-3 days they become very potent antigen presenting cells 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. Recent studies demonstrate that cultured human Langerhans cells have enhanced antigen presenting capacity. The other major focus of this laboratory has been the study of the function of class II MHC bearing keratinocytes which appear in humans and mice during cell-mediated reactions in the skin. We have demonstrated that these cells can 1) present peptide fragments to T cell hybridomas, 2) serve as targets for class II specific cytotoxic T lymphocytes, and 3) induce secondary alloreactive T cell responses, although the first and third are performed only poorly. Recent studies demonstrate that these class II bearing keratinocytes induce specific immunological unresponsiveness in cloned T cells and in vivo in contact sensitization.

Project Description

Major Findings:

We have found that when murine epidermal Langerhans cells are cultured for 2-3 days they become very potent antigen presenting cells for the generation of allogeneic and autologous T cell responses as well as for T cell responses to hapten modified self and for protein antigens. When cultured, these cells express much greater amounts of Class II antigens than do freshly prepared cells. Utilizing these cells in primary immune responses in vitro, we found that the T cells responded preferentially to the hapten to which they were "primed." We have produced T cell lines from these in vitro sensitized cells and virtually all of them produce IL-4 as opposed to IL-2. We have found that during the first few stimulations, the primed T cells produce mainly IL-2, however after multiple stimulations they produce mainly IL-4 and not IL-2. Utilizing this in vitro sensitization system, we have recently attempted to generate primary in vitro responses to tumor-associated antigens. We have utilized the Friend Leukemia Virus associated gp-70 protein but have been unable to detect any antigen processing or presentation by cultured or freshly prepared epidermal Langerhans cells despite being able to induce primary responses to soluble protein antigens.

We have also continued 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 then attempt to generate primary in vitro responses to viral-or tumor-associated antigens. As well, we are assessing the role of class II-bearing human keratinocytes in the activation or suppression of specific T cell responses.

During this past year, we have also sought to resolve two very important, though controversial, pieces of information with regard to Langerhans cells. The first is the question of whether freshly-prepared Langerhans cells express class I MHC molecules and the second is whether when cultured, they can actually process as well as present soluble protein antigens for the activation of T cells. With regard to the former, in collaborative studies with Ms. S. Sharrow and Dr. A. Singer, EIB, DCBD, we have found that Langerhans cells express markedly diminished amounts of H-2K whereas they express normal, if not increased, amounts of H-2D compared to keratinocytes. The potential significance of this finding may relate to the ability of Langerhans cells to function as targets and/or stimulators of CTL responses. With regard to the ability of cultured Langerhans cells to process antigen, we have found that although there appears to be a strain specific difference in this function, freshly prepared cells from all strains can process antigen and present the relevant peptides after culture.

We have also investigated the very earliest events which occur after skin is exposed to haptens and other chemicals, and have found that within 24 hours there is "activation" of Langerhans cells as demonstrated by their expressing

greatly increased amounts of class II MHC on their surfaces, as well as their becoming much more potent antigen presenting cells than are "unstimulated" Langerhans cells. We are currently studying the early activating signals.

Other major studies include 1) those characterizing the functions of class II-MHC bearing keratinocytes, and 2) the assessment of the effect of cyclosporine A on the antigen-presenting and accessory cell functions of Langerhans cells and on the activation of T cells. With regard to the former, we have found that class II bearing keratinocytes function
1) as targets for class II specific cytotoxic T cells, 2) as weak stimulators in secondary allogeneic T cell proliferative responses, and 3) poorly in the presentation of small peptide antigens to T cell hybridomas. We have also demonstrated that these class II bearing keratinocytes when hapten-coupled, induce specific immunological unresponsiveness in hapten-specific T cell clones. Our most recent studies indicate that class II bearing keratinocytes induce tolerance to various haptens in vivo.

Studies assessing the effects of cyclosporine A (CSA) on antigen presenting and accessory cell functions of Langerhans cells have demonstrated that CSA interacts with Langerhans cells after a 2 hour incubation and renders them (Langerhans cells) relatively unable to perform many of the T cell stimulatory functions. In addition, CSA has a profound effect on the proliferation of epidermal cells. We have also studied the effects of dexamethasone on epidermal Langerhans cells. From these in vitro studies, it appears that dexamethasone is cytolytic to a subpopulation of Langerhans cells. In studies comparing the effects of dexamethasone and cyclosporine on the activation of T cells, we have demonstrated a differential effect of the two. That is, using various activation signals, it is clear that phorbol and ionomycin activation of T cells cannot be inhibited by dexamethasone, whereas it is completely blocked by cyclosporine.

Publications:

Kusuda M, Gaspari AA, Chan C-C, Gery I, Katz SI. Expression of Ia antigen by ocular tissues of mice treated with interferon gamma, Invest Opthalmol vis Sci 1989;30:764-768.

Hall RP, Sanders ME, Duquesnoy RJ, Katz SI, Shaw SS. Alterations in HLA-DP and -DQ antigen frequency in patients with dermatitis herpetiformis, J Invest Dermatol 1989;93:501-505.

Hauser C, Yokoyama WM, Katz SI. Characterization of primary T helper cell activation and T helper cell lines stimulated by hapten-modified, cultured Langerhans cells J Invest Dermatol 1989;93:649-655.

Furue M, Kawakami Y, Kawakami T, Katz SI. Differential inhibition of T cell activation pathway by dexamethasone and cyclosporine, Transplantation 1990;49:560-564.

Rosenberg AS, Katz SI, Singer A. Rejection of skin allografts by $CD4^+$ T cells is antigen specific and requires expression of target alloantigen on $Ia^-$ epidermal cells, J Immunol 1989;43:2452-2456.

Furue M, Katz SI, Kawakami T, Kawakami Y. Coordinate expression of SRC family protooncogenes in T cell activation and its modulation by cyclosporine, J Immunol 1990;144:736-739.

Gaspari AA, Huang CM, Davey RJ, Bondy C, Lawley TJ, Katz SI. Prevalence of thyroid abnormalities in patients with dermatitis herpetiformis and HLA-B8/-DR3 controls, Am J Medicine 1990;88:145-150.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 03659-16 D

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Therapy of Skin Cancer, Disorders of Keratinization, and Cystic Acne

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

P.I.: G.L. Peck, Senior Investigator, Dermatology Branch, DCBD, NCI

OTHER: J.J. DiGiovanna, Expert Scientist, Dermatology Branch, DCBD, NCI
I. Tokar, Registered Nurse, Dermatology Branch, DCBD, NCI
K. Kraemer, Senior Investigator, Lab. Molecular Carcinogenesis

COOPERATING UNITS *(if any)*

Cancer Prevention Studies Branch, DCPC, NCI, NIH, Bethesda, Maryland 20892

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER |
|---|---|---|
| 2.8 | 2.8 | |

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.)*

Seven patients with xeroderma pigmentosum were entered into a cancer chemoprevention study using isotretinoin: results indicate significant partial inhibition of new tumor formation during therapy and a marked increase in new tumor formation after therapy. Long-term therapy with low-dose isotretinoin is now being used for cancer chemoprevention in these patients. Suicidal ideation is a potential problem in patients with chronic disfiguring dermatoses, such as Darier's disease. Seven of 11 patients with Darier's disease had a history of suicidal thoughts, plans or attempts. In a control group of 11 patients with lamellar ichthyosis and similar disorders, 3 patients had suicidal thoughts or plans. One patient with multiple skin cancers developed an adverse drug reaction to fenretinide, a synthetic retinoid. The reaction included diarrhea, elevated liver function tests and a morbilliform skin eruption. Two patients with symtomatic, etretinate-resistant, Darier's disease were successfully treated with deep split-thickness skin excision using as electrosurgical unit. Healing was rapid and recurrence of disease has been minimal. A case of Darier's disease, well-controlled with etretinate, markedly worsened on two separate occasions after ingestion of lithium and improved after its discontinuation. Seven patients developed symptoms of sever depression during treatment with oral isotretinoin. All depressive symtoms resolved completely within 2 to 7 days after discontinuation of the drug.

Project Description

Major Findings:

The efficacy of isotretinoin at high dose as a chemopreventive agent is being studied further in a series of 7 patients with xeroderma pigmentosum and nevoid basal cell carcinoma syndrome. Preliminary results indicate significant partial, but not complete, efficacy in inhibiting new tumor formation. Furthermore, impressive increases in the rate of new tumor formation have been observed after stopping therapy. Low-dose isotretinoin is now being used in these patients in order to minimize toxicity.

Two patients with symtomatic, etretinate-resistant, Darier's disease were successfully treated with deep split-thickness skin excision using as electrosurgical unit. Healing was rapid and recurrence of disease has been minimal.

A case of Darier's disease, well-controlled with etretinate, markedly worsened on two spearate occasions after ingestion of lithium and improved after its discontinuation.

Seven patients developed symtoms of severe depression during treatment with oral isotretinoin. All depressive symtoms resolved completely within 2 to 7 days after discontinuation of the drug. One patient who was rechallenged with a second course of isotretinoin at a lower dose, again eveloped depressive symtoms that remitted on discontinuation. There was no predictable relationship between dose or duration of drug administration and the onset of depressive symptoms. None of the patients had signs of benign intracranial hypertension during their depressive episodes, and none had a pretreatment history of a major depressive episode indicating that depression is a rare, idiosyncratic side effect of isotretinoin.

Suicidal ideation is a potential problem in patients with chronic disfiguring dermatoses, such as Darier's disease. Seven of 11 patients with Darier's disease had a history of suicidal thoughts, plans or attempts. In a control group of 11 patients with lamellar ichthyosis and similar disorder, 3 patients had suicidal thoughts or plans.

One patient with multiple skin cancers developed an adverse drug reaction to fenretinide, a synthetic retinoid. The reaction included diarrhea, elevated liver function tests and a morbilliform skin eruption.

Publications:

Sarnoff DS, Peck GL. Therapy of basal cell carcinoma. In: Magrath IT, ed. New Directions in Cancer Treatment. New York: Springer-Verlag, 1989;541-545.

Milton GP, DiGiovanna JJ, Peck GL. Treatment of nevus comedonicus with ammonium lactate lotion, J Am Acad Dermatol 1989;20:324-328.

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DiGiovanna JJ, Zech LA, Ruddel ME, Gantt G, Peck GL. Etretinate: persistent serum concentrations after long-term therapy, Arch Dermatol 1989;125:246-251.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 03667-06 D

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecules Defined by Autoantibody - Mediated Skin Diseases

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

P.I.: John R. Stanley, M.D., Medical Officer, Dermatology Branch, DCBD, NCI

OTHER: Neil Korman, M.D., Medical Staff Fellow, Dermatology Branch, DCBD, NCI
Toshihiro Tanaka, M.D., Visiting Fellow, Dermatology Branch, DCBD, NCI
Masayuki Amagai, M.D., Visiting Fellow, Dermatology Branch, DCBD, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 5 | 4 | 1 |

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [X] (b) Human tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

B

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

The general and long-term goal of my laboratory is to study autoantibody-mediated skin diseases in order to further our understanding not only of the pathophysiology of these diseases but also of the structure and function of normal epidermis and epidermal basement membrane zone. Specifically, antibodies in these diseases define molecules in the normal epidermis. We are characterizing the antigens defined by three of these diseases: bullous pemphigoid (BP), pemphigus vulgaris (PV), and pemphigus foliaceus (PF). BP antigen 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. With antibody screening of a λgt11 expression cDNA library derived from cultured human keratinocytes, we have isolated a partial cDNA clone encoding the C-terminal end of this antigen. In order to find a cDNA with the full length coding sequence, we have used portions of this partial cDNA as primers and probes to construct and screen new cDNA libraries. We have thus cloned overlapping cDNAs encoding almost the total molecule. Antibodies raised against fusion proteins and synthetic peptides from sequences coding the carboxy-domain bind the hemidesmosome plaque, inside basal cells, as determined by immunoelectron microscopy. 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 have also characterized, by immunochemical methods, the PV and PF antigen complexes extracted from normal human epidermis, and shown that they contain the adhering junction molecules desmoglein (PF antigen) and plakoglobin (both antigens). Thus, in diseases of defective cell adhesion, autoantibodies are directed against adhering junction components. Patients with drug-induced pemphigus have antibodies against these same molecules.





GPO 914-918

Project Description

Major Findings:

Overlapping cDNAs encoding almost the entire approx. 230 kD bullous pemphigoid (BP) antigen have been cloned.

Antibodies raised against fusion proteins and synthetic peptides encoded by cDNAs reflecting the carboxy-domain of BP antigen bind the basement membrane of epidermis, as determined by indirect immunofluorescence, immunoprecipitate the 230 kD BP antigen on immunoblots, and bind the plaque of the hemidesmosome inside the basal cell, as determined by immunogold electron microscopy.

Sequence analysis indicates that BP antigen is similar in structure, charge periodicity, and amino acid sequence to desmoplakin I, a desmosomal plaque protein.

Antibodies from pemphigus foliaceus (PF) patients bind a complex of polypeptides that includes desmoglein, a core desmosomal glycoprotein, and plakoglobin, a protein found in the dense plaques of both desmosomes and adherens junctions.

Antibodies from pemphigus vulgaris (PV) patients bind a complex of polypeptides that contains a 130 kD glycoprotein and plakoglobin.

The diagnosis of both sporadic and drug-induced PF and PV can be made by immunoprecipitation of their respective antigens.

Publications:

Matsuoka LY, Wortsman J, Stanley JR: Epidermal autoantibodies in erythema multiforme. J Am Acad Dermatol 1989;21:677-680.

Korman NJ, Eyre RW, Klaus-Kovtun V, Stanley JR: Demonstration of an adhering-junction molecule (plakoglobin) in the autoantigens of pemphigus foliaceus and pemphigus vulgaris. New Engl J Med 1989;321:631-5.

Tanaka T, Korman NJ, Shimizu H, Eady RAJ, Klaus-Kovtun V, Cehrs K, Stanley, JR: Production of rabbit antibodies against carboxy-terminal epitopes encoded by bullous pemphigoid cDNA. J Invest Dermatol 1990;94:617-623.

Stanley JR, Is pemphigus an anti-adhering junction-autoimmune disease? J Dermatol Sci (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01-03668-02 D

PERIOD COVERED

October 1, 1989 to June 30, 1990

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

Molecular and Cell Biology of Immunoreactive Molecules in the Skin

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

P.I.: S. Wright Caughman, M.D., Dermatology Branch, DCBD, NCI

Other: Klaus Degitz, M.D., Guest Researcher, Dermatology Branch, DCBD, NCI
Lian-Jie Li, M.D., Visiting Fellow, Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(if any)*

Cell biology and Metabolism Branch, NICHD, NIH
European Molecular Biology Laboratories, Heidelberg, W. Germany
Department of Dermatology, USUHS, Bethesda, MD

LAB/BRANCH

Dermatology Branch

SECTION

NCI, NIH, Bethesda, MD 20892

INSTITUTE AND LOCATION

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 3 | 3 | 0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

ICAM-1 is a cell surface glycoprotein that serves as the major ligand for LFA-1, an anchoring receptor on all T cells whose interaction with ligand is critical for proper T-cell function. The regulation of ICAM-1 expression is critically important in both normal inflammatory and pathologic processes. We have established the kinetics and molecular regulatory level for cytokine-induced de novo ICAM-1 expression by human keratinocytes (HK) in culture. We have established an in vitro leukocyte adhesion assay for resting and blast T cells to cultured HK and have demonstrated that the marked increase in adhesion of T cells to IFN-$\gamma$-treated HK is primarily due to the induction of HK ICAM-1 expression. We have further explored the regulatory mechanisms of ICAM-1 gene expression and shown that ICAM-1 expression in HK is induced by short-term exposure to cycloheximide, implicating the existence of a trans-acting protein with a short half-life that is involved in the constitutive repression of ICAM-1 in HK. We have shown that the kinetics for IFN-$\gamma$-induced expression of HLA-DR$\beta$ by HK (DR) is different from those for ICAM-1. Additionally, TGF-$\beta$ enhances the induced expression of ICAM-1 and suppresses the induced expression of DR. We have isolated a human genomic clone containing the 5' flanking transcriptional regulatory regions of ICAM-1, which includes consensus sequences for promoter and enhancer elements, and have established the transcription start site by primer extension and S1 nuclease protection assays. A 282 bp fragment including the cap site at its 3' end is a potent functional promoter and can drive the expression of a heterologous reporter gene to a degree comparable to the SV40 promoter and enhancer. We are currently analyzing additional 5' flanking and intronal regions for repressor and enhancer functions as well as for consensus and functional elements for cytokine induced transcriptional activation.



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

Projection Description

Major Findings:

An in vitro leukocyte-HK adhesion assay has been established, and has been utilized to demonstrate that the IFN-$\gamma$-induced upregulation of T cell binding to HK is due to de novo expression of ICAM-1 by HK.

Exposure of HK to cycloheximide induces the expression of ICAM-1 mRNA, implicating the existence of a trans-acting protein with a short half-life that is involved in the constitutive repression of ICAM-1 in HK.

IFN-$\gamma$-induced expression of ICAM-1 and DR follow different kinetics. Furthermore, TGF-$\beta$ differential modulates the expression of these two IFN-$\gamma$-inducible genes in that ICAM-1 expression at the level of both mRNA and surface protein is enhanced, while DR expression is suppressed.

The transcriptional regulatory region of the human ICAM-1 gene has been cloned and characterized:

A 17.4 kb genomic lambda phage clone has been isolated, restriction mapped and restriction fragments subcloned.

Multiple subclones have been sequenced and analyzed, including a 2.05 kb subclone containing the first exon and 1350 bp of the 5' flanking region.

The transcription start site (cap site) has been established by primer extension and S1 nuclease protection assays and is located 40 bp 5' of the translation start codon of the first exon.

A promoter has been identified within the first 275 bp 5' of the cap site of the ICAM-1 gene and has been demonstrated to posses potent promoter function in a heterologous reporter gene construct and transient transfection and expression system.

Collaborative studies with Mattheus Hentze, EMBL, and RD Klausner and JB Harford, CBMB, NICHHD, have continued. While the regulatory IRE-binding protein can bind to IRE structures located more 3' from the cap site than is normally found in most characterized ferritin cDNAs, it appears that translational regulation that is dependent upon IRE function may require the IRE to be located within a certain distance of the cap site.

Collaborative studies with Kin B. Yancey, Department of Dermatology, USUHS, have demonstrated that human keratinocytes and A431 cells, a human squamous cell carcinoma cell line, synthesize and secrete the complement components C3 and factor $\beta$ that are identical to those expressed by reference HepG2 cells. Keratinocytes thus may serve as a previously unrecognized source of pro-inflammatory complement components.

Publications:

Caughman SW, Li L-J, Degitz K. Characterization and functional analysis of interferon-$\gamma$-induced intercellular adhesion molecule-1 in human keratinocytes and A431 cells. J Invest Dermatol (in press).

Degitz K, Caughman SW. The T cell antigen receptor. Dermatologic Clinics (in press).

Shimada S, Caughman SW, Blueston 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 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 03669-01 D

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (90 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: Aimin Tang, M.D., Visiting Fellow, Dermatology Branch, DCBDC, NCI

COOPERATING UNITS (if any)
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LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 2.83 | 1.83 | 1.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The major focus of this laboratory has been to determine how ultraviolet B (UV-B) radiation modulates the epidermal immune system. We have found that low doses of UV-B radiation administered to epidermal cells (EC) in vitro completely inhibit the ability of freshly isolated Langerhans cells (fLC) to support the mitogenic response of T cells to anti CD3 antibodies. This inhibitory effect appears to result from the direct action of UV-B on LC and is not due to cytotoxicity. LC that have been cultured for 24-72 hours (cLC) become progressively more resistant to UV-B radiation, an observation which correlates with the progressive increase in the expression of the intercellular adhesion molecule ICAM-1 by cLC. We have found that anti-murine ICAM-1 antibodies inhibit the proliferative response of T cells to anti CD3 antibodies supported by fLC or cLC, confirming that the ICAM-1/LFA-1 interaction is critical in this system. We have also found that exposure of fLC to low doses of UV-B radiation in vitro prevents them from expressing increased levels of ICAM-1 after a 24 hours culture period. Finally, irradiated fLC do not form clusters with T cells in response to anti CD3 suggesting that ICAM-1 is functionally deficient as well. We are also studying the effect of UV-B radiation on protein antigen processing in a model system where expression of costimulatory molecules (eg. ICAM-1) is unimportant. In collaborative studies, we are examining the accessory cell activity of EC from essential fatty acid (EFA) deficient mice. EC from EFA deficient mice are better stimulators of T cell responses to protein antigens and alloantigens than EC from normal mice. LC number and class II antigen expression by LC is unaltered in EFA deficient mice, as is cytokine production. The most striking difference between EC from EFA deficient and normal mice is that some EFA deficient keratinocytes (KC) express high levels of class II antigens. We have suggested that EFA deficient KC serve as "costimulators" of LC dependent T cell responses.

Project Description

Major Findings:

Exposure of animals and human beings to levels of ultraviolet B (UV-B) radiation which can be easily achieved through recreational or occupational sun exposure alters immune function within the epidermis and in some cases systemically. These perturbations probably result from direct effects of UV-B radiation on immunocompetent cells residing in the epidermis and perhaps also from the release of eicosanoids, cytokines and other immunomodulatory factors from keratinocytes (KC) in response to UV-B. These soluble mediators may act locally or extracutaneously. The major focus of this laboratory is in trying to determine precisely how UV-B radiation inhibits the accessory cell function of the epidermal Langerhans cell (LC).

The proliferative response of unprimed murine splenic T cells which have been exhaustively depleted of accessory cells to anti-T cell receptor antibodies (anti-CD3 antibodies) can be reconstituted by adding back small numbers of LC (or epidermal cells (EC) containing LC). We have found that exposure of EC to low doses of UV-B radiation ($\leq$ 200 $J/M^2$) in vitro completely inhibits the ability of LC to support T cell proliferation in this assay. This inhibition appears to result from a direct effect of UV-B on LC because 1) purified LC (> 50% LC) are inhibited by UV-B, 2) the addition of UV-B treated KC to purified LC does not inhibit LC function and 3) culture supernatants harvested from UV-B irradiated EC are not suppressive in the proliferation assay when unirradiated EC are used as a source of accessory cells. In addition, the effect of UV-B on LC does not appear to result from simple cytoxicity.

We have observed that LC which have been cultured in vitro for 24-72 hours undergo striking surface phenotypic and functional alterations and simultaneously become resistant to the effects of low dose UV-B radiation. The level of UV-B resistance and the rate of its acquisition correlates with the increased expression of the adhesion molecule ICAM-1 on the surface of cultured LC (cLC) as assessed by flow cytometry. The ability of monoclonal anti-murine ICAM-1 antibodies (in addition to anti LFA-1 and anti Fc receptor antibodies) to block the proliferative response of T cells to anti-CD3 antibodies when freshly isolated LC (fLC) or cLC are used as accessory cells suggests that modulation of ICAM-1 levels on LC by UV-B radiation may explain the inhibitory capacity of UV-B in this system.

We have found that UV-B irradiated LC express several-fold lower levels of cell surface ICAM-1 than do unirradiated LC after a 24 hour culture period. The observation that UV-B irradiated LC are unable to serve as the nidus for the large T cell - LC clusters that typically form during the course of the assay is also consistent with a decreased level of functional ICAM-1 expression on UV-B irradiated LC. Using murine or perhaps human macrophage cell lines as a model for LC and molecular biologic techniques, we hope ultimately to determine exactly how UV-B radiation interferes with the expression of this important intercellular adhesion molecule.

Previous studies have also suggested that UV-B radiation inhibits the processing of protein antigens, but they are not definitive (perhaps in part because of the effects of UV-B radiation on adhesion molecule expression detailed above). To address this question we are utilizing a murine T cell hybridoma (Hd-1) specific for a small, well characterized protein antigen (hen egg lysozyme; HEL) as a responding T cell and an MHC matched B cell lymphoma (A20) and hybridoma cells (LK) in addition to EC as antigen presenting cells (APC). Activation of Hd-1 cells is determined by assaying the amount of lymphokine produced utilizing an IL-2 dependent T cell clone (CTLL). Because T cell hybridomas can be triggered by processed antigen associated with the relevant class II antigen (in absence of any other costimulatory signals) and a peptide containing the HEL determinant recognized by the Hd-1 cells has been synthesized, effects of UV-B on antigen processing can be studied in isolation from those on such things as cytokine production or adhesion molecule expression.

In preliminary experiments, we have found that low levels of UV-B radiation (eg. 200 $J/m^2$) inhibit A20 proliferation and are ultimately cytotoxic. Despite this, antigen processing does not appear to be inhibited by UV-B radiation in experiments which employ paraformaldehyde (PFA)-fixed A20 cells (which because they are fixed are not sensitive to the delayed cytotoxic effects of UV-B radiation) as antigen presenting cells. Irradiated antigen-pulsed PFA-fixed A20 cells stimulate Hd-1 cells to produce IL-2 as well as identically treated unirradiated A20 cells, irrespective of whether exposure to antigen occurs before or after UV-B exposure. Similar experiments utilizing EC as antigen presenting cells are in progress.

In collaboration with Dr. James Lefkowith at Washington University School of Medicine, we are continuing studies of antigen presenting cells in the epidermis of essential fatty acid (EFA) deficient mice. We have found that EC from EFA deficient mice are several-fold better stimulators of accessory cell dependent protein antigen and alloantigen specific T cell proliferative responses than EC from age and sex matched mice fed a normal diet. These differences are not due to increased numbers of LC or increased levels of class II antigens on LC and do not appear to result from alterations in cytokine production by EFA deficient EC. A striking difference between EC from EFA deficient and normal mice is the expression of relatively high levels of class II antigens by a subpopulation of EFA deficient KC (up to 30% of all KC). KC from normal animals are devoid of class II antigens. We have suggested that EFA deficient KC serve as more effective "costimulators" of T cell responses than normal EC, perhaps because they express high levels of class II antigens or other surface molecules (ICAM-1 for example), but have not yet ruled out the possibility that EFA deficient LC themselves have enhanced APC activity.

ANNUAL REPORT OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1989 through September 30, 1990

The clinical research program of the Metabolism Branch is directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. In this area, a broad range of immunological investigations are carried out in patients with immunodeficiency diseases that are associated with a high incidence of neoplasia, as well as in patients with malignancy, especially T- and B-cell leukemias. These studies are directed toward defining the factors involved in the control of the human immune response. Major efforts in this area are directed toward: 1) studies of the arrangement of the immunoglobulin genes and T-cell antigen-specific receptor genes and the rearrangements and deletions of these genes that are involved in the control of the synthesis of immunoglobulins and T-cell receptors; one focus in this area is the study of novel transforming genes that translocate into the T-cell receptor locus in T-cell tumors; 2) the characterization of transacting regulatory factors that mediate lymphocyte specific gene transcription; the scientific focus of this area is the purification of the transactivating factors, the cloning of the genes encoding these factors and the definition of their mode of action at a molecular level; 3) somatic gene therapy for human genetic immunodeficiency diseases; 4) genetic control of the immune response; one emphasis of this area is the development of a novel method for predicting molecular structures recognized by T-cells and the applications of this algorithm to the development of vaccines aimed at preventing AIDS and malaria; 5) identification, purification, and molecular genetic analysis of the multichain interleukin-2 receptor on normal and malignant lymphocytes; one emphasis of this area is the development of different forms of IL-2 receptor directed therapy; 6) analysis of action of immunoregulatory cells including helper T cells, suppressor T cells, and macrophages that regulate antibody responses and on studies of disorders of these immunoregulatory cell interactions and of leukemias of these immunoregulatory cells; and 7) isolation and characterization of biological response modifiers and oligosaccharides that regulate the human immune response. The second major goal of the Metabolism Branch is to determine the physiological and biochemical effects that a tumor produces on the metabolism of the host. Both patients with neoplastic diseases as well as those with nonneoplastic disorders that facilitate the development of techniques for the study of cell membranes, homeostatic mechanisms, and metabolic derangements of biochemical control mechanisms are being investigated. Within this area, one special emphasis is placed on the normal growth factors especially insulin-like growth factors that play a role in the hormonal control of normal and malignant growth.

## MOLECULAR ANALYSIS OF TRANSLOCATIONS OF TRANSFORMING GENES TO THE LOCUS OF T-CELL ANTIGEN RECEPTOR GENES

Dr. Waldmann has exploited illegitimate chromosomal translocations in T cell leukemia involving the T cell receptor genes to clone elements involved in the regulation of normal and malignant cell proliferation. Dr. Glenn Begley and Dr. Waldmann, in collaboration with Dr. Ilan Kirsch, has identified a putative

transforming gene which they call SCL-1 that is expressed in hematopoietic tissues. The gene was identified through the molecular cloning of a translocation between chromosomes 1 and 14;t(1:14)(p33;q11) that occurred in a human stem cell leukemia that was able to differentiate into myeloid and lymphoid lineages. As a consequence of the translocation, a novel fusion transcript was generated between the sequences on chromosome 1 and chromosome 14. The gene on chromosome 1 manifested a DNA binding motif shared with the Drosophila daughterless gene and the myc and MyoD proteins. This gene was expressed in fetal liver, normal regenerating bone marrow, and CD34-positive and CD33-negative bone marrow stem cells, and may be important for hematopoietic stem cell development and oncogenesis.

MOLECULAR ANALYSIS OF TRANS-ACTING FACTORS THAT MEDIATE LYMPHOID SPECIFIC GENE TRANSCRIPTION

As an approach to understanding development in the B lymphocyte lineage, Dr. Louis Staudt has characterized trans-acting factors that mediate lymphoid-specific gene transcription. As a model he has studied the lymphoid-specific transcription of immunoglobulin (Ig) genes. Oct-2 is a transcription factor expressed in B lymphocytes which is critical for the lymphoid-specific expression of immunoglobulin genes. Dr. Staudt's studies on Oct-2 in the past year have focused on his observation that Oct-2, in addition to being expressed in all B lymphocytes, is also expressed in some T lymphocytes, myeloid cells and in the fetal spinal cord. In T lymphocytes, Oct-2 is induced during activation of T lymphocytes by a variety of stimuli including cognate antigen. These findings suggest that Oct-2 controls gene expression in other cells besides B lymphocytes and must therefore cooperate with other B cell restricted transcription factors to fully account for lymphoid-specific gene expression.

The molecular cloning of Oct-2 helped to identify a new class of transcription factors that share two structural domains, a homeobox and POUbox. Dr. Staudt has molecularly cloned and characterized a new member of this POU-homeobox family, termed Oct-3. Oct-3 binds to the same DNA motif as Oct-2, the octamer, but 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 cells of the germ line including primordial germ cells and oocytes. These findings place Oct-3 in marked contrast with previously studied homeobox genes which are not expressed in pluripotent stem cells or in the germ line but rather are expressed in cells that have differentiated along one of the somatic lineages. Oct-3, therefore, may be important for maintaining the pluripotent state and for committing a stem cell to the germ line lineage.

SOMATIC GENE THERAPY FOR HUMAN GENETIC DISEASE

Dr. Michael Blaese has continued his research program to develop gene transfer for the therapy of human disease. He and his collaborators, W. French Anderson and Steven A. Rosenberg, have now performed the first authorized clinical experiment in which a foreign gene has been introduced into man. A recombinant murine retroviral vector containing the bacterial gene encoding Neomycin resistance (neoR) was used to label tumor infiltrating lymphocytes (TIL) used in the treatment of patients with malignant melanoma. These neoR-expressing TIL were reinfused into the patients to permit Dr. Blaese and his associates to study the survival and trafficking patterns of TIL in an attempt to determine whether they can identify

any characteristics which correlate with the clinical efficacy of the TIL in inducing tumor remission. The next phase of these studies will involve the insertion of genes encoding various cytokines into TIL in an attempt to improve their tumoricidal activity for a variety of tumors. Dr. Blaese's laboratory has prepared vector constructs with the genes for various cytokines including IL-1$\beta$, TNF and IL-6. Murine TIL expressing the introduced gene for IL-1$\beta$, for example, were shown to have a 70-fold enhancement in tumor cell lysis in vitro. The growth of tumor cells transduced to express these genes was greatly inhibited in normal animals but was unaffected in sub-lethally irradiated animals suggesting that recruitment of host effector elements by the cytokine-producing tumors is an important factor in their anti-tumor effect in vivo. Major studies of the biological effects of cells constitutively secreting these molecules are also underway as are studies of the potential utility of using lymphocytes as cellular vehicles for gene therapy of non-malignant diseases such as ADA deficiency SCID and hemophilia. Non-transformed peripheral blood T cells from ADA deficient SCID patients have been grown to high numbers using anti-CD3 and rIL-2 stimulation and the human ADA gene has been successfully introduced into these cells. A clinical protocol for the treatment of ADA(-)SCID with gene-treated cultured T cells has now been approved by the Human Gene Therapy Subcommittee of the Recombinant DNA Advisory Committee. With the expected early approval by two additional Federal regulatory agencies, the first use of gene transfer for the therapy of human disease could begin as early as this autumn.

Dr. Blaese has also continued his work with the compound Succinylacetone (SA) which he, Richard Hess and Donald Tschudy discovered to have potent immunosuppressive properties. The most recent work with this drug has involved studies of solid organ and bone marrow transplantation in rats, pigs, primates and dogs. SA has now been demonstrated to greatly prolong skin allograft survival in rats and to permit indefinite survival of totally mismatched cardiac allografts in rats. Studies of heterotopic cardiac allografts in rhesus and cynomolgus monkeys have shown very significant graft prolongation in these animals as well. In dogs receiving total allogeneic bone marrow transplantation, Reiner Storb at the Fred Hutchinson Cancer Center has demonstrated that SA is the most effective single immunosuppressive agent so far tested in preventing lethal graft versus host disease.

THE GENETIC CONTROL OF THE IMMUNE RESPONSE: APPLICATION TO THE DESIGN OF SYNTHETIC VACCINES

Dr. Jay Berzofsky has studied the mechanisms by which T cells recognize antigens on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, and the application of these principles to the design of synthetic vaccines for AIDS and malaria. Using a large series of variant peptides with single amino acid substitutions to identify residues that interact with the MHC molecule or with the T-cell receptor, he found that a peptide can bind to the same MHC molecule in more than one position or conformation, indicating that the role of a given amino acid residue in binding MHC cannot be defined independently of the T cell involved. In the case of HIV, Dr. Berzofsky found that the same peptide can also bind to both class I and class II MHC molecules for recognition by CD8 cytotoxic T lymphocytes (CTL) and CD4 helper T cells, respectively. Thus, this peptide can provide help for induction of CTL to itself. He has mapped the residues responsible for this peptide to interact with class I MHC molecules and with CTL, and are doing so for class II MHC molecules. A single amino acid seems to contribute most of the energy responsible for the specificity

differences between non-crossreactive CTL specific for different HIV isolates. This peptide is also recognized by CTL from HIV-infected and uninfected immunized human volunteers, in association with two common human HLA types, A2 and A3, making it broadly useful in a vaccine. Dr. Berzofsky also identified a conserved CTL epitope in the HIV-1 reverse transcriptase recognized by CD8 cells from both mice and 5 of 12 HIV-infected humans. Thus, CTL epitopes immunodominant for murine T cells are often seen by human CTL, making the mouse a useful model to identify CTL epitopes useful in humans. The finding that both this site and the envelope CTL site are recognized by CTL and also helper cells from mice or the same MHC type may contribute to their immunodominance. He has identified multideterminant helper T-cell regions of the HIV envelope that are broadly recognized by mice and humans also. These may be useful in diagnosis of exposed individuals who are still seronegative, as well as in vaccine development. Dr. Berzofsky has shown that some of the helper peptides can elicit help for an enhanced antibody response to HIV envelope in monkeys the first time the monkeys are exposed to a suboptimal dose of whole envelope protein, and that humans immunized with a recombinant gp160 vaccine make much higher levels of IL-2 in response to these peptides than do HIV-infected individuals. Thus, vaccine induced immunity can exceed that produced by natural infection. Dr. Berzofsky has developed a way to get purified nonliving subunit vaccines, which do not normally induce CD8 CTL, to do so reproducibly by incorporating them into ISCOMs. This may make it possible for the first time for subunit vaccines to induce all 3 types of specific immunity, CTL as well as helper T cells and antibodies. Finally, he has identified an immunodominant CTL epitope from a mouse malaria, and has shown that CTL specific for this peptide can eliminate malaria parasites from infected liver cells.

THE MULTI-CHAIN IL-2 RECEPTOR: MOLECULAR CHARACTERIZATION AND USE AS A TARGET FOR IMMUNOTHERAPY

Antigen stimulated activation of resting T cells induces the synthesis of interleukin-2 as well as high affinity IL-2 receptors that are not expressed on resting T cells. Dr. Waldmann previously identified two peptides that bind IL-2, the 55 kD protein reactive with the anti-Tac monoclonal antibody and a novel 70/75 kD (p75) protein reactive with a monoclonal antibody termed Mik$\beta$1. Dr. Waldmann proposed a multichain model for the high affinity receptor in which both p55 $\alpha$ and the p75 $\beta$ IL-2-binding proteins are associated in a receptor complex. The addition of the anti-Tac antibody inhibits IL-2-induced proliferation of IL-2 dependent malignant T-cell lines bearing the high affinity receptor. In contrast, the addition of Mik$\beta$1, an antibody that blocks IL-2 binding to the p75 $\beta$ chain was relatively ineffective in inhibiting IL-2 induced proliferation of such cells. However, when both anti-IL-2R$\alpha$ and IL-2R$\beta$ chain antibodies were present, IL-2 induced proliferation was almost completely abolished. Furthermore, the addition of an antibody that blocks the interaction of the IL-2 alpha and beta chains complements the anti-IL-2R$\beta$ chain antibodies in inhibiting IL-2 induced proliferation. From these and other observations, a hierarchial model of IL-2 action can be proposed for the high affinity receptor in which IL-2 must initially interact with the p55 $\alpha$ protein with subsequent communication of the IL-2 signal from the IL-2 p55 complex to the p75 $\alpha$ chain. Previously using flow cytometric resonance energy transfer and lateral diffusion measurements, Dr. Waldmann demonstrated that a 95 kD protein identified by two monoclonal antibodies interacts physically with the 55 kD/IL-2R$\alpha$ protein of the high-affinity IL-2 receptor. Over the past year, in conjunction with Dr. Jack Burton, he purified the p95 protein and defined its amino acid sequence and thus showed that this protein is ICAM-1. This association of ICAM-1 with the IL-2 receptor may facilitate the paracrine IL-2-

mediated stimulation of T cells expressing IL-2 receptors by augmenting homotypic T/T cell interaction, by receptor-directed focusing of IL-2 release by helper T cells, and by focusing IL-2 receptors of the physically linked cells to the site of LFA-1/ICAM-1/IL-2 receptor interaction.

A state of T cell activation reflected by a marked degree of spontaneous proliferation in vitro exists among patients with HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP). Dr. Waldmann wished to define the mechanism by which the circulating cells from HAM/TSP are driven, thus gaining an insight into the pathogenesis of this HTLV-I associated disease. Using a modification of the polymerase chain reaction, Drs. Craig Tendler, Steven Greenberg and Waldmann demonstrated that the circulating mononuclear cells of such patients express the HTLV-I transactivator gene PX. There was an up-regulation of IL-2 and IL-2Rα transcripts in HAM/TSP that paralleled the coordinate mRNA expression of the PX transactivator. In addition, IL-2 and IL-2Rα serum levels in this disorder were elevated. Finally, the marked degree of in vitro spontaneous proliferation in HAM/TSP was profoundly inhibited by specific anti-IL-2 receptor or anti-IL-2 blocking antibodies. Collectively these results suggest that immune activation in HAM/TSP is virally driven by the transactivation and coordinate expression of IL-2 and the IL-2Rα protein. This deregulated autocrine process may contribute to the development of inflammatory nervous system damage in HAM/TSP.

One of Dr. Waldmann's most critical contributions was his recognition that the IL-2 receptor represents an extraordinarily versatile therapeutic target. He entered this area to exploit his observations that T cells in patients with certain lymphoid malignancies and select autoimmune disorders as well as individuals rejecting allografts express the IL-2 receptors identified by the anti-Tac monoclonal antibody whereas normal resting cells and their precursors do not. He reasoned that agents that could eliminate Tac-expressing leukemic cells or activated T cells involved in other disease states could be effective therapy for these disorders yet would retain the Tac negative mature, normal T cells and their precursors. Dr. Waldmann and his coworkers have developed a variety of therapeutic reagents. Over the past year Dr. Waldmann has extended the use of the unmodified anti-Tac monoclonal antibody in the treatment of patients with adult T cell leukemia. This therapy has had no toxic complications. Six of sixteen patients underwent remissions, in four cases complete, lasting from one to over eleven months. In addition to their use in the therapy of patients with adult T cell leukemia, IL-2 receptor directed antibodies were evaluated in organ allograft protocols. The survival of cardiac and renal allografts was prolonged in cynomolgus monkeys treated with anti-Tac. In light of these encouraging results, in a randomized prospective trial, anti-Tac was added to the treatment of one of two patient groups of 40 patients each following renal transplantation who received otherwise identical immunosuppression. There was no increased toxicity associated with the administration of anti-Tac but there was a significant reduction of early rejection episodes following renal transplantation. In order to improve the effectiveness of IL-2 receptor directed therapy, cytotoxic agents were developed that could be conjugated to anti-Tac and were effective when bound to the surface of Tac-expressing cells. Specifically in conjunction with Dr. Otto Gansow, Dr. Waldmann showed using in vitro assays and in vivo cardiac allograft and xenograft models that $^{212}Bi$, an alpha-emitting or $^{90}Y$, a beta-emitting radionuclide conjugated to anti-Tac by use of bifunctional chelates were effective and specific immunocytotoxic agents for the elimination of Tac expressing cells. The administration to cynomolgus monkeys of $^{90}Y$ anti-Tac led to a marked prolongation of the survival of both cardiac xenografts and allografts when compared with their

survival in untreated animals or in animals treated with unmodified anti-Tac. The use of mouse anti-Tac is hindered by the host immune response to the murine antibody and by the lack of effector cytotoxic capacity of the monoclonal. Therefore in conjunction with Dr. Cary Queen, Dr. Waldmann and his coworkers have constructed a "humanized" anti-Tac antibody by placing the complementarity determining regions of the murine anti-Tac antibody into the human variable region framework and IgG1 constant regions. It is hoped that these "humanized" antibodies will be minimally immunogenic. They retained high binding affinity and were very effective in inhibiting IL-2 mediated events. Furthermore, they added the new functional activity of antibody dependent cellular cytoxicity that was absent in the parent mouse anti-Tac. With this new ADCC capacity it is hoped that there will be a substantial improvement in the performance of the antibody in vivo that should translate into an increase in efficacy in therapy of T cell leukemia/lymphoma. In this regard, Dr. Waldmann observed that "humanized" hyperchimeric anti-Tac prolonged cardiac allograft survival in cynomolgus monkeys. The graft survival observed in the group treated with "humanized" anti-Tac was significantly prolonged over that of the group treated with murine anti-Tac. No toxicity was observed. Thus "humanized" anti-Tac provides a versatile therapeutic reagent with possible applications in adult T cell leukemia and other malignancies and in allograft, graft versus host, and autoimmune settings. Thus the clinical application of IL-2 receptor directed therapy represents a new perspective for the treatment of certain neoplastic diseases and autoimmune disorders and for the prevention of allograft rejection.

Dr. David Nelson has identified a soluble form of the p55 component (Tac protein) of the human interleukin-2 receptor in the supernatants of activated T cells, B cells, and monocytes in vitro and in the serum of normal individuals in vivo. Elevated levels of soluble Tac protein were found in the sera of patients with human lymphoproliferative disorders including the adult T cell leukemia (ATL), hairy cell leukemia (HCL), non-Hodgkin's lymphoma and the acquired immunodeficiency syndrome (AIDS). In ATL and HCL patients, the serum level of Tac protein was indicative of tumor burden and favorable responses to therapy were associated with reductions in the serum level of Tac protein. Elevations of Tac protein in serum were also indicative of allograft rejection episodes in patients with liver and heart-lung transplants. Patients with autoimmune diseases and other immune disorders of unknown etiology (sarcoidosis, Kawasaki disease) also had elevated levels of serum Tac protein. The measurement of soluble Tac protein in various body fluids is useful in monitoring certain neoplastic and immune-mediated events in vivo.

## ISOLATION AND CHARACTERIZATION OF BIOLOGICAL RESPONSE MODIFIERS AND OLIGOSACCHARIDES THAT REGULATE THE HUMAN IMMUNE RESPONSE

Dr. Andrew Muchmore continues to pursue two basic interests. The first concerns regulation of the immune response during pregnancy and the second concerns the characterization of the role that carbohydrate structures may play in intracellular and intercellular communication. Based on his studies with Uromodulin which he purified, characterized and ultimately molecularly cloned from human 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 activity. Dr. Muchmore 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. His primary aim is to characterize the minimal structure required for biologic activity. The second goal 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. These compounds appear to directly regulate gene transcription and are able to modulate the affinity and activity of transcriptionally regulatory proteins including the oncogene products c-fos and c-jun. These studies point the way towards 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.

## INSULIN-LIKE GROWTH FACTOR (IGF-I AND IGF-II) RECEPTORS

Dr. Peter Nissley previously reported that the tissue insulin-like growth factor-II/mannose 6-phosphate receptor was high in fetal tissues and then declined sharply in postnatal tissues. In order to determine whether receptor expression reflected mRNA levels, he measured mRNA concentration in tissues from 20-day gestation fetuses and from 20-day postnatal animals. Total RNA was analyzed by Northern blotting using as a probe the rat IGF-II/Man-6-P receptor cDNA clone K3 kindly provided by Drs. David Morgan and William Rutter. A major mRNA band of 9.2 kilobases was found in all tissues. By Northern blotting, fetal tissues contained greater amounts of receptor mRNA than corresponding postnatal tissues. To provide a more quantitative comparison between fetal and postnatal tissues, Dr. Nissley employed a solution hybridization/RNAase protection assay. The rank order of mRNA concentrations in fetal tissues corresponded to the rank order for expression of the receptor protein. There was a decline of mRNA concentration in the postnatal tissues which ranged from 14-fold for heart to 2-fold for lung. These results suggest that the production of IGF-II/Man-6-P receptor is controlled at the level of transcription and/or mRNA degradation in most tissues. Immunoprecipitation of growth factor receptors has been used successfully to identify receptor-associated proteins. These proteins are candidates for being important components in the signaling pathway following binding of ligand to the receptor. In order to employ this immunoprecipitation technique on the IGF-I receptor, Dr. Nissley first set out to develop monoclonal antibodies directed against this receptor. He purified the IGF-I receptor from human placenta by successive affinity chromatography steps on a wheat germ lectin affinity column, an insulin-Sepharose column (to exclude insulin receptors), and a receptor antibody (alpha IL-3) affinity column. The purified receptor was used to immunize mice. Two of six mice developed serum antibodies and spleens from these animals were used to develop hybridomas. Positive hybridomas were identified by ligand blocking and immunoprecipitating assays. These hybridomas have been cloned, expanded and injected into mice to produce ascites fluids. These fluids and purified IgGs from the fluids are being screened for ability to immunoprecipitate the receptor from metabolically labeled cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB-04002-21

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Defects in Immunoregulatory Cell Interactions in Patients with Immune Dysfunctions

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

| | | | |
|---|---|---|---|
| PI: | Thomas A. Waldmann, M.D. | Branch Chief | MET, NCI |
| | Claude Kasten-Sportes, M.D. | Medical Staff Fellow | MET, NCI |
| | Richard P. Junghans, M.D. | Medical Staff Fellow | MB, DCT, NCI |
| | Erich Roessler, M.D., Ph.D. | Medical Staff Fellow | MET, NCI |
| | Steven J. Greenberg, M.D. | Biotechnology Fellow | MET, NCI |
| | Jack Burton, M.D. | Biotechnology Fellow | MET, NCI |
| | Angus Grant, Ph.D. | Biotechnology Fellow | MET, NCI |

COOPERATING UNITS *(if any)*

Laboratory of Molecular Biology, NCI
Radiation Oncology Branch, NCI

LAB/BRANCH
Metabolism Branch

(More professional personnel listed on next page)

SECTION

INSTITUTE AND LOCATION
DCBDC, NCI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS: | PROFESSIONAL | OTHER |
|---|---|---|
| 11 | 9 | 2 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors "B" 100%

☐ (a2) Interviews

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

Resting T cells do not express high-affinity IL-2 receptors, but receptors are rapidly expressed on T cells after activation with antigen or mitogen. We have identified two peptides that bind IL-2, the 55 kD peptide reactive with the anti-Tac monoclonal and a novel 70/75 kD (p75) peptide. We have proposed a multichain model for the high-affinity IL-2 receptor in which both the p55 Tac and the p75 IL-2 binding peptide are associated in the receptor complex. Using crosslinking, coprecipitation, and fluorescence energy transfer techniques, additional p22, p35, p40, p95, and p180 peptides have been identified that may be associated with the 55 kD Tac peptide in the multichain receptor. T cells in patients with certain lymphoid malignancies, select autoimmune disorders as well as individuals rejecting allografts express the IL-2 receptors identified by the anti-Tac monoclonal antibody whereas normal resting cells do not. To exploit this differential expression, a wide variety of IL-2 receptor-directed therapeutic reagents have been generated including unmodified anti-Tac, toxin conjugates of anti-Tac, radioisotopic chelates of anti-Tac as well as hyperchimeric "humanized" anti-Tac antibodies that are entirely human except for the small hypervariable segments from the mouse antibody. The clinical application of these highly potent IL-2-receptor directed agents represents a new therapeutic perspective for certain neoplastic diseases, autoimmune disorders, and for the prevention of allograft rejection.



PHS 6040 (Rev. 1/84) GPO 904-917

Continuation of Professional Personnel for PHS 6040

| | | |
|---|---|---|
| Craig Tendler, M.D. | Guest Researcher | MET, NCI |
| Roger Garcia, M.D. | Guest Researcher | MET, NCI |
| Verena Bier, M.D. | Fogarty Fellow | MET, NCI |

## Project Description

Major Findings:

Dr. Waldmann has exploited illegitimate chromosomal translocations in T cell leukemia involving the T cell receptor genes to clone elements involved in the regulation of normal and malignant cell proliferation. Dr. Glenn Begley and Dr. Waldmann, in collaboration with Dr. Ilan Kirsch, has identified a putative transforming gene which they call SCL-1 that is expresed in hematopoietic tissues. The gene was identified through the molecular cloning of a translocation between chromosomes 1 and 14;t(1:14)(p33;q11) that occurred in a human stem cell leukemia that was able to differentiate into myeloid and lymphoid lineages. As a consequence of the translocation, a novel fusion transcript was generated between the sequences on chromosome 1 and chromosome 14. The gene on chromosome 1 manifested a DNA binding motif shared with the Drosophila daughterless gene and the myc and MyoD proteins. This gene was expressed in fetal liver, normal regenerating bone marrow, and CD34-positive and CD33-negative bone marrow stem cells, and may be important for hematopoietic stem cell development and oncogenesis.

Antigen stimulated activation of resting T cells induces the synthesis of interleukin-2 as well as high affinity IL-2 receptors that are not expressed on resting T cells. Dr. Waldmann previously identified two peptides that bind IL-2, the 55 kD protein reactive with the anti-Tac monoclonal antibody and a novel 70/75 kD (p75) protein reactive with a monoclonal antibody termed Mik$\beta$1. Dr. Waldmann proposed a multichain model for the high affinity receptor in which both p55 $\alpha$ and the p75 $\alpha$ IL-2-binding proteins are associated in a receptor complex. The addition of the anti-Tac antibody inhibits IL-2-induced proliferation of IL-2 dependent malignant T-cell lines bearing the high affinity receptor. In contrast, the addition of Mik$\beta$1, an antibody that blocks IL-2 binding to the p75 $\beta$ chain was relatively ineffective in inhibiting IL-2 induced proliferation of such cells. However, when both anti-IL-2R$\alpha$ and IL-2R$\beta$ chain antibodies were present, IL-2 induced proliferation was almost completely abolished. Furthermore, the addition of an antibody that blocks the interaction of the IL-2 alpha and beta chains complements the anti-IL-2R$\beta$ chain antibodies in inhibiting IL-2 induced proliferation. From these and other observations, a hierarchial model of IL-2 action can be proposed for the high affinity receptor in which IL-2 must initially interact with the p55 $\alpha$ protein with subsequent communication of the IL-2 signal from the IL-2 p55 complex to the p75 $\beta$ chain. Previously using flow cytometric resonance energy transfer and lateral diffusion measurements, Dr. Waldmann demonstrated that a 95 kD protein identified by two monoclonal antibodies interacts physically with the 55 kD/IL-2R$\alpha$ protein of the high-affinity IL-2 receptor. Over the past year, in conjunction with Dr. Jack Burton, he purified the p95 protein and defined its amino acid sequence and thus showed that this protein is ICAM-1. This association of ICAM-1 with the IL-2 receptor may facilitate the paracrine IL-2-

mediated stimulation of T cells expressing IL-2 receptors by augmenting homotypic T/T cell interaction, by receptor-directed focusing of IL-2 release by helper T cells, and by focusing IL-2 receptors of the physically linked cells to the site of LFA-1/ICAM-1/IL-2 receptor interaction.

A state of T cell activation reflected by a marked degree of spontaneous proliferation in vitro exists among patients with HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP). Dr. Waldmann wished to define the mechanism by which the circulating cells from HAM/TSP are driven, thus gaining an insight into the pathogenesis of this HTLV-I associated disease. Using a modification of the polymerase chain reaction, Drs. Craig Tendler, Steven Greenberg and Waldmann demonstrated that the circulating mononuclear cells of such patients express the HTLV-I transactivator gene PX. There was an up-regulation of IL-2 and IL-2Rα transcripts in HAM/TSP that paralleled the coordinate mRNA expression of the PX transactivator. In addition, IL-2 and IL-2Rα serum levels in this disorder were elevated. Finally, the marked degree of in vitro spontaneous proliferation in HAM/TSP was profoundly inhibited by specific anti-IL-2 receptor or anti-IL-2 blocking antibodies. Collectively these results suggest that immune activation in HAM/TSP is virally driven by the transactivation and coordinate expression of IL-2 and the IL-2Rα protein. This deregulated autocrine process may contribute to the development of inflammatory nervous system damage in HAM/TSP.

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administration to cynomolgus monkeys of $^{90}Y$ anti-Tac led to a marked prolongation of the survival of both cardiac xenografts and allografts when compared with their survival in untreated animals or in animals treated with unmodified anti-Tac. The use of mouse anti-Tac is hindered by the host immune response to the murine antibody and by the lack of effector cytotoxic capacity of the monoclonal. Therefore in conjunction with Dr. Cary Queen, Dr. Waldmann and his coworkers have constructed a "humanized" anti-Tac antibody by placing the complementarity determining regions of the murine anti-Tac antibody into the human variable region framework and IgG1 constant regions. It is hoped that these "humanized" antibodies will be minimally immunogenic. They retained high binding affinity and were very effective in inhibiting IL-2 mediated events. Furthermore, they added the new functional activity of antibody dependent cellular cytoxicity that was absent in the parent mouse anti-Tac. With this new ADCC capacity it is hoped that there will be a substantial improvement in the performance of the antibody in vivo that should translate into an increase in efficacy in therapy of T cell leukemia/lymphoma. In this regard, Dr. Waldmann observed that "humanized" hyperchimeric anti-Tac prolonged cardiac allograft survival in cynomolgus monkeys. The graft survival observed in the group treated with "humanized" anti-Tac was significantly prolonged over that of the group treated with murine anti-Tac. No toxicity was observed. Thus "humanized" anti-Tac provides a versatile therapeutic reagent with possible applications in adult T cell leukemia and other malignancies and in allograft, graft versus host, and autoimmune settings. In summary, the clinical application of IL-2 receptor directed therapy represents a new perspective for the treatment of certain neoplastic diseases and autoimmune disorders and for the prevention of allograft rejection.

Honors and Awards:

1989 Milken Family Medical Foundation Distinguished Basic Scientist Award
1990 Distinguished Immunologist Lecture, University of California Medical Center, San Francisco, CA
1990 Robert A. Cooke Memorial Lectureship, The American Academy of Allergy and Immunology
1990 Honor Award for Original Contribution to Human Retrovirology, HTLV-I Society
1990 Jeffrey Modell Foundation Lifetime Achievement Award
1990 Ireland Cancer Center Lecture, Case Western Reserve University
1990 Doctor Honoris Causa, University Medical School, Debrecen, Hungary

Publications:

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Ramos EL, Leggat JE, Milford EL, Kirkman RL, Tilney NL, Strom TB, Shapiro ME, Waldmann TA, Carpenter CB. In vivo anti-interleukin-2 receptor (anti-Tac) therapy is immunosuppressive, but not tolerogenic. Transactions of the Assoc of Amer Phys 1989;CII:231-9.

Begley, CG, Aplan PD, Haynes B, Waldmann TA, Kirsch IR. The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. Proc Natl Acad Sci USA 1989;86:10128-32.

Kirkman RL, Shapiro ME, Carpenter CB, Milford EL, Ramos EL, Tilney NL, Waldmann TA, Zimmerman CE, Strom TB. Early experience with anti-Tac in clinical renal transplantation. Transplantation Proceedings 1989;21:1766-8.

Kozak RW, FitzGerald DJP, Atcher RW, Goldman CK, Nelson DL, Gansow OA, Pastan I, Waldmann TA. Strategies for the removal of alloresponsive T cells to human transplantation antigens: toxin or radionuclide conjugated anti-IL-2 receptor (Tac) monoclonal antibody immunotherapy. J Immunol 1990;144:3417-23.

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Waldmann TA. Organization of the Immune System. Sauder DN, ed. In: Dermatologic Clinics. W B Saunders. In Press.

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Greenberg SJ, Tendler CL, Manns A, Bartholomew CF, Hanchard B, Blattner WA, Waldmann TA. Altered cellular expression in human retroviral associated leukemogenesis. Blattner W, ed. Human Retrovirology HTLV. New York: Raven. In Press.

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Waldmann TA, Grant A, Tendler C, Greenberg S, Goldman C, Bamford R, Junghans RP, Nelson D. Lymphokine receptor-directed therapy: a model of immune intervention. J Clin Immunol. In Press.

Kirkman RL, Shapiro ME, Carpenter CB, McKay DB, Milford DL, Ramos EL, Tilney NL, Waldmann TA, Zimmerman CE, Strom TB. A randomized prospective trial of anti-Tac monoclonal antibody in human renal transplantation. Transplantation. In Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04015-19 MET

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Development and Function of Humoral and Cellular Immune Mechanism

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

| | | | |
|---|---|---|---|
| PI: | R. Michael Blaese, M.D. | Section Chief | MET, NCI |
| | Kenneth Culver, M.D. | Medical Staff Fellow | MET, NCI |
| | W. French Anderson, M.D. | Chief | MHB, NHLBI |
| | Steven A. Rosenberg, M.D., Ph.D. | Chief | SB, NCI |
| | Scott Freeman, M.D. | IPA, Univ. Rochester Sch Med/ | MET, NCI |
| | Kimberly Leichtling, M.D. | Special Volunteer | MET, NCI |
| | Gary Parenteau, M.D. | Medical Staff Fellow | CSB, NHLBI |

COOPERATING UNITS (if any)

MHB, NHLBI SB, NCI

LAB/BRANCH
Metabolism Branch

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 6 | 3 | 3 |

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither "B" 100%

☒ (a1) Minors

☐ (a2) Interviews

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

Dr. Michael Blaese has continued his research program to develop gene transfer for the therapy of human disease. He and his collaborators, W. French Anderson and Steven A. Rosenberg, have now performed the first authorized clinical experiment in which a foreign gene has been introduced into man. A murine retroviral vector containing the bacterial gene encoding Neomycin resistance (neoR) was used to label tumor infiltrating lymphocytes (TIL) used in the treatment of patients with malignant melanoma. These neoR expressing TIL were reinfused into the patients to study the survival and trafficking patterns of TIL in vivo. The next phase of these studies will involve insertion of genes encoding various cytokines into TIL in an attempt to improve their tumoricidal activity for a variety of tumors. Major studies of the biological effects of cells secreting these molecules are also underway as are studies of the potential utility of using lymphocytes as cellular vehicles for gene therapy of non-malignant diseases such as ADA deficiency SCID. Non-transformed peripheral blood T cells from ADA deficient SCID patients have been grown using anti-CD3 and rIL-2 stimulation and the human ADA gene has been successfully introduced into these cells. A clinical protocol for the treatment of ADA(-)SCID with gene-treated cultured T cells has now been approved by the Human Gene Therapy Subcommittee. With the expected early approval by two additional Federal regulatory agencies, the first use of gene transfer for the therapy of human disease could begin as early as this autumn.

Work has also continued with the immunosuppressive compound Succinylacetone (SA) studying solid organ and bone marrow transplantation in rats, pigs, primates and dogs. SA greatly prolongs skin allograft survival in rats and permits indefinite survival of totally mismatched cardiac allografts in rats. In dogs SA is the most effective single immunosuppressive agent so far tested in preventing lethal graft versus host disease.

Project Description

Major Findings:

A major effort of the Cellular Immunology Section has continued to focus on the development of techniques of gene transfer which will be useful for the application of gene therapy in man. Several systems have been studied including retroviral mediated gene transfer into bone marrow progenitor cells, cultured fibroblasts, transformed T cells and primary antigen specific IL-2 independent T cell lines from mice, rats, monkeys and IL-2 dependent T cells from man. We have been successful in transferring the gene for adenosine deaminase into ADA deficient T cell lines and totally correcting the biochemical defect in these cells in vitro. Using autologous bone marrow transplantation in lethally irradiated monkeys, we have been successful in transferring the gene for human ADA into these monkeys. Expression of the human enzyme in the peripheral blood mononuclear cells of the recipient animals was detected within 45 days of transplantation and persisted for another 100 days. These results are very encouraging, but the level of expression of human enzyme was low and not sustained for a long enough period to permit application of present techniques to the therapy of human disease. To avoid some of the problems encountered with bone marrow gene therapy, we have been developing the alternative approach of using lymphocytes as cellular vehicles for gene transfer. Lymphocytes have several potential advantages for gene therapy. Cultured lymphocytes are readily infected with retroviral vectors, can be selected for appropriate gene expression in culture, and can be thoroughly tested before reintroduction into the body. The antigen specificity of lymphocytes can also be utilized to expand the population of gene transduced cells in vivo by simple immunization or to target the introduced gene to certain specific sites in the body such as directing antineoplastic factors to the sites of tumor metastases. We have shown that murine antigen specific T cells transduced with the genes for neomycin resistance and human ADA will survive for many weeks after reintroduction into intact animals and will continue to express both genes at high levels. As an initial clinical application of gene transfer with lymphocytes, we joined with W. French Anderson and Steven A. Rosenberg in a major collaborative study. We used an introduced NeoR gene as a cell marker to permit evaluation of the in vivo distribution and survival of tumor infiltrating lymphocytes (TIL) given to treat patients with metastatic malignant melanoma. The gene marked TIL have been found to remain in the patients' circulation for at least 21 days and for as long as 183 days after infusion. In addition, they have been identified as homing to tumor deposits in detectable numbers as early as 2 days after infusion. These encouraging preliminary results have now led us to propose the use of ADA gene corrected autologous T cells in the treatment of children with ADA deficiency SCID. Trials of this first use of gene transfer for therapy should begin within six months.

We have continued with our long-time interest in the Wiskott-Aldrich syndrome with studies of platelet function before and after splenectomy, changes in platelet cell membrane glycoproteins occurring after splenectomy, detailed lymphocyte phenotype analysis of both T cells and B cells, family genetic linkage analysis using X-chromosome RFLPs, and carrier detection based on the pattern of X-inactivation found in peripheral cells of the carriers. This latter study is based on the observation that the affected boys have defects in T cell, B cell, monocyte, granulocyte, and platelet function and yet the carrier females have no detectable

defects in any of these lineages. Since all females are mosaics for traits inherited on the X-chromosome because of random inactivation of one of her two X-chromosomes (lyonization) occurring in the early embryo, carriers might be expected to demonstrate abnormalities unless all their cells were derived from precursors in which the chromosome carrying the defective gene was inactivated. We have studied this question using RFLPs for the probes to the X-linked genes PGK and HPRT to distinguish the maternal from the paternally inherited X-chromosome and methylation sensitive restriction endonucleases to distinguish the active from the inactive X-chromosomes. Whereas approximately half the cells from normal females express the genes on the paternally derived X and the remaining express genes from the maternally derived X-chromosome, the T cells, B cells, and granulocytes from obligate carriers of the WAS gene were found to be using only 1 of the 2 chromosomes in all their peripheral cells. This strikingly unbalanced pattern of X-inactivation was not seen in any of the more than 80 normal females studied and thus appears to represent a reliable marker for the carrier state in these females.

Our studies of the immunosuppressive compound succinylacetone have continued to provide exciting information about this most potent of immunosuppressive agents. Succinylacetone is a seven carbon organic acid. It is an inhibitor of the second step of heme biosynthesis and has been shown to have inhibitory effects on the growth of certain tumors in vitro. We initially demonstrated that the compound appeared to have potent immunosuppressive activity in the rat because it was capable of preventing the rejection of tumor allografts. More recent studies showed that succinylacetone behaves as a profound suppressive agent for T cell mediated immune functions. It not only blocks tumor and other forms of allograft rejection but also prevents graft vs. host disease induced in adult F1 animals after the injection of parental strain lymphoid cells. In a model of bone marrow transplantation in fully allogeneic adult rats, succinylacetone treatment not only prevented lethal graft vs. host disease in the transplanted animals but also permitted normal engraftment and full reconstitution. The striking finding is that the treatment required to prevent graft vs. host disease was limited to 24 days of duration and yet the animals remained fully chimeric for at least 300 days post bone marrow transplantation. Immune competence returned to the transplanted animals in a timely fashion, and survival in the recipients of allogeneic cells was equivalent to the survival in transplant recipients receiving syngeneic bone marrow. Succinylacetone has been shown to have profound effects on the B lymphocyte system as well. Initially, it was shown to inhibit the production of antibodies to sheep erythrocytes in rats treated for a period of 2 weeks. We have extended these initial studies to demonstrate that the drug has immunosuppressive effects in mice as well. Using a series of model antigens that are both helper T cell dependent and helper T cell independent type I and type II antigens, we have shown that succinylacetone interferes with the production of antibodies to all classes of antigen. Therefore, its inhibitory effects cannot be explained solely by the inhibition of T cell function. Thus, succinylacetone is one of the very few agents that has profound effects on the production of antibodies and immunoglobulin. Our studies of this agent as a potentially useful immunosuppressive for clinical application have been very encouraging. We have found it to completely prevent experimental autoimmune uveitis in the rat. SA also permits totally allogenic cardiac transplants to survive indefinitely in rats. Skin allografts in rats, a much more difficult immune response to suppress, were also greatly augmented by SA treatment.

In monkeys, SA treatment was able to totally inhibit antibody production as well as prolong cardiac allograft survival. In dogs, SA has been shown to be the most effective single agent so far tested for the prevention of acute GVHD in total mismatched donor-recipient pairs. The mechanism of action of succinylacetone is unclear. Although the agent has an antiproliferative effect to T cells and B cells in vitro, the dose required in vitro also inhibited the proliferation of non-lymphoid cells, such as fibroblasts and tumor cells. The observation that bone marrow engraftment proceeds normally in the presence of profoundly immunosuppressive effects also suggest that this is not a general antiproliferative type agent. Preliminary studies of adding various growth factors to cells in vivo and in vitro have been unable to overcome the immunosuppressive effects of the drug. We have extensively analyzed histology and blood chemistries in animals treated with toxic doses of succinylacetone and have found no consistent pathological finding other than a profound depletion of the lymphoid areas in all tissues. With 14 days of high dose succinylacetone therapy, the thymus of treated animals almost totally involutes, giving the histological appearance of the thymus seen in infants with one of the forms of SCID. Thus succinylacetone, in addition to being a profound and potentially useful clinical immunosuppressive drug, may provide us with a mechanism for studying a variety of cellular pathways.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04016-17 MET

PERIOD COVERED

October 1, 1989 through September 30, 1990

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 |
| | Mark Sklar | Medical Staff 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

DCBD, NCI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER |
|---|---|---|
| 5.25 | 2.75 | 2.5 |

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors "B" 100%

☐ (a2) Interviews

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

We have investigated the interaction of the two classes of ligands (lysosomal enzymes and IGF-II) which bind to the IGF-II/Man-6-P receptor. Highly purified β-galactosidase which we isolated from bovine testis inhibited $^{125}$I-IGF-II binding to pure IGF-II/Man-6-P receptor with the concentration that was required for half-maximal inhibition being 25 nM. The inhibition by β-galactosidase was reversed by mannose-6-phosphate and the dose response curve for the reversal by mannose-6-phosphate was typical for binding to the IGF-II/Man-6-P receptor. Acidic forms of β-galactosidase were isolated by ion exchange chromatography on DEAE-Sephacel. These acidic forms of β-galactosidase which had been previously shown to exhibit enhanced cellular uptake also showed increased potency in inhibiting the binding of $^{125}$I-IGF-II to the receptor. Scatchard analysis of binding of IGF-II to the receptor in the presence of concentrations of β-galactosidase which caused partial inhibition of $^{125}$I-IGF-II binding, demonstrated that β-galactosidase decreased the binding affinity for IGF-II. We have shown that IGF-II is a substrate for Cathepsin C. Cathepsin C cleaves Ala-Tyr dipeptide from the N-terminus. Comparison of the native IGF-II with the desdipeptidyl IGF-II for binding to the IGF-II/Man-6-P receptor, the IGF-I receptor, and IGF binding proteins showed equipotency of the two species of IGF-II. We have also attempted to confirm a report that in the 1854 rat cell line which produces IGF-II and has IGF-II receptors, IGF-II is an autocrine growth factor for of these cells in serum free media, acting through the IGF-II/Man-6-P receptor. We found that neither IGF-I or IGF-II could support the multiplication of the 1854 cell line under serum-free conditions and, using an antibody which blocks the binding of IGF-II to the IGF-II/Man-6-P receptor, we were unable to demonstrate a role for this receptor in signaling the growth response.

Project Description

Major Findings:

Developmental expression of the rat insulin-like growth factor - II/mannose 6-phosphate receptor mRNA

We previously reported that the IGF-II/Man-6-P receptor was developmentally regulated and variably expressed in multiple tissues in the rat. The receptor expression was high in fetal tissues and then declined sharply in postnatal tissues. In fetal heart, receptor expression approached 2% of total extractable protein. In order to determine whether receptor expression reflects mRNA levels, we measured mRNA concentration in tissues from 20-day gestation fetuses and in tissues from 20-day postnatal animals. Fresh tissues were immediately placed in liquid nitrogen and total RNA was isolated using the guanidinium thiocyanate/cesium chloride method. Total RNA was analyzed by Northern blotting using as a probe the rat IGF-II/Man-6-P receptor cDNA clone K3 kindly provided by Drs. David Morgan and William Rutter. A major mRNA band of 9.2 kilobases was found in all tissues and a very minor 3.7 kilobase band was also noted in many tissues. A 9.2 kilobase mRNA is consistent with the size of the IGF-II/Man-6-P receptor protein and is in agreement with the few published reports on the IGF-II/Man-6P receptor mRNA. By Northern blotting, fetal tissues contained greater concentrations of receptor mRNA than corresponding postnatal tissues. To provide a more quantitative comparison between fetal and postnatal tissues, we employed a solution hybridization/RNAase protection assay. The plasmid used for generation of $^{32}$P-labeled RNA transcripts was constructed by subcloning a 0.5 kilobase fragment of clone K3 into pGem 4Z. Protected RNA:RNA hybrids were electrophoresed on 8% urea-acrylamide gels. The gels were scanned using a computer driven imaging system so that the radioactivity of the band representing the protected probe could be measured in each lane. The rank order for receptor mRNA abundance among fetal tissues was: heart> lung> limb> kidney> liver> brain. This rank order corresponds to the rank order for expression of the receptor protein in fetal tissues. There was a decline of mRNA concentration in the postnatal tissues which ranged from 14-fold in heart to 2-fold in lung. These results suggest that the production of IGF-II/Man-6P receptor is controlled at the level of transcription and/or mRNA degradation for most tissues. For several tissues (lung, kidney, liver) the postnatal decline in receptor protein was more dramatic than the decline in receptor mRNA. This result suggests that for these tissues there may be an element of translational control of receptor production.

Monoclonal antibodies to the insulin-like growth factor-I receptor

Immunoprecipitation of growth factor receptors has been used successfully to identify receptor-associated proteins. These proteins are candidates for being important components in the signaling pathway following binding of the ligand to the receptor. There is only one monoclonal antibody to the IGF-I receptor that has been described (alpha IR-3) and this hybridoma is not available because of commercial constraints. We therefore sought to develop additional monoclonal antibodies to the IGF-I receptor to allow us greater flexibility in attempting to use immunoprecipitation to identify receptor-associated proteins. In order to increase the amount of antigen which could be used to immunize mice, we decided to purify the IGF-I receptor from human placenta, a well-characterized source for the

IGF-I receptor. Microsomal membranes were prepared by differential centrifugation and the receptor was released from the membranes by treatment with Triton X-100. The initial purification step was chromatography on a wheat germ lectin affinity column. Insulin receptors were then eliminated by chromatography on an insulin-Sepharose column. The flow-through from this column was applied to an alpha IR-3 affinity column and eluted under alkaline conditions. Analysis of the purified receptor by polyacrylamide gel electrophoresis and silver staining indicated that the receptor was the major component of this preparation. These receptor preparations were used to immunize mice and two out of six animals developed receptor antibodies in their sera. spleens from these animals were used to develop hybridomas. Hybridoma cultures were screened for antibody production with both a blocking assay which assessed the ability to block the binding of $^{125}$I-IGF-II and an assay designed to detect antibodies directed against portions of the receptor outside of the binding site. Cells in positive wells were expanded, cloned, expanded again, and injected into mice for production of ascites fluids. These ascites fluids and immunoglobulins purified from them are currently being tested for ability to immunoprecipitate receptors from metabolically-labeled cells.

Publications:

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PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 04017-12 MET

PERIOD COVERED
October 1, 1989 through September 30, 1990

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

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

PI: David L. Nelson Head, Immunophysiology Section MET, NCI
Dr. William D. Schwieterman, Medical Staff Fellow MET, NCI
Dr. Hariclia Litou, 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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.8 | 3 | 1.8 |

CHECK APPROPRIATE BOX(ES)

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

"B" 100%

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

Studies were performed to examine the maturation and regulation of the human immune response in normal individuals and in patients with congenital and acquired immune deficiency states associated with a high frequency of cancer. The interaction of the T-cell derived lymphokine interleukin-2 (IL-2) with its cell membrane receptor (IL-2R) plays a pivotal role in the establishment and maturation of the immune response. Elevated levels of soluble IL-2R (Tac protein) were found is the serum of several retroviral associated diseases including the adult T cell leukemia (ATL), hairy cell leukemia (HCL), the acquired immune deficiency syndrome (AIDS), and patients with Kawasaki disease. Elevated serum levels of soluble Tac protein were also observed in patients with "autoimmune" disorders and patients undergoing allograft rejection episodes. Favorable responses to therapy in ATL and HCL were associated with reductions in serum Tac protein. Measurement of Tac protein in serum is thus useful in the diagnosis and management of certain cancer patients and in monitoring the state of immunologic activation in humans in vivo. In a continuing effort to define the nature of certain immunodeficiency disorders and to gain insight into human B-lymphocyte maturation, we have initiated molecular genetic studies of a kindred with X-linked hypogammaglobulinemia and isolated growth hormone deficiency. Mapping studies by restriction fragment length polymorphism have shown that this disease is due to a gene distinct from that causing the more common form of X-linked agammaglobulinemia or Bruton's disease.

Project Description

Major Findings:

The cell membrane receptor for the T-cell derived lymphokine, interleukin-2 (IL-2) is a multichain structure consisting of at least two proteins with molecular weights of 55 and 75 kilodaltons (kDa). Hybridoma-derived monoclonal antibodies binding to distinct epitopes on the 55 kDa molecule have been produced and this chain has been termed the Tac protein. The soluble Tac protein is 10 kDa smaller in size than the cell-membrane form of this molecule and specifically binds IL-2, albeit at low affinity (20 nM).

Using two monoclonal antibodies directed against distinct epitopes on the Tac protein, we constructed a "sandwich" enzyme-linked immunoassay (ELISA) to quantitatively measure soluble Tac molecules. Soluble Tac protein was measurable in the serum and urine but not cerebrospinal fluid of all normal individuals. Since soluble Tac protein was produced by activated normal lymphocytes and monocytes as well as constitutively produced by certain tumor cell lines, we undertook studies to measure the level of Tac protein in body fluids of certain patients with cancer and other patients whose immune system might be undergoing activation in vivo.

Elevated levels of soluble Tac protein were observed in diseases associated with human retroviral infections including the adult T cell leukemia (ATL), hairy cell leukemia (HCL), the acquired immune deficiency syndrome (AIDS), and patients with Kawasaki disease. Elevations of soluble Tac protein were also observed in several groups of patients with neurological diseases including multiple sclerosis and schizophrenia. The measurement of Tac protein from the human IL-2 receptor in various body fluids is useful in the diagnosis and management of patients with neoplastic and inflammatory disorders.

While the genes encoding foreign antigen binding immunoglobulin molecules on B-cells (Ig) have been molecularly cloned, our knowledge of the regulation and maturation of the expression of these genes is still very limited. One example of abnormal expression of these genes occurs in the primary immunodeficiency diseases such as X-linked hypogammaglobulinemia and isolated growth hormone deficiency where B-cell maturation is apparently blocked at the pre-B-cell level. Therefore a molecular understanding of the cause of disordered gene expression in these patients might provide important information as to the regulation of the expression of immunoglobulin genes during normal development. Mapping studies by restriction fragment length polymorphism have shown that X-linked hypogammaglobulinemia and isolated growth hormone deficiency is due to a gene distinct from that causing the more common form of X-linked agammaglobulinemia or Bruton's disease. Currently, studies are underway to examine more closely how these patients differ from those with Bruton's disease.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 04018-14MET

PERIOD COVERED

October 1, 1989 - September 30, 1990

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. | Biotechnology Fellow | MET, NCI |
| | Neeraja Sathyamoorthy, Ph.D. | Visiting Fellow | MET, NCI |
| | Jean Decker, B.S. | Chemist | MET, NCI |
| | Michael Dipre, M.D. | Clinical Associate | MET, NCI |

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolism Branch

SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 5 | 4 | 1 |

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 importance of carbohydrate structure in the regulation of diverse biologic responses is becoming more and more apparent to a number of investigators. In recognition of this growing trend, a new journal (Glycobiology, Oxford Press) has been launched to report, in a timely manner, advances in this rapidly emerging field. I have been asked by Dr. Gerald Hart to be an editor for this journal. Advances in the laboratory have also occurred. Dr. Mishra's studies have confirmed that defined mannose structures are able to disrupt the association of the heterodimeric c-fos-c-jun-transcriptionally active protein complex's ability to bind to its consensus recognition site. Using a growth hormone expression vector we have shown that these same mannose structures are able to regulate gene expression (i.e., growth hormone production) under the regulatory control of fos-jun responsive elements. Dr. Michael Dipre has markedly extended our technology in the purification of these mannose compounds and, using HPLC methodology, we are making progress in defining the minimal carbohydrate structure required for cellular activation. Dr. Sathyamoorthy is continuing her studies on the in vitro and in vivo activity of these compounds, but more recently has begun a project to purify a unique circulating alpha mannosidase found in normal human serum. We will utilize this enzyme for structural studies and will also screen several disease states (most notable, chronic mucocutaneous candidiasis) for levels of this enzyme.

Project Description

Major Findings:

1) The immunosuppressive oligosaccharides of uromodulin have been purified and structurally characterized.

2) A panel of related oligosaccharides have also been purified and structurally characterized.

3) These compounds have been shown to

a. block T cell responsiveness in vitro.

b. induce a delayed inflammatory response in vitro.

c. induce $PGE_2$ 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 AP-1, AP-3 and TNF-1 and are able to directly regulate gene transcription.

6) We have also shown that IL-2 and TNF directly bind to class II antigens and can regulate their expression in vitro. This binding is mediated by oligosaccharides expressed by class II molecules.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 04020-13 MET

PERIOD COVERED
October 1, 1989 - September 30, 1990

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 |
| Anne Hosmalin, M.D. | Visiting Fellow | MET, NCI |
| Richard England, M.D., Ph.D. | Medical Staff Fellow | MET, NCI |
| Mutsunori Shirai, M.D., Ph.D. | Special Volunteer | MET, NCI |
| Marika Kullberg | Special Volunteer | MET, NCI |
| Donna Barnd, Ph.D. | IRTA Fellow | MET, NCI |

COOPERATING UNITS (if any)

| | | |
|---|---|---|
| Louis H. Miller, M.D. | Chief, Malaria Section | LPD, NIAID |
| Gene H. Shearer, Ph.D. | Section Chief | EIB, NCI |
| Robert Gallo, M.D. | Lab Chief | LTCB, NCI |

LAB/BRANCH
Metabolism Branch (More professional personnel listed on next page)

SECTION
Molecular Immunogenetics and Vaccine Research Section

INSTITUTE AND LOCATION
DCBDC, NCI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 11.5 | 9.5 | 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 have studied the mechanisms by which T cells recognize antigens presented on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules and the application of these principles to the design of synthetic vaccines for AIDS and malaria. We found that a peptide can bind to the same MHC molecule in more than one position or conformation, indicating that the role of a given amino acid residue in binding MHC cannot be defined independently of the T cell involved. In the case of HIV, we found that the same peptide can also bind to both class I and class II MHC molecules for recognition by CD8 cytotoxic T lymphocytes (CTL) and CD4 helper T cells, respectively. Thus this peptide can provide help for induction of CTL to itself. A single amino acid seems to contribute most of the energy responsible for the specificity differences between noncrossreactive CTL specific for different HIV isolates. This peptide is also recognized by CTL from HIV-infected and uninfected immunized human volunteers, in association with two common human HLA types, A2 and A3, making it broadly useful in a vaccine. We have also identified a conserved CTL epitope in the HIV-1 reverse transcriptase recognized by CD8 cells from both mice and humans, which is also recognized by helper T cells. Thus, CTL epitopes seen by murine T cells are often seen by human CTL and also by helper cells. We have identified multideterminant helper T-cell regions of the HIV envelope that are broadly recognized by both mice and humans also. We have shown that some of the helper peptides can elicit help for an enhanced antibody response to HIV envelope in monkeys, and that humans immunized with a recombinant gp160 vaccine make much higher levels of IL-2 in response to these peptides than do HIV-infected individuals. Thus, vaccine induced immunity can exceed that produced by natural infection. We have developed a way to get purified nonliving subunit vaccines, which do not normally induce CD8 CTL, to do so reproducibly by incorporating them into ISCOMs. This may make it possible for subunit vaccines to induce all 3 types of specific immunity.

Continuation Sheet for PHS 6040

Other Cooperating Units:

| | | |
|---|---|---|
| Ronald N. Germain, M.D., Ph.D. | Section Chief | LI, NIAID |
| Bernard Moss, M.D., Ph.D. | Lab Chief | LVD, NIAID |
| Walter Weiss, M.D. | Lt. Cdr. | ID, NMRI |
| Stephen Hoffman, M.D. | Cdr. | ID, NMRI |
| Sanjai Kumar, Ph.D. | Visiting Fellow | LPD, NIAID |
| Mario Clerici, M.D. | Visiting Fellow | EIB, NCI |

## Project Description

Major Findings:

We have been studying the mechanisms by which T cells recognize antigens on the surface of other cells in association with major histocompatibility complex (MHC)-encoded molecules, the factors that determine which antigenic structures are more likely to be recognized, and the application of these principles to the design of synthetic vaccines for AIDS and malaria. T cells recognize antigen after it has been proteolytically processed into fragments or unfolded forms which then associate with MHC molecules. Each of these steps can influence which antigenic determinants are seen by T cells.

To study antigen processing, we are comparing the peptide fragments produced by the natural processing of myoglobin by antigen presenting cells with the fragments produced by proteolytic cleavage with cathepsin B, which we had previously implicated as a major enzyme involved in processing, and cathepsin D, which had been implicated by others. We have also been developing ways of reisolating processed antigen from different organelle fractions of antigen-pulsed antigen presenting cells and studying their ability to stimulate T cells without further processing. These studies, if successful, should help determine the intracellular sites of processing and the cell-biological pathways involved. They should also allow us to compare naturally processed antigen with the synthetic peptides that correspond to the minimal antigenic sites.

At the level of antigen presentation by class II MHC molecules, we have studied a large series of 36 peptide variants, each with a single amino acid substitution, to try to determine which residues are involved in binding to the MHC molecule, and which are involved in binding to the receptor of the T cell. We studied the ability of these peptides to stimulate two different T-cell clones specific for the same antigenic site, and the ability of the peptides to compete with each other for binding to the class II MHC molecule. It has generally been assumed that each peptide would have a particular set of amino acid residues that interacted with a given MHC molecule, independent of which T cell was recognizing the peptide-MHC complex. However, we asked what would be expected if the same peptide could bind in more than one way to the same MHC molecule. We would predict that we could find examples of substitutions among these residues that would behave as though the residue interacted with the MHC molecule when one T cell was responding, but would appear to interact with the receptor of the other T cell. Among the 36 substituted peptides, we found several such examples, which constitute

the first proof that a single peptide can bind in more than one conformation, orientation, or position within the same MHC peptide-binding groove. Therefore, one cannot define which residues of a peptide interact with a given MHC molecule without specifying which T cell is responding. The data suggest that it may even be possible for two peptides to bind at once to different subsites within the groove, a possibility made plausible by the recent crystal structure data of Garrett et al. Such binding, rather than producing competition, may actually be able to enhance recognition in certain circumstances.

We have also been applying these and other new basic discoveries on T-cell recognition to the design of synthetic vaccines for malaria and AIDS. In the case of malaria, in collaboration with the groups of Lou Miller (NIAID) and Steve Hoffman (NMRI), we have identified a major antigenic site recognized by cytotoxic T cells (CTL) specific for the circumsporozoite protein (CSP) of a mouse malaria, Plasmodium yoelii. Moreover, the CTL specific for this peptide can eliminate malaria parasites from murine liver cells infected with P. yoelii sporozoites in vitro, in an antigen-specific and genetically restricted manner. In addition to demonstrating the potentially protective role of this antigenic site, this result demonstrates for the first time that sporozoite-infected liver cells express processed fragments of the CSP on their surface, despite the inability to detect these with antibodies. We have also been studying protection against the blood stages of P. vinckei, which is mediated by CD4 T cells, but also depends on nonspecific changes in the spleen induced by the parasite. We have prepared several CD4 T cell lines and clones specific for P. vinckei lysed parasites, including some IL-2 producing T cells and some T-cell lines that preferentially produce IL-4. We are starting to study these for the ability to protect nude mice in adoptive transfer studies. If any T-cell lines or clones protect, we can use these with "T-cell Western blots" and also P. vinckei gene libraries now being prepared by Sanjai Kumar, in order to identify the antigenic proteins that lead to protection.

In the case of HIV, we have extended our studies of the immunodominant CTL antigenic site of the HIV envelope protein we identified earlier. We have found that a single amino acid interchange between two isolates of HIV-1 is sufficient to reciprocally interchange the specificities of the peptides for CTL lines that do not crossreact between these two isolates of HIV. This result not only identifies an important contact residue for interaction with the CTL receptor, but also indicates that a single amino acid can contribute most of the contact energy for defining the specificity of T-cell recognition. We have also found that this same 15-residue peptide can restimulate CTL precursors without the need for exogenous lymphokine. This makes it useful in vaccine development, but also indicates that it can induce T-cell help for induction of CTL against itself. We showed that his help is due to CD4 T cells that see the peptide in association with a class II MHC molecule, and which help CD8 CTL precursors that see the same peptide in association with a class I MHC molecule. Thus, the response to this site is under dual genetic control, and we can show this with recombinant strains of mice. We are currently studying whether the peptide uses similar residues to interact with these two classes of MHC molecule. In collaboration with Drs. Ammar Achour and Daniel Zagury of Paris, we have found that this peptide is recognized by human CD8 CTL from 5/5 volunteers immunized with a recombinant vaccinia virus expressing the HIV-1 envelope gene. These volunteers did not share any single class I HLA molecule, and using transfected cells expressing only one HLA molecule, we could

show that this peptide was being presented in association with at least two different human HLA molecules, A2 and A3. Since both of these are common in the population, this peptide should be recognized by a large fraction of the US population. In collaboration with Drs. Mario Clerici and Gene Shearer, we found that indeed more than half of the HIV-infected humans studied had CD8 CTL that recognized this peptide on autologous target cells, and very frequently in association with HLA-A2.

With regard to helper T cells specific for the HIV envelope, in collaboration with Mario Clerici and Gene Shearer, we have studied individuals immunized with a recombinant gp160 trial vaccine candidate. Not only did these individuals, who were not infected with HIV, make IL-2 in response to several helper T-cell epitopes we had defined, but also they made much higher responses than infected but asymptomatic individuals. This contrast indicates that it is possible to achieve much higher levels of T-cell immunity with an artificial vaccine than are produced by natural infection, and gives hope that a vaccine could be protective. Also, we found that we could immunize rhesus monkeys with these helper T-cell antigenic peptides and elicit helper T-cell immunity that resulted in a sizable antibody response the first time the animals were exposed to suboptimal doses of intact envelope protein, whereas the control animals made no antibody in responses to this suboptimal dose. Therefore, these peptides can induce help in vivo in a primate, and may be able to enhance immunity on exposure to virus. In addition, we have been studying larger peptides that we showed last year contained overlapping antigenic determinants recognized by helper T cells from mice of multiple haplotypes. These longer peptides have now been synthesized, and we have found that some of them indeed elicit T-cell responses from mice of multiple haplotypes as predicted. They also stimulate IL-2 production by T cells from a large fraction of infected humans studied in collaboration with Clerici and Shearer. Thus, these larger peptides may be useful both for diagnostic purposes in individuals who may have been exposed but are not yet seropositive, and for vaccine development.

We have also identified an immunodominant CTL epitope in the reverse transcriptase of HIV-1, which is recognized by CD8 CTL from mice in association with class I MHC molecules. We have used a nested series of peptides to define the boundaries of this antigenic site, and then have tested the optimized peptide with peripheral blood T cells from infected humans, in collaboration with Mario Clerici and Gene Shearer. Five of 12 infected patients had CTL that killed autologous targets incubated with this peptide, in fresh peripheral blood without restimulation in vitro, despite the fact that they did not all share any single HLA molecule. Therefore, this antigenic site will also be useful for a vaccine recognized by a large proportion of individuals of different HLA types. In addition, because this comes from a conserved portion of a conserved protein necessary for viral replication, this site should not be subject to the same escape mutation that has plagued both neutralizing antibodies and CTL specific for the envelope. Also, we have identified three immunodominant helper T cell epitopes in HIV-1 reverse transcriptase. Strikingly, one of these corresponds to the same site was recognized by both helper and cytotoxic T cells from mice of the same haplotype, and suggests that it may be more than a coincidence. Either such concordance contributes to immunodominance of that site, or pressure to maintain this type of concordance could contribute to linkage disequilibrium between class I and class II MHC loci.

Finally, it has been notoriously difficult to immunize with anything other than a live vector to elicit CD8 cytotoxic T cells recognizing antigen in association with class I MHC molecules. this has been a major drawback to subunit vaccines. To overcome this problem, we have collaborated with Bror Morein of Uppsala, Sweden who has incorporated HIV-1 envelope protein into immunostimulatory complexes (ISCOMs) containing cholesterol and Quil A, a mixture of natural detergent-like molecules from the bark of a tree. These 35-nm particles have been shown to be highly immunogenic for antibody production. We tested their ability to immunize mice, and found that gp160 in ISCOMs could elicit CD8 CTL specific for gp160 in association with a class I MHC molecule, whereas the same preparation of recombinant gp160 in complete or incomplete Freund's adjuvant or in saline did not. We showed that this was not due to peptide contamination, and that the CD8 precursors had to be immunized in vivo with the ISCOMs. The CTL produced were specific for the same epitope as those elicited by vaccination with a live recombinant vaccinia virus expressing gp160. We also showed that this method was generalizable to other proteins, such as influenza hemagglutinin. Thus, it may finally be possible to regularly induce CD8 cytotoxic T cells as well as helper T cells and antibodies with a non-living subunit vaccine.

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Patent:

Good MF, Berzofsky JA, Miller LH. US Patent 4,886,782: Improved malarial immunogen.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB-04024-3 MET

PERIOD COVERED

October 1, 1989 - September 30, 1990

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

Control of Gene Expression in Lymphoid Development

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

| | | | |
|---|---|---|---|
| PI: | Louis M. Staudt, M.D., Ph.D. | Senior Staff Fellow | MET, NCI |
| | Timothy Behrens, M.D. | Special Volunteer | MET, NCI |
| | Hon-Sum Ko, M.D. | Fogarty Visiting Fellow | MET, NCI |
| | Mitchell Rosner | Special Volunteer | MET, NCI |
| | Alessandra Vigano, Ph.D. | Fogarty Visiting Fellow | MET, NCI |
| | Sharon Doll, Ph.D. | IRTA Fellow | MET, NCI |
| | Peggy Scherle, Ph.D. | IRTA Fellow | MET, NCI |

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolism Branch

SECTION

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 7 | 6 | 1 |

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.)

As an approach to understanding development in the B lymphocyte lineage, Dr. Louis Staudt has characterized trans-acting factors that mediate lymphoid-specific gene transcription. As a model he has studied the lymphoid-specific transcription of immunoglobulin (Ig) genes. Oct-2 is a transcription factor expressed in B lymphocytes which is critical for the lymphoid-specific expression of immunoglobulin genes. Dr. Staudt's studies on Oct-2 in the past year have focused on his observation that Oct-2, in addition to being expressed in all B lymphocytes, is also expressed in some T lymphocytes, myeloid cells and in the fetal spinal cord. In T lymphocytes, Oct-2 is induced during activation of T lymphocytes by a variety of stimuli including cognate antigen. These findings suggest that Oct-2 controls gene expression in other cells besides B lymphocytes and must therefore cooperate with other B cell restricted transcription factors to fully account for lymphoid-specific gene expression.

The molecular cloning of Oct-2 helped to identify a new class of transcription factors that share two structural domains, a homeobox and POUbox. Dr. Staudt has molecularly cloned and characterized a new member of this POU-homeobox family, termed Oct-3. Oct-3 binds to the same DNA motif as Oct-2, the octamer, but 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 cells of the germ line including primordial germ cells and oocytes. These findings place Oct-3 in marked contrast with previously studied homeobox genes which are not expressed in pluripotent stem cells or in the germ line but rather are expressed in cells that have differentiated along one of the somatic lineages. Oct-3, therefore, may be important for maintaining the pluripotent state and for committing a stem cell to the germ line lineage.

Project Description

Major Findings:

Oct-2 is a transcription factor expressed in B lymphocytes that binds the octamer DNA motif in immunoglobulin (Ig) promoters. Several lines of evidence indicate that Oct-2 is critical for the lymphoid-specific expression of immunoglobulin (Ig) genes. Expression of Oct-2 in non-lymphoid cells by transient transfection of Oct-2 cDNA expression vectors will activate cotransfected immunoglobulin promoter constructs which are normally inactive in these cells. This basic observation has been extended to an analysis of the transcriptional activation properties of variant forms of the Oct-2 protein. Antibodies specific for Oct-2 showed that at least 6 different Oct-2 protein species can be separated by SDS polyacrylamide gel electrophoresis. Analysis of Oct-2 cDNAs has demonstrated at least 5 different protein coding regions that are generated by alternative splicing and/or alternative polyadenylation and cleavage of the Oct-2 gene. One interesting Oct-2 variant has a different carboxy terminal domain that is missing the proline/glycine/serine/threonine rich region that is present in other forms of Oct-2 and which has been shown to be critical for transcriptional activation by Oct-2. This Oct-2 variant binds DNA but does not activate transcription suggesting that it could function as a naturally occurring "dominant negative" version of Oct-2 that might antagonize the function of other forms of Oct-2.

Oct-2 is expressed in virtually all B lymphocytes and during all defined stages of B cell development but in addition is expressed in some T lymphocytes, myeloid cells and in the fetal spinal cord. We have found Oct-2 expression in pro-B cells that have not yet rearranged Ig genes, in pre-B cells that have begun the rearrangement process, in surface Ig bearing mature B cells and in plasma cells. In addition, Oct-2 was detected in lymphoid-myeloid precursor cells that are capable of differentiating in vitro into either pre-B or mature macrophages, depending on the culture conditions. Interestingly, Oct-2 expression was maintained in both the pre-B and macrophage progeny of the precursor cells. In T lymphocytes, Oct-2 is induced during activation of T lymphocytes by a variety of stimuli, including mitogens and cognate antigen. The T cell line Jurkat can be stimulated to express Oct-2 by treatment with phorbol ester (PMA) and a mitogen (PHA). The stimulation of Oct-2 occurs over a long time course, first appearing at 4 hours and plateauing at 24 hours after stimulation. A similar increase in Oct-2 expression was seen in both helper T cell and cytotoxic T cell clones stimulated in vitro by antigen and antigen presenting cells. These findings suggest that Oct-2 may play a role in the long-term changes in gene expression that accompany T cell activation. Outside of the hematopoietic system, Oct-2 expression was found in the day 14 fetal spinal cord. These findings suggest that Oct-2 controls gene expression in other cells besides B lymphocytes. The role of Oct-2 in lymphoid-specific gene expression must therefore be to cooperate with other B cell restricted transcription factors.

The molecular cloning of Oct-2 helped to identify a new class of transcription factors that share two structural domains, a homeobox and POUbox. We have molecularly cloned and characterized a new member of this POU-homeobox family, termed Oct-3. Oct-3 was originally identified as an octamer binding protein found in nuclear extracts of undifferentiated embryonal carcinoma (EC) cell lines that

had a different molecular weight than Oct-2 or the ubiquitously expressed octamer binding protein, Oct-1. Oct-2 and Oct-3 displayed identical DNA binding characteristics and thus we reasoned that Oct-3 would be encoded by a gene containing a homeobox homologous to the Oct-2 homeobox. The Oct-3 gene was cloned by low stringency screening of a cDNA library prepared from undifferentiated EC cells with an Oct-2 homeobox probe. The full length Oct-3 cDNA encodes a 41 kD protein that contains a POUbox and homeobox that are, respectively, 75% and 68% identical in amino acid sequence to the corresponding domains in Oct-2. Outside of the POU-homeobox domain, Oct-3 is not homologous to previously cloned genes. Oct-3 does have proline-glycine rich regions at both its amino and carboxy termini which might function as transcriptional activation domains by analogy to the role of similar domains in other transcription factors. Oct-3 binds to the same DNA motif as Oct-2, the octamer motif, and can transactivate immunoglobulin promoters as efficiently as Oct-2.

Oct-3's novel expression pattern distinguishes it from previously cloned transcription factors. Oct-3 is expressed in undifferentiated, but not differentiated, EC and embryonic stem (ES) cells. ES cells are true totipotent stem cells that can give rise to all somatic and germ line lineages of a mouse. Since ES cells are derived from the inner cell mass of the murine blastocyst we studied Oct-3 expression in mouse development by in situ hybridization. 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 mRNA was detected in all cells of the early embryo through the 3.5 days post coitum (dpc) blastocyst stage. Oct-3 expression was seen both in the inner cell mass and the trophectoderm of the 3.5 dpc blastocyst but by 4.5 dpc, Oct-3 expression in the trophectoderm had decreased. At this stage, the primitive ectodermal cells within the ICM, which are pluripotent in developmental potential, expressed Oct-3 strongly compared to the primitive endoderm. After implantation, at the 6.5 dpc egg cylinder stage, Oct-3 expression was restricted to the pluripotent primitive ectodermal cells. During gastrulation, Oct-3 was found to be down-modulated in mesodermal cells of the primitive streak that differentiated from the primitive ectoderm. Oct-3 expression continued in the primitive ectoderm until 8.5 dpc and thereafter Oct-3 was not observed in somatic cells. The feature common to all Oct-3 expressing cells in the early embryo is that they retain the capacity for differentiation along multiple lineages. During later embryogenesis, Oct-3 expression was seen in primordial germ cells and, in the adult, was found in the testis and in maturing oocytes. These findings place Oct-3 in marked contrast with previously studied homeobox genes which are not expressed in pluripotent stem cells or in the germ line but rather are expressed in cells that have differentiated along one of the somatic lineages. Oct 3, therefore, may be important for maintaining the pluripotent state and for committing a stem cell to the germ line lineage.

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SUMMARY REPORT
IMMUNOLOGY BRANCH
October 1989 - September 1990

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. Therefore, no new intra MHC recombinants were detected during the past year.

2. DEVELOPMENT OF MODELS FOR THE INDUCTION OF SPECIFIC TOLERANCE TO MHC PRODUCTS

The induction of intentional mixed chimerism across MHC barriers has been investigated as a means of producing specific transplant tolerance (5021). The use of mixtures of T-cell depleted syngeneic plus allogeneic bone marrow has been found to produce permanent, specific tolerance with full immunocompetence. Reconstitution of irradiated animals with a mixture containing T cell depleted syngeneic marrow plus non-T-cell depleted allogeneic marrow has been found to produce complete allogeneic chimerism while avoiding GVHD. This model has been found to have potential usefulness in the treatment of leukemia, since the procedure does not appear to eliminate the graft-vs-leukemia (GVL) effect.

In order to decrease the toxicity of ablative radiation regimens used to prepare radiation chimeras, the use of monoclonal anti-T-cell subset antibodies as a substitute for part of the irradiation has been explored. Treatment with such antibodies in vivo has been found to deplete mature T-cells effectively from all peripheral lymphoid tissues, but not from the thymus. Selective thymic irradiation has therefore been added to this regimen, and has produced long-term mixed chimerism and specific tolerance across an MHC barrier with a non-lethal preparative regimen. This regimen has now been successfully applied to rat-mouse xenogeneic chimeras as well. 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, and 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) a fact was still seen in animals treated by this new protocol.

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 $\alpha$ and $\beta$ genes. Hybridization with the human DQ$\alpha$ cDNA probe revealed a single fragment in each of the enzymatic digests performed, and showed limited but definite polymorphism. Hybridization with the human DR$\alpha$ cDNA probe showed a set of bands clearly distinct from those revealed with DQ$\alpha$, and showed no polymorphism among the herds. In contrast to the unique hybridization patterns of class II $\alpha$ probes, hybridization with class II $\beta$ probes showed extensive cross hybridization. Polymorphism between all three haplotypes was apparent in many of the digests.

A genomic library has been constructed from the $SLA^c$ 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 $\alpha$ and $\beta$ genes have been identified. Sequences of the cDNA clones have been carried out, as have studies of expression following transformation into mouse cell lines. 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.

5. STUDIES OF BONE MARROW AND ORGAN TRANSPLANTATION IN MINIATURE SWINE

Without exogenous immunosuppression, miniature swine matched for class II and transplanted across a class I plus minor antigen difference have been found to frequently develop specific systemic tolerance. The mechanism of this tolerance has been investigated in vitro (5023) and has been found to involve a specific depletion of class I reactive helper T-cell populations. Also, consistent with a lack of T-cell help is the fact that recipients of class II matched renal allografts which went on to accept such grafts long-term showed a brief rejection crisis at approximately fourteen days during which there was a rise in IgM antibodies to SLA class I antigens, which subsided spontaneously without conversion from IgM to IgG.

Because the response to ablative regimens in miniature swine is much more similar to that of man than is the response of rodents, establishment of a bone marrow transplant regimen in this model has been carried out as a pre-clinical step (5023). Ablative radiation regimens have been established similar to those used for HLA identical sibling transplants in man, and have been found satisfactory to permit MHC matched bone marrow allografts. A new regimen with fractionated irradiation has been found necessary to permit successful transplantation of MHC mismatched bone marrow. This regimen has been found not sufficient to permit T-cell depleted bone marrow grafts to be accepted. Non-T-cell depleted bone marrow grafts across full MHC barriers were found to produce aggressive lethal GVHD. Addition of T-cell depleted autologous bone marrow to the non-T-cell depleted allogeneic inoculum has been attempted and, in some cases, has permitted engraftment while avoiding lethal GVHD. These data may have potential applicability to clinical transplantation. 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 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 05021-18 I

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

| | | | |
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| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 5.0 | 4.0 | 1.5 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Studies are being directed toward understanding the major histocompatibility complex, the structure and function of the products of this complex, and manipulations of immune responses to these products. Current studies include: 1) Characterization of major histocompatibility antigens: Congenic resistant strains of mice are developed, maintained, and used in serologic and immunochemical analyses of the MHC products of the mouse; 2) Studies of monoclonal antibodies to H-2 and Ia antigens: Hybridoma cell lines are produced by fusion of immune mouse spleen cells with mouse myeloma cells. The monoclonal anti-H-2 and anti-Ia antibodies produced by these hybridomas are analyzed by serologic and immunochemical means and are used to further characterize the fine structure of the MHC; 3) 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.



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

Project Description

Major Findings: 1) Fusion of spleen cells from mice hyperimmunized against H-2 antigens and boosted 2-3 days before fusion has been found to give satisfactory results in the production of anti-H-2 hybridomas. About 70 stable hybridomas have so far been produced, most of which detect H-2 or Ia antigens of a variety of haplotypes. Panel testing has indicated that most of these antibodies react with public specificities of the H-2 and Ia antigens, while a few appear to detect private specificities. Numerous crossreactions have been detected using these monoclonal antibodies, which define a variety of new public H-2 and Ia specificities.

2) Using a new MHC recombinant strain (C3H.KBR) we have detected an unusually high responsiveness to Qa antigens. Hybridomas prepared using appropriately immunized spleen cells from this strain yielded 11 new monoclonal antibodies directed against several epitopes of the Qa antigens. Immunoprecipitation studies using these antibodies indicate that at least two different molecules are detectable. In vivo injection of these antibodies has also been found to have profound effects on circulating T-cells. Two of these antibodies of the same immunoglobulin class but directed toward different epitopes of Qa-2 have been found to have different in vivo T-cell depletion characteristics. In vitro studies show that the antibody causing rapid depletion of T-cells in vivo causes much less cell surface modulation of Qa-2 than does the antibody leading to slow depletion. This difference may indicate that the antibody causing modulation reacts with the site on Qa-2 involved in its physiologic ligand binding.

3) A new recombinant which occurred during the backcrossing of A.TH has permitted, for the first time, an analysis of A.TH anti-A.TL reactivity in the absence of a Qa-1 antigen difference. Our results with skin grafting experiments indicate that in the absence of a Qa-1 difference, there is marked prolongation of skin graft survival. These studies suggest that a class II difference alone may be insufficient to cause skin graft rejection, and that class II antigens may actually function by presenting other antigenic differences (e.g., minor antigens or Qa antigens) to the immune system.

4) Mice reconstituted with mixtures of syngeneic plus either allogeneic or xenogeneic bone marrow have been found to manifest specific hyporeactivity to donor type skin grafts at 8 weeks following reconstitution. In the case of mixed allogeneic reconstituted animals, both host and donor lymphoid elements are found in the peripheral circulation, while only host elements can be found in the mixed xenogeneic reconstituted animals. In vitro assays have been performed in order to examine the mechanism of transplantation tolerance in these animals. Evidence for generation of high levels of natural suppressor (NS) cells in the early weeks following grafting has been obtained. Characterization of these suppressor cells by antibody reactivity indicates that they are not T cells. They are derived predominantly from the host component of mixed marrow grafts and may be involved in the elimination of those alloreactive cells which otherwise would cause GVH.

5) Because in vitro assays using spleen cells from mixed chimeras showed maximal suppressive activity at approximately 8 days following radiation and bone marrow reconstitution, delayed administration of non-T-cell-depleted allogeneic marrow to animals irradiated and reconstituted with T-cell-depleted syngeneic marrow was performed. This procedure was found to markedly reduce the GVH produced by allogeneic T cells and to result in long-term completely allogeneic chimeras.

6) Elimination of T cells from the syngeneic component, but not the allogeneic component of a mixed bone marrow inoculum has been found to produce completely allogeneic chimeras which are, however, protected from lethal GVHD. By adding the EL4 murine leukemia to the syngeneic component of such a reconstituting inoculum, it has been found that this procedure does not eliminate the strong graft-vs-leukemia (GVL) effect of non-T-cell depleted allogeneic bone marrow. The GVL effect has also been seen when non-T-cell depleted allogeneic marrow was added as late as 8 days following the T-cell depleted syngeneic marrow plus leukemia.

7) 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-NK1.1 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

ZO1 CB 05023-18 I

PERIOD COVERED

October 1, 1989 to September 30, 1990

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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| | C. LeGuern | Expert | IB, NCI |
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| | K. Nakajima | Visiting Fellow | IB, NCI |

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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.)*

Three herds of miniature swine, each homozygous for a different set of histocompatibility antigens at the MHC have been developed. Current projects include: 1) Assessment of survival of organs and tissue transplants among and between members of these herds as a model for human transplantation; 2) Assessment of the immunologic parameters involved in tolerance to allografts in this species; 3) Detection and characterization of intra-MHC recombinants: Five intra-MHC recombinants have been obtained and have been or are being bred to homozygosity. Kidney transplants utilizing these new recombinants have shown that selective matching for Class II antigens frequently permits long-term kidney graft survival across a Class I difference; 4) Bone marrow transplants in miniature swine: the effect of mixing autologous plus allogeneic marrow in the reconstituting inoculum are being examined. This modality is being assessed as a specific preparative regimen for allogeneic organ transplantation; 5) Production and characterization of monoclonal antibodies reactive with subsets of pig lymphocytes: antibodies corresponding to many of the OKT series in man have been identified (including T4, T8, and T11). The effects of these antibodies on in vitro and in vivo transplantation immunity are being assessed, and they are also being used to assess mechanism of tolerance; and 6) Analysis of class II MHC genes: Southern blot analyses using cDNA probes from human class II genes have been performed and indicate that genes corresponding to each of the major human class II loci are present in the pig genome. In addition, a genomic library and two cDNA libraries have been constructed and screened with these probes. Class II genes from the pig herds have been isolated and characterized by hybridization and by sequence analysis. These genes have also been used in vitro and in vivo for transfection studies, and have been incorporated into retroviral constructs for transduction of bone marrow cells.

Project Description

Major Findings: 1) Breeding of further generations has continued to be successful. We therefore now have three different herds of swine, each homozygous for a different SLA antigen, as well as five lines bearing intra-MHC recombinants.

2) Transplants of kidneys between recombinant and non-recombinant haplotypes have permitted an evaluation of the effects of selective class I and/or class II matching on vascular allograft survival. A non-MHC-linked gene determining immune responsiveness has been detected and determined to control rejection of vascular grafts across non-MHC differences. In animals bearing the non-rejector gene at this locus, vascular grafts are accepted across minor differences and also across class I plus minor differences, but not across class II differences. These studies indicate, therefore, that class II matching is of major importance for survival of vascular grafts.

3) Skin grafts are promptly rejected across class I or class II differences. However, following successful kidney transplants in the class II matched situation, donor skin grafts are specifically prolonged, indicating that acceptance of the vascular graft induces systemic tolerance.

4) The mechanism of systemic tolerance in class II matched kidney allografts has been examined. Studies on the generation of CTLs in vitro indicate that such tolerant animals cannot generate a CTL response across a class I only difference, as can ltheir non-grafted counterparts. The defect appears to involve helper independent class I reactive CTLs, since addition of other helper cells (such as anti-class II reactive populations) leads to normal anti-class I CTL activity. In addition, non-T suppressor cells have been identified as contributing to the defect. The role of local suppressor cells is suggested by studies of graft infiltrating lymphocytes.

5) Southern blot analyses of lymphocyte DNA from each of the miniature swine herds and recombinant lines have been performed. The data indicate that swine possess a single class II $\alpha$ chain gene corresponding to that of the human DR $\alpha$ chain, DQ $\alpha$ chain, and DP $\alpha$ chain. Of these, only the DQ $\alpha$ chain appears to be polymorphic within these herds. Approximately 5-7 class II $\beta$ chain genes have been revealed by these analyses, which show both polymorphism and extensive cross-hybridization between $\beta$ chains of the three major loci.

6) A complete EMBL-3 phage library has been constructed from the c haplotype, and class II genes corresponding to those detected in the Southern blot analysis have been isolated and characterized.

7) cDNA libraries from the c and d haplotypes have been constructed and screened with human class II probes. cDNA clones corresponding to $DQ\alpha^c$, $DQ\beta^c$, $DQ\alpha^d$, $DQ\beta^d$, $DR\alpha^d$, and $DR\beta^d$ have been isolated. Several of these genes have been successfully transfected into mouse L cells, leading to cell surface expression of the swine class II antigens on these cell lines.

8) Analysis of the derived amino acid sequence of the SLA $DR\beta^d$ cDNA gene has led to a surprising finding. Comparison of this sequence with sequences of a panel of human DR$\beta$ genes indicates that at the amino acid level all three hypervariable

regions of the swine sequence are more similar to the corresponding regions of the human DR1$\beta$ allele than are the sequences of any other human DR$\beta$ alleles. At the DNA level, silent substitutions show no differences between different human $\beta$ alleles. These data therefore suggest a high level of selection leading to the amino acid sequence homology between these species.

9) A series of monoclonal antibodies reactive with a variety of swine lymphocyte surface antigens have been prepared. One of these antibodies recognizes an SLA antigen of the SLAdd haplotype, at least two others react with selective T-cell subpopulations, and at least one antibody is reactive with macrophages. Further characterization of these antibody reactivities is in progress.

10) A protocol which permits bone marrow engraftment across MHC differences has been obtained. Using this protocol, fully allogeneic bone marrow grafts led to lethal GVHD. Using a mixture of T-cell depleted autologous bone marrow plus non-T-cell depleted fully MHC mismatched bone marrow, alloengraftment has been obtained in 17 of 19 animals to date. In 9 of these animals clinical GVHD was self-limited, with resolution by the third week. These data indicate that, as in the murine model, T-cell depleted autologous bone marrow has an anti-GVHD effect when administered as part of the mixed bone marrow reconstitution inoculum. The longest survivor to date has lived 154 days, dying of probable radiation damage to the lungs. 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 genes along with constitutive or homologous promoters. These constructs have been effective in transducing both murine and porcine cell lines, leading to expression. Culture systems for bone marrow stem cells are in progress, with the intention of transducing these as well with the new retroviral constructs.

Publications:

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SUMMARY REPORT
EXPERIMENTAL IMMUNOLOGY BRANCH
October 1989 - September 1990

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) transplantation biology; 6) tumor immunology; and 7) flow cytometry. This report briefly summarizes research efforts in each of the foregoing areas during the past year. More detailed information on specific accomplishments can be found in the individual annual reports cited by number in the text.

1. LYMPHOCYTE DIFFERENTIATION AND REGULATION

Dr. Alfred Singer's laboratory has examined the role of CD4 and CD8 accessory molecules in T cell differentiation and function. His laboratory found that antibody induced engagement of CD4 on immature $CD4^+8^+$ (double positive) murine thymocytes caused the immature double positive thymocytes to markedly increase their expression of T cell receptor (TCR) by 3-5 fold (9268), as opposed to the reduction in TCR expression that occurs upon antibody induced CD4 engagement on mature $CD4^+8^-$ (single positive) thymocytes. Since increased TCR expression is a necessary step in the differentiation of immature double positive thymocytes into mature single positive thymocytes, these studies indicate a critical role in T cell differentiation for CD4 engagement on developing thymocytes (9268). Dr. Singer's studies to elucidate the molecular basis for the increased expression of T cell receptor on immature thymocytes in response to CD4 engagement revealed that the great majority of receptor proteins are retained and degraded within the endoplasmic reticulum of immature thymocytes, and that the amount of TCR retained and degraded in the endoplasmic reticulum is regulated by CD4-mediated signals (9268). Indeed, Dr. Singer's laboratory demonstrated that anti-CD4 mAb increases TCR expression by thymocytes because it blocks the generation of tonic CD4-mediated inhibitory signals (9268). Thus, the increase in TCR expression occurring during development does not result from increased synthesis, but rather from decreased degradation of newly synthesized and assembled TCR complexes. Furthermore, the low expression of TCR on immature thymocytes results from active signals mediated by cell surface CD4 molecules. These studies imply that increased TCR expression and function during thymic ontogeny requires release from CD4-mediated inhibition.

Dr. Alfred Singer's laboratory also found that intra-thymic CD4-mediated signals could be revealed by examining the phosphorylation status of the zeta chain of the T cell receptor complex (9268). Consistent with the presence of a tonic CD4 signal in immature double positive thymocytes, Dr. Singer's laboratory found that TCR-zeta is already phosphorylated in immature thymocytes resident in the thymus, but that they spontaneously dephosphorylate upon being separated from thymic epithelium (9268).

Dr. Alfred Singer's laboratory has also examined the role of T cell receptor expression in thymocyte development. In studies in mice which are genetically unable to express T cell receptor (SCID mice), Dr. Singer's laboratory observed

that $TCR^-$ SCID thymocytes are arrested at the $CD4^-8^-$ stage of differentiation. Interestingly, if $TCR^+$ cells were introduced into the SCID thymus, the SCID thymocytes remained $TCR^-$ but would differentiate into $CD4^+8^+$ cells (9273). These studies indicate a role for functional $TCR^+$ cells in initiating the differentiation of $TCR^-$ $CD4^-8^-$ thymocytes, a function that might be performed by $TCR(\gamma\delta)$ in normal T cell ontogeny.

Dr. Alfred Singer's laboratory has analyzed a rare thymocyte subpopulation characterized as $CD4^-CD8^-$ $TCR\alpha\beta$ (9273). These cells have a markedly skewed TCR repertoire, but the skewing does not reflect the accumulation of non-deleted T cells expressing autoreactive TCR. Moreover, their TCR repertoire is not clearly molded by their self-antigens. Finally, these cells have a mature phenotype in that they are $Qa2^+$ and sensitive to cyclosporin treatment (9273).

Dr. Alfred Singer's laboratory has examined the mechanisms by which T cell tolerance is induced in the thymus. His laboratory found that the thymus is able to induce either deletion or inactivation of T cells expressing potentially autoreactive TCR. The alternative outcomes are mediated by two distinct cell types that can be distinguished by their sensitivity to $\gamma$-irradiation (9273).

Members of the b1 (VLA) integrin subfamily of adhesion molecules mediate cell interaction primarily with extracellular matrix (ECM) proteins. Dr. Stephen Shaw's laboratory (9257) has investigated the expression and function of b1 integrins on resting human CD4+ T lymphocytes. Analysis of T cell adhesion to purified human laminin (LN) and human fibronectin (FN) reveals three b1 integrin receptor/ligand interactions: VLA-6/LN, VLA-4/FN, and VLA-5/FN. Two levels of regulation of b1 integrin function on T cells were observed: 1) strong adhesion to LN and FN occurs only after acute activation of T cells with the phorbol ester PMA or crosslinking of the CD3/T cell receptor complex with CD3 mAb; 2) greater adhesion to FN and LN by "memory" T cells (i.e., previously stimulated by exposure to antigen) compared to "naive" T cells correlates with greater expression of VLA-4, VLA-5, and VLA-6 on memory T cells. VLA-4 and VLA-5 on T cells interact with distinct sites on FN that can be specifically inhibited by FN peptides: VLA-5-dependent binding is blocked by RGD peptides while VLA-4-dependent binding is specifically blocked by a peptide derived from the IIICS region ("CS-1 peptide"). Furthermore, immunologic relevance of T cell VLA/ECM interactions is suggested by the ability of both LN and FN to provide a costimulatory signal to CD3-mediated T cell activation. These studies should prove to be seminal ones in understanding the interaction of T cells with ECM.

Identifying and subsequently characterizing T cell subsets has been essential to our current understanding of cellular function and interactions in the immune system. Studies from the laboratory of Dr. Stephen Shaw continue to elucidate the phenotypic heterogeneity of normal human T cells (9257) by defining two further subpopulations within human memory T cells using a mAb specific for CD45RB. Data on further diversity of human CD4+ T cells is being systematically accumulated to enable a much more complete classification of T cell subsets. Limited preliminary studies have been undertaken to characterize CD4 T cells subsets in HIV-infected individuals.

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 approximately 50% 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 anti-viral 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.

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$\beta$ 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$\beta$ expression was carried out by quantitating mRNA corresponding to 16 V$\beta$ gene products, as well as with monoclonal antibodies specific for 9 of these V$\beta$ families, in a large panel of inbred strains. Strain-specific deletions in 9 of the 16 V$\beta$ families were detected and were shown to be related to the expression of multiple MHC and non-MHC self determinants. These findings indicate that maintenance of tolerance to a variety of self determinants results in substantial deletions in available TCR

Vβ repertoire (9265). The self determinants that function as ligands for Vβ-specific T cell deletions were shown generally to represent the products of non-MHC-encoded genes in association with MHC gene products. Ligands responsible for deletion of Vβ11- and Vβ12-expressing T cells were characterized and were shown to represent a previously uncharacterized Mls "superantigen" capable of inducing a strong response by allogeneic T cells. Thus, the set of Mls superantigens appears to be more extensive than was previously appreciated, and these antigens play a critical role as self determinants in shaping the TCR repertoire by negative selection.

The role of the thymus in TCR negative selection was analyzed by examining TCR Vβ expression in T cells which have matured in congenitally athymic nude mice. It was found that deletions of Vβ3 and Vβ11 that normally occur in mice expressing appropriate non-MHC products in association with MHC class II determinants fail to occur in the CD4+ and CD8+ T cells of athymic mice, demonstrating that efficient negative selection is thymus-dependent (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 cyclosporine 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.

2. CELL BIOLOGY OF IMMUNE RESPONSES

Studies in Dr. Alfred Singer's laboratory have identified both $CD4^+$ and $CD8^+$ lymphokine-secreting T-helper cells that initiate anti-MHC responses in vivo and in vitro (9256). Studies to identify any second signals involved in the activation of these two phenotypically distinct populations of T-helper cells revealed that IL-1 and IL-6 are biologically active co-factors that together with ligand, either MHC class I or class II, will trigger either $CD4^+$ or $CD8^+$ Th cells to secrete IL-2 (9256).

Dr. Alfred Singer's laboratory has examined the molecular requirements for MHC recognition by class I allospecific T-killer cells (9256). These studies have identified a segment of the alpha-helical loop of the class I molecule to be a necessary docking site for most Kb-specific T cells.

It is now widely accepted that T cell activation often requires not only occupancy of the T cell receptor but costimulatory interactions of other molecules. These molecules have remained largely undefined. Studies from the laboratory of Stephen Shaw (9257) demonstrate that LFA-1 interaction with its ligand ICAM-1 is such a costimulatory interaction. The experimental approaches

to elucidate this range include: 1) a simple model system designed to be as relevant as possible to antigen-specific recognition: biochemically purified ICAM-1 and T cell receptor cross-linking by anti-CD3 mAb OKT3 immobilized on plastic; 2) a more complex one to assess relevance to cell-mediated costimulation: resting T cells stimulated by OKT3 on beads in the presence of costimulation from activated fixed monocytes (plus IL1/IL6). The findings from these and other model systems demonstrate a potent costimulation from LFA-1/ICAM-1. These results, interpreted in light of a large body of literature, indicate that LFA-1/ICAM-1 is of major physiologic importance as a costimulatory signal.

Dr. Hodes' laboratory has analyzed the mechanisms mediating the function of T helper cells in T dependent B cell antibody responses (9266). A role of both specific and non-specific T cell derived signals was demonstrated for activation of B cell antibody responses. It was shown that the supernatants of activated cloned T helper cells were capable of replacing these T cells in the antigen specific and MHC restricted induction of B cell IgG antibody responses. Two distinct and synergizing activities were identified in this supernatant: IL4, and an antigen-specific and MHC-restricted soluble factor. It was demonstrated that this latter factor can be affinity purified employing monoclonal antibodies specific for T cell receptor V$\beta$8 determinants. Biochemical analysis of 35S-methionine metabolically labeled cell-free material has shown that a disulfide-linked heterodimer indistinguishable from the cell surface TCR $\alpha\beta$ heterodimer is released into the supernatant of multiple murine as well as human T cell lines studied. The $\alpha\beta$ dimer in this material is associated with the CD3$\epsilon$ chain and is not associated with other cell surface molecules such as MHC class I products. It was shown that in its native state the TCR material is associated with lipid, presumably in the form of cell membrane. Further analysis of metabolically labelled cell-free material demonstrated the presence of CD4 molecules that are not lipid associated and that are of a lower molecular weight than the CD4 molecules isolated from the T cell surface.

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 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. Thus, CD44 expression on activated B cells may serve as an adhesion molecule playing a role in B cell trafficking in vivo.

Dr. Segal's laboratory has used bispecific antibodies to study the cellular mechanisms of cytotoxicity and antigen presentation. Bispecific antibodies with specificities for targeting structures on cytotoxic cells and for cell surface structures on target cells redirect the specificities of cytotoxic cells. Work in Dr. Segal's laboratory has recently identified the subsets of cells in human lymphocytes that contain cytotoxic activity targetable through $\gamma/\delta$ TcR chains (9254). These studies showed that there are two subsets of targetable $\gamma/\delta^+$ T

cells separable by buoyant density, and further divisible by cell surface markers. The two subsets have different activation requirements and can mediate potent cytotoxic activity against many types of target cells. In general, the $\gamma/\delta^+$ and $\alpha/\beta^+$ T cells are similarly distributed in PBL with respect to buoyant density, activation requirements, and ability to mediate MHC unrestricted cytotoxicity. These results suggest that $\gamma/\delta^+$ T cells have similar cytotoxic function as $\alpha/\beta^+$ T cells in PBL.

Dr. Segal's laboratory has also used bispecific antibodies to target antigens to antigen presenting cells (APC) in vitro and in vivo (9290). Bispecific antibodies that bind antigen to MHC molecules or sIg on B cells greatly enhance the efficiency with which the antigen is presented to T cells. In vitro, antigen bound to sIg is rapidly internalized and then slowly processed and presented to T cells. Antigen bound to MHC molecules is taken up slowly, and is presented less efficiently than antigen bound to sIg on B cells. Antigen bound to $Fc_\gamma R$ or B200 on B cells is not presented. These studies are important because they define the processing pathways by which resting B cells present antigen to T cells.

Dr. Segal's laboratory has also demonstrated that immunization of mice, as measured by antibody production, is greatly enhanced when the antigen is injected with bispecific antibodies (9290). Bispecific antibodies with specificity for MHC molecules and $Fc_\gamma R$, but not sIgD, mediate this effect, suggesting that B cells are not involved in the enhancement of immunogenicity of antigen in the primary immunization. These observations should have practical use in immunizing people or animals against viral, tumor, or parasitic antigens.

3. SIGNAL TRANSDUCTION

In order to understand structure function relationships in T cell receptor mediated signal transduction, Dr. Weissman's laboratory has undertaken site-specific mutagenesis of the T cell receptor zeta subunit. The zeta subunit was chosen for study as it is a substrate for a receptor activated protein tyrosine kinase and undergoes multiple, apparently cooperative, phosphorylations in response to receptor activation. The effects of these mutations have been analyzed by expression of the mutated proteins in zeta negative T cell hybridomas. Mutagenesis of Lysine150, which has been postulated to be a key residue in a nucleotide binding consensus sequence, does not effect receptor mediated signalling as evaluated by tyrosine phosphorylation of the zeta subunit itself. In contrast, mutagenesis of either the last tyrosine of zeta or the penultimate tyrosine to phenylalanine residues resulted in deficient tyrosine phosphorylation of zeta. Although tyrosine phosphorylation is effected by both of these mutations each appears to result in a distinct functional phenotype as defined by interleukin-2 production and growth inhibition in response to prolonged stimulation. These results suggest that the last two tyrosines of zeta are either substrates for a receptor activated protein tyrosine kinase or affect the ability of other tyrosines to be substrates for a protein tyrosine kinase (9293).

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 has for the study of T cell hybridomas. 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 zeta 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. Studies using antibodies directed against accessory signalling molecules suggests that the conditions required to maintain phosphorylation in permeabilized cells vary depending on what surface molecule is being stimulated (9292).

Dr. Weissman's laboratory has also examined the signalling role of the zeta chain in natural killer cells, which are CD3 negative. His laboratory has been able to demonstrate that the zeta 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 zeta. 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 zeta 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 zeta (9294).

The role of "second messengers" mediating activation of T cells through the TCR/CD3 complex was analyzed in cloned T cell populations by Dr. Hodes (9281). A cloned T cell population that was maintained by repeated stimulation in vitro with IL2 alone was capable of responding to subsequent stimulation with anti-CD3 antibody by proliferating and by strong phosphatidyl inosotol (PI) hydrolysis and increased intracellular calcium concentration. In contrast, the same cloned line maintained by stimulation with specific antigen and antigen-presenting cells responded to anti-CD3 stimulation by proliferating, but without measurable PI hydrolysis or calcium response. The signalling pathways utilized by cloned T cells were therefore influenced by prior stimulation through the TCR.

4. STRUCTURE, REGULATION AND FUNCTION OF GENES INVOLVED IN IMMUNE RESPONSES

Studies carried out in Dr. Dinah Singer's laboratory have demonstrated that the swine class I MHC multigene family contains only seven members, and as such is the smallest known family. Despite the small size of the family, three sub-groups of genes can be discerned based on their sequence homologies. The three sub-groups defined by DNA sequence also display discrete patterns of expression (9270,9276). In order to investigate the molecular basis for the differential patterns of expression, Dr. Dinah Singer's laboratory has begun to generate and analyze transgenic mice containing isolated swine class I genes (9276). For one gene encoding a classical transplantation antigen, PD1, it has been demonstrated that the pattern of expression of the swine gene in the transgenic parallels that observed in situ in the pig. Lines of transgenic mice containing various constructs of another class I gene, PD7 have been generated. Analysis of PD7 expression in these mice, as a function of the various regulatory elements present, reveals the presence of a lymphoid tissue specific silencer which contributes to the regulation of PD7 in vivo (9276).

Dr. Dinah Singer's laboratory has initiated studies to characterize DNA sequence elements and cognate trans acting factors associated with class I MHC genes (9270, 9276). A series of negative and positive regulatory DNA sequences elements within the 5' flanking region of the classical transplantation antigen gene, PD1, have been identified. One of the silencer elements associated with this gene consists of a regulatory DNA complex in which reside overlapping negative and positive elements. Analysis of this complex has 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.

Class I MHC gene expression is known to be induced by immunomodulators. Recent studies in Dr. Dinah Singer's laboratory have demonstrated that ethanol, at physiologically attainable levels, is able to act as an immunomodulator by elevating steady state RNA and cell surface levels. Measurement of class I MHC antigen on peripheral blood lymphocytes in acutely intoxicated individuals showed a highly significant increase over controls (9285). Analysis of the effect of ethanol on internal organs in a mouse model of alcoholism revealed that class I MHC RNA levels are significantly elevated in ethanol-fed mice, but not in control-fed mice. In addition, acute cellular depletion of splenocytes was noted, with a selective sparing of the T lymphocyte population. These findings suggest a model in which autoimmune mechanisms may play a role in alcohol related diseases.

Dr. Dinah Singer's laboratory has identified a highly divergent member of the murine class I MHC gene family, M1, which has been used to define a new MHC subregion, M (9279). M1 appears to represent a direct descendant of a primordial class I gene, which antedates speciation. Analysis of M1 expression has revealed that expression of M1 is controlled by a strong silencer element located within a 16 kb genomic fragment containing the M1 gene.

Studies in Dr. Weissman's laboratory have focused on elucidating the genomic organization of the human zeta gene. Studies have determined that the cDNA encoded for by zeta is encoded on at least 8 distinct exons that span at least 15kB of genomic DNA. Although the transcription initiation sites have not yet been determined with certainty, there was no evidence of classic TATA or CAAT boxes. There is a large first exon whose full size is yet to be determined. A variable number tandem repeat restriction fragment polymorphism exists within the zeta gene. This polymorphism has been localized to a 6KB BamH1 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. Hybridization of genomic clones encoding zeta with an oligonucleotide based on the specific murine eta cDNA sequence has revealed an area of strong hybridization in the 3' region of the zeta gene. This finding lends credence to the suggestion that zeta and eta are in fact alternative splice products of the same primary transcript (9291).

5. TRANSPLANTATION BIOLOGY

Studies from Dr. Alfred Singer's laboratory have focused on the basic cellular mechanisms responsible for initiating and mediating transplantation responses in vivo (9275). Dr. Alfred Singer's laboratory found that in vivo skin allograft rejection responses required the initial activation of lymphokine secreting Th

cells and the subsequent activation of lymphokine responsive Tk cells. These two T cell functions could either be meditated by interactions between distinct populations of Th and Tk cells, or, alternatively, could be mediated by a activation of a single population of dual function T cells that both secreted lymphokine and differentiated into cytolytic effector cells (9275). Consistent with Tk cells performing the effector function of allograft rejection, Dr. Singer's laboratory found that the effector mechanism of in vivo allograft rejection is highly antigen-specific, destroying cells expressing alloantigens but sparing cells within the graft that do not. Surprisingly, Dr. Singer's laboratory found a similar degree of antigen-specificity for Ia-restricted $CD4^+$ T-effector cells, even though most of the epidermal cells within a skin allograft are MHC class II negative (9275). It was likely from these findings that $Ia^-$ keratinocytes are induced by such agents as interferon-$\gamma$ to express Ia determinants during the in vivo rejection response, making them targets for Ia-restricted T-effector cells. Indeed, Dr. Singer's laboratory found that monoclonal antibodies specific for murine interferon-$\gamma$ blocked the rejection of Ia-disparate skin allografts, demonstrating a requirement for endogenously produced interferon-$\gamma$ presumably to induce Ia expression on keratinocytes (9275).

To ascertain that the immune mechanisms involved in rejection of skin allografts are also involved in rejection of other types of allografts, Dr. Alfred Singer's laboratory has examined the cellular basis for rejection of fetal pancreas allografts and isolated Islet cell allografts (9275). These studies revealed that the cellular basis for rejection of fetal pancreas allografts is identical to that involved in rejection of skin allografts (9275). In contrast, rejection of isolated Islet cell allografts is accomplished entirely by $CD4^+$ T cells, and not by $CD8^+$ T cells (9275). The basis for the limited rejection response against isolated Islet cell allografts is thought to be the absence of donor APC in the Islet cell allografts capable of activating $CD8^+$ Th cells (9275).

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 allogeneic 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.

Graft-versus-host disease (GvHD) is dependent in its generation on the presence of immune competent T cells in the donor marrow with specificity for allogeneic

antigens of the host. One approach to preventing GvHD is the removal of these T cells from the marrow inoculum. Monoclonal antibodies with specificity for cell surface antigens expressed by human T cells have been used in Dr. Gress' laboratory (9271) to deplete normal T cells from allogeneic marrow and to remove malignant T cells from autologous marrow. A clinical protocol has been developed to assess the feasibility of utilizing these marrows in the treatment of aggressive T cell malignancies in combination with marrow ablative chemotherapy and radiotherapy. The initial stage of a phase one study has been completed with definition of a new chemotherapy regimen, the efficacy of which is under evaluation.

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL), may play a significant role in mediating allogeneic marrow graft rejection. In a murine model system developed in Dr. Gress' laboratory (9287), it was found that: 1) a cloned CTL population was sufficient to reject an allogeneic marrow graft; and 2) the mechanism by which these marrow grafts were rejected was specific for MHC gene products expressed by the donor marrow and corresponded to the cytotoxic specificity of the clone. One approach to inhibit such graft rejection by CTL is administration of suppressor cells in vivo. Because IL-2 enhances suppressor activity of T cell depleted donor marrow, such marrow was evaluated for its ability to engraft sublethally irradiated, MHC-disparate hosts. In the presence of IL-2 administration to the host, enhancement of engraftment was observed. A second approach to inhibit marrow graft rejection by host CTL is by the administration of monoclonal antibodies with specificity for CTL surface antigens. Administration of anti-CD3 to mice results in T cell activation within hours of administration, manifest by secretion of colony stimulating factors (CSF) detectable in the serum for 24-48 hours. The injection into sublethally irradiated hosts of anti-CD3 monoclonal antibody with donor marrow results in allogeneic chimerism. If the antibody is given seven days before the marrow, such facilitation of engraftment is not observed, although host T cell responses are suppressed.

Studies in murine marrow chimeras have suggested that MHC mismatched marrow transplantation is associated with immunoincompetence of the T cell arm of the immune response. To evaluate whether T cell maturation processes are comparable in mouse and man, the development of T cell responses in a primate, the rhesus monkey, has been studied in other experiments from Dr. Gress' laboratory (9288). The time course of $CD4^+$ and $CD8/CD28^+$ T cell reconstitution and the time course of the development of in vivo T cell immunocompetence (as defined by the ability to respond to an organ allograft) correlated with the number of T cells infused in autologous marrow, and did not correlate with marrow cell dose or with time of hematopoietic reconstitution, raising the possibility that residual T cells in the infused T cell depleted marrow played a central role in the generation of subsequent T cell populations. This would imply that the generation of T cells in marrow grafted primates may not be the same as in the mouse. Such differences would potentially have important implications for the transplantation of HLA-mismatched marrow in man. Studies of T cell regeneration in patients receiving T cell depleted autologous marrow demonstrate the regeneration of $CD4^+$ and $CD8^+/CD28^+$ subsets of T cells which appear, by preliminary evaluation, to be oligoclonal rather than polyclonal in nature.

6. TUMOR IMMUNOLOGY

Drs. Segal and Wunderlich have used targeted cytotoxic cells to specifically kill tumor cells in vitro and block the growth of tumor cells in vivo, in mouse models (9250,9254). Targeting is done using bispecific antibodies that react with the tumor cells and with triggering sites on effector cells. These laboratories have shown that a targetable tumor growth inhibition activity is mediated by human peripheral blood T and K cells both in vivo and in vitro. This activity 1) is different from cytotoxicity as measured in a $^{51}Cr$ release assay, 2) is mediated by factors secreted by targeted T or K cells, and 3) is able to block the growth of bystander tumor cells. Indeed, it has been demonstrated that targeted PBL block the growth of bystander tumor cells in Winn assays in vivo and in tumor growth inhibition assays in vitro, but fail to lyse bystander tumor cells in vitro. This is an extremely important observation, because it suggests that targeted PBL can block the growth of tumor cells to which they do not bind, as long as they are in the vicinity of tumor cells that are bound by the bispecific antibody. Under these circumstances, tumor cells should not be able to escape the anti-tumor effects of targeted cytotoxic cells simply by expressing low amounts of the tumor antigen, or by being inaccessible to the targeted cell.

Drs. Segal and Wunderlich have also demonstrated that human PBL, targeted with bispecific antibodies, block the growth of human ovarian carcinoma cells established in the peritoneal cavity of nude mice (9250,9254). In assays lasting two weeks, targeted PBL activated in different ways are highly effective in eradicating tumor. In long term studies, targeted PBL prolong host survival, but the effects are not yet optimized. These results are relevant to clinical studies now being initiated in the Netherlands and Italy, for treating ovarian carcinoma in cancer patients with targeted cytotoxic cells.

Studies from Dr. Wunderlich's laboratory have identified a mechanism for elevating the state of activation of both MHC-independent cytotoxic activity and targetable T-cell activity against tumor cells (9250). They have found that exposure of mouse splenocytes to nanogram levels of bacterial lipopolysaccharide (LPS) in vitro, elevates antitumor activity resulting from T cells targeted with bispecific antibodies. Both native and detoxified LPS exhibit this activity. With the addition of selected polyanions, MHC-nonrestricted antitumor cytotoxic activity also increases. The mechanism 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. Under normal circumstances, this mechanism may be initiated in vivo by LPS-activation of B cells in the intestinal wall.

7. FLOW CYTOMETRY

The Experimental Immunology Branch flow cytometry laboratory is managed by Susan Sharrow (9255). The laboratory currently supports over 80 individual research projects for more than 55 investigators. These investigations involve quantitative single cell analysis of parameters associated with cell types freshly prepared from different species/tissues, as well as spectrum of in vitro cultured cells. Basic research support is provided to members of the EIB and the Immunology Branch, as well as to other members of DCBDC. Currently supported projects include a broad range of applications in the areas of

cellular immunology, immunochemistry, transplantation biology, clinical immunology, immunogenetics, tumor immunology, and molecular immunology. These include, but are not limited to, the following studies: a) in vivo and in vitro analyses of intra-cellular signaling via T cell surface molecules, b) characterization of T cells from animals with genetic or induced immune dysfunction; c) studies of the pathogenesis of graft-versus-host disease; d) analyses of the correlations between T cell memory and the quantitative expression of cell surface adhesion molecules; e) investigations of T cell ontogeny and differentiation; f) studies of mechanisms of T cell repertoire generation; g) analyses of transplantation antigens and of gene regulation of expression of these antigens; h) investigations involving mechanisms and manipulations of antigen-presentation processes; and i) analyses of the mechanisms involved in marrow graft rejection versus acceptance.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09250-25 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Cell-Mediated Cytotoxicity

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

| | | | |
|---|---|---|---|
| PI: | John Wunderlich | Senior Investigator | EIB, NCI |
| Others: | David Segal | Senior Investigator | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER |
|---|---|---|
| 2.0 | 2.0 | 0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Bispecific antibodies have been used to redirect the antigen specificity of PBL from normal human donors, so that they become cytotoxic for an allogeneic ovarian carcinoma cell line. The altered lymphocytes can kill the tumor cells in vitro and block intraperitoneal growth of an established tumor in immunodeficient mice. The degree of antitumor activity depends on the types of lymphocytes in the donor sample, the procedure used to make the bispecific antibodies, and the techniques used to activate the lymphocytes in vitro prior to use in the experiments.

A natural mechanism for nonspecifically activating MHC-independent cytotoxic cells and CTL is indicated by results of in vitro studies carried out with mouse cells. The mechanism is initiated by polyanions and low levels of lipopolysaccharides, such as may occur from naturally resident bacterial flora. The response to these activating factors utilizes ancillary cells, primarily B lymphocytes and T-helper cells, in addition to the precursors to the activated cytotoxic cells.



PHS 6040 (Rev 1/84)

GPO 914-918

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 human lymphocytes to human tumor cells, and b) MHC-nonrestricted cytotoxicity mediated by activated killer (AK) cells.

1. Retargeting the human immune system against human tumor cells. Earlier, we established both in vitro and in vivo models for redirecting the immune specificity of peripheral blood T cells from normal donors, so that they would react with an ovarian carcinoma cell line. The change in immune specificity is mediated through covalently linked bispecific antibodies that react on one hand with the T cell receptor as a triggering site and on the other hand with tumor cell-surface antigens. Irrespective of their native antigen specificity, CTL that are attached to target cells through these bispecific antibodies, kill the target cells. Both types of antibodies, which are combined to form the bispecific reagent, are murine monoclonal antibodies. The criteria for choosing an antitumor antibody includes highly selective binding to tumor cells in contrast to normal cells. Redirected cytotoxic T lymphocytes (CTL) destroy tumor cells both in vitro and in vivo. In the latter case, when given intraperitoneally they block the growth of ovarian carcinoma cells established 4-6 days earlier in the peritoneal cavity of immunodeficient nude mice. About 60% of the treated mice have tumor-free peritoneal lavage fluid 2 weeks later, relative to about 20% of mice treated with a variety of control procedures.

Progress this year with retargeting human T cells against human tumor cells has been made in the following areas. 1) Bispecific antibodies generated by hybrid hybridomas, or quadromas, are more active in vitro than those produced by chemically crosslinking the two types of monoclonal antibodies. 2) Effector T cells from peripheral blood T cells, preactivated in vitro by anti-CD3 and recombinant IL-2, are more active in retargeting experiments in vitro than those activated by either reagent alone. The additional cytotoxic activity results primarily from activation of resting, dense T cells by anti-CD3 plus IL-2. In contrast to low density T cells, the high density cells cultured with IL-2 alone cannot be recruited as cytotoxic cells with bispecific antibodies. 3) Survival studies lasting 6 months have demonstrated that retargeted lymphocytes from some donors are able to protect against tumor growth. The lymphoctyes were preactivated with anti-CD3 plus IL-2. Why protection does not result from treatment with retargeted cells from all donors is not clear.

2. MHC-nonrestricted cytotoxicity of murine activated killer (AK) cells against tumors. Previously, we established that potent MHC-non restricted cytotoxicity against tumor cells is generated by culturing murine splenocytes for 5 days with small amounts of lipopolysaccharide (LPS) and selected polyanions. Most of the cytotoxicity, referred to here as activated killer (AK) cell activity, results from activated NK cells. The response requires cell cooperation between accessory cells, T-helper cells, and precursors to the cytotoxic cells. Enhanced production of IL-2 or interferon, which can elevate AK cell activity when added exogenously, is not required for the response.

Results of experiments conducted this year indicate that the response may have a role in maintaining AK cell activity in situ. These experiments required the

ability to both deplete and reconstitute individual cellular components involved in the in vitro response. They also required the ability to rapidly inactivate extracellular LPS and polyanions in a fashion not toxic for cells. These steps were successfully developed and applied this year.

We have found that picogram levels of LPS, combined with polyanions, are able to stimulate in vitro generation of AK activity. These results are in sharp contrast to the microgram levels of LPS previously used alone by others for activating NK cells. The LPS activity is directed primarily at the accessory cells, which are mainly radiation-sensitive B lymphocytes that can be enriched by Percoll density gradient fractionation combined with depletion of both T cells and NK cells. Although LPS is a well-established B cell mitogen, proliferation of these cells is not necessary for the response. Thus, isolated B cells, whose proliferation is blocked by mitomycin C, still provide the necessary accessory cell activity when added to a mixture of T-helper cells and AK precursor cells, together with LPS and polyanions. Exposure of cells to LPS for as little as an hour is effective, but the exposure must occur within the first day of a 5-day response in order to generate near-maximum responses. By contrast, exposure to polyanions can occur as late as 3 days into the response. LPS is known to stimulate release of TNF$\alpha$ and interferon $\alpha/\beta$, and both of these factors may be involved in the response, insofar as both factors synergize with polyanions to stimulate generation of AK cell activity. Active sources of naturally occurring polyanions have yet to be identified, but the numerous possibilities include native polynucleotides, heparin, chondroitin sulfate, hyaluronic acid, and polyglycans. Their target cells among the types of cells participating in the AK cell response, have not been identified.

LPS and polyanions also induce polyclonal activation of murine CTL activity. This conclusion follows from experiments in which splenocytes, cultured for 5 days with LPS and polyanions, generate polyclonal CTL that are targetable with bispecific antibodies that bridge T-cell receptors and target cell antigens.

Our results are consistent with the following model. B lymphocytes exposed to low levels of naturally occurring LPS, such as in Peyer's patches in the intestinal mucosa, are activated as accessory cells. The activated B cells cooperate directly with T-helper cells, NK cells, and polyanions at the site of activation to generate AK cells and to nonspecifically activate CTL, or they migrate to distant parts of the body to carry out this function.

Proposed Course of Project:

1. Retargeting the immune system against tumor cells. We plan to pursue the following issues regarding retargeting the immune system against tumor cells. 1) The effect of retargeted human T cells on the survival of immunodeficient mice bearing ovarian carcinoma cells will be continued in the laboratory of Drs. Colnagi and Mezannica at the Instituto Nazionale Tumori in Milan, Italy. 2) A syngeneic mouse model for retargeting the immune system against syngeneic tumors is being set up in our laboratory. The most promising tumor system, in which both the antitumor monoclonal antibodies and the antibody-producing hybridoma cell lines are available, involves C3H carcinomas induced by mammary tumor viruses. 3) We plan to start work on hybridizing selected receptors on CTL, using variable region genes of antitumor monoclonal antibodies spliced to constant region genes of triggering sites.

2. MHC-nonrestricted cytotoxicity of activated killer (AK) cells against tumors. We plan to investigate the following issues concerning generation of AK cells. 1) What is the cellular target of polyanions, and what are the polyanions, occurring naturally in situ, that synergize with low levels of endotoxins such as LPS in order to maintain an activated state of AK and CTL cells? These issues will be addressed by cell depletion and addback experiments, combined with activation of selected cell populations by LPS and/or polyanions. 2) Can differences in the capacity to generate AK activity that are mouse strain dependent, be associated with a specific cell population? Preliminary results indicate that accessory cells account for the differences. This issue will be addressed by cell depletion and addback experiments in which participating cell types are mixed between MHC-compatible high and low responder strains. 3) Among the cytokines released by stimulation with polyanions and LPS, which are responsible for direct activation of the cytotoxic precursor cells? This issue will be addressed by adding purified recombinant cytokines to NK cells isolated from normal splenocytes.

Publications:

Garrido MA, Perez P, Titus JA, Valdayo MJ, Winkler DF, Barbieri SA, Wunderlich JR, Segal DM. Targeted cytotoxic cells in human peripheral blood lymphocytes. J Immunol 1990;144:2891-2898.

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 Research 1990;50:1-6.

Garrido MA, Valdayo MJ, Winkler DF, Titus JA, Perez P, Segal D, Wunderlich J. Refocusing the immune system to react with human tumors. Developments in Biological Standardization 1990;71:33-42.

Wunderlich JR, Shearer GM. The $^{51}$Cr-release assay for lysis of target cells by cytotoxic T-lymphocytes (CTL). In: Coligan JE et al Ed. Current protocols in immunology. 1990; in press.

Wunderlich JR, Hodes RJ. Principles of tumor immunity: biology of cellular immune responses. In DeVita Jr., V.T., Hellman, S., and Rosenberg, S.A. Biologic therapy of cancer: principles and practice. Lippincott, Philadelphia. 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09251-20 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Target cell damage by immune mechanisms

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

| | | | |
|---|---|---|---|
| PI: | P.A. Henkart | Senior Investigator | EIB, NCI |
| Others: | J. Shiver | Staff Fellow | EIB, NCI |
| | S. Winslow | Microbiologist | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS. | PROFESSIONAL | OTHER |
|---|---|---|
| 2.0 | 2.5 | 0.5 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

In order to test the granule exocytosis model for lymphocyte cytotoxicity, we have examined the function of a non-cytotoxic cell which possesses a well-defined regulated secretory system after transfection for the granule cytolysin of cytotoxic T lymphocytes. The cells chosen for this are RBL, a rat mucosal mast cell tumor line, which degranulates in response to cross-linking its IgE Fc receptor. In order to allow transfection, a cDNA cloned for the cytolysin was inserted into the mammalian cell expression vector pSVL. This construct was co-transfected with the neo-containing pSV3 using calcium phosphate, and RBLs grown in G418 to select for transfectants. Northern blots revealed a low but positive level of cytolysin expression in the surviving cells, which were then cloned by limiting dilution. Clones were individually screened by Northerns and those with the highest expression were selected for further study (RBL-cy). Homogenates of RBL and RBL-cy were fractionated on Percoll gradients, and the dense granule fractions examined for cytolysin activity. The calcium dependent hemolytic activity characteristic of cytolysin was found in RBL-cy but not RBL granules. Rbl-cy were found to have a potent cytolytic activity against TNP-RBC in the presence of an IgE anti-DNP, giving 50% lysis at an E/T of about 1. Under these conditions, cytotoxic activity was undetectable in RBL, as was the activity of RBL-cy in the absence of IgE. RBL-cy cytotoxic activity was completely abolished in the presence of EGTA, as expected from the granule exocytosis model. Bystander RBC labeled with $^{51}Cr$ but not TNP-modified were not lysed by RBL-cy in the presence of an excess of co-pelleted unlabelled TNP-RBC, thus reproducing the classic sparing of bystanders by CTL. TNP modified tumor targets were lysed only to a marginal level by RBL-cy under conditions giving excellent lysis of TNP-RBC.





GPO 914-918

Project Description

Major Findings:

In order to test the granule exocytosis model for lymphocyte cytotoxicity, we have examined the function of a non-cytotoxic cell which possesses a well-defined regulated secretory system after transfection for the granule cytolysin of cytotoxic T lymphocytes. The cells chosen for this are RBL, a rat mucosal mast cell tumor line, which degranulates in response to cross-linking its IgE Fc receptor. In order to allow transfection, a cDNA cloned for the cytolysin was inserted into the mammalian cell expression vector pSVL. This construct was co-transfected with the neo-containing pSV3 using calcium phosphate, and RBLs grown in G418 to select for transfectants. Northern blots revealed a low but positive level of cytolysin expression in the surviving cells, which were then cloned by limiting dilution. Clones were individually screened by Northerns and those with the highest expression were selected for further study (RBL-cy). Homogenates of RBL and RBL-cy were fractionated on Percoll gradients, and the dense granule fractions examined for cytolysin activity. The calcium dependent hemolytic activity characteristic of cytolysin was found in RBL-cy but not RBL granules. Rbl-cy were found to have a potent cytolytic activity against TNP-RBC in the presence of an IgE anti-DNP, giving 50% lysis at an E/T of about 1. Under these conditions, cytotoxic activity was undetectable in RBL, as was the activity of RBL-cy in the absence of IgE. RBL-cy cytotoxic activity was completely abolished in the presence of EGTA, as expected from the granule exocytosis model. Bystander RBC labeled with $^{51}Cr$ but not TNP-modified were not lysed by RBL-cy in the presence of an excess of co-pelleted unlabelled TNP-RBC, thus reproducing the classic sparing of bystanders by CTL. TNP modified tumor targets were lysed only to a marginal level by RBL-cy under conditions giving excellent lysis of TNP-RBC.

Proposed course:
We plan on trying various manipulations of this system to accomplish the lysis of tumor cells. The first one is to co-transfect genes for the CTL granzymes into the RBL-cy, and we are currently creating the appropriate expression vector for granzyme A. We will then proceed with granzyme B. In addition, we will collaborate with Paul Millard at Cornell University to examine RBC killing by RBL-cy using time lapse video microscopy with calcium localization.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09252-19 E

PERIOD COVERED
October 1, 1989 to September 30, 1990

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

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

| | | | |
|---|---|---|---|
| PI: | R. J. Hodes | Senior Investigator | EIB, NCI |
| Others: | S. A. Rosenberg | Chief | SB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH
Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL | OTHER |
|---|---|---|
| 0.1 | 0.1 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

D

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

A controlled, randomized trial comparing immunotherapy to chemotherapy in stage I and stage II malignant melanoma has been initiated. A total of 181 patients have entered the trial, which is closed to further accrual of patients. Preliminary evaluation of data has demonstrated no significant effect of adjuvant therapies on clinical course.



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

Project Description

Major Findings:

As of Dec. 1, 1982, 181 patients had been randomized into this protocol. The state of the trial is summarized in the following table:

| | Control | MeCCNU | BCG | BCG & Vaccine |
|---|---|---|---|---|
| Total Patients Entered | 43 | 46 | 47 | 45 |
| Recurrence | 32 | 25 | 38 | 25 |
| Deaths | 24 | 23 | 27 | 21 |

In vitro assays have only been carried to the point of indicating that patients are being effectively immunized with the vaccine (see Project No.Z01-CB-05016-10 I). In addition, PPD tests indicate that all patients receiving BCG have converted to a positive skin test.

Proposed Course of Project:

No further patient accrual will occur. All treatment has been completed and, for those patients already on study, follow-up as described by the IB-2 protocol will be continued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09254-16 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 | Senior Investigator | EIB, NCI |
| Others: | D. Mezzanzanica | Guest Researcher | EIB, NCI |
| | S. Andrew | Visiting Fellow | EIB, NCI |
| | J. Wunderlich | Senior Investigator | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 0.5 | 1.0 |

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

☐ (a1) Minors

☐ (a2) Interviews

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

1. Bispecific antibodies with specificity for TcR $\gamma/\delta$ chains can induce human PBL subsets to lyse surrogate target cells. Not all targetable activity is in the $\gamma/\delta$+ T cells.

2. Targeted human PBL exhibited an anti-tumor activity mediated by factors secreted into the medium. This activity leads to the eradication of bystander tumor cells, and appears to operate both in vitro and in vivo.

3. Targeted human PBL can block intraperitoneal growth of established human ovarian carcinoma in nude mice. The potency of the effect is donor dependent, and varies with the type of bispecific antibody used, the mode of activation of the PBL, and the treatment schedule.



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

Project Description

Major Findings:

Targeting with $\gamma/\delta$+ T cells

In order to test which subsets of TcR $\gamma/\delta$+ cells in normal human PBL are cytotoxic, we targeted isolated PBL subsets using an anti-$\gamma/\delta$ mAb (11F2) crosslinked to an anti-target cell antibody. Targetable cytotoxic activity was mediated by fresh unstimulated PBL. The amount of this lysis rarely approached the amount which resulted from anti-CD3-targeted lysis. $\gamma/\delta^+$ T cells were found in both high and low density Percoll fractions, were $CD4^-$ and expressed variable percentages of CD8 and CD56. These cells required rIL2 to maintain their activity. The high buoyant density cells initially contained no targetable $\gamma/\delta$ cytotoxic activity. When the high density cells were activated with immobolized OKT3 or 11F2 in the presence of rIL2 both anti-CD3 and anti-$\gamma/\delta$ cytotoxic activities were generated. These $\gamma/\delta^+$ cytotoxic cells did not express CD4 or CD56, but were both $CD8^+$ and $CD8^-$. Although the $\gamma/\delta$+ T cells were potent mediators of targeted cytotoxicity, it is clear that much of the targetable activity in PBL was mediated by CD3+, $\gamma/\delta$- T cells, ie, $\alpha/\beta$+ T cells. In general, targetable $\gamma/\delta$ activity is distributed similarly in PBL to the targetable $\alpha/\beta$ activity.

Targeted tumor neutralization

Targeted tumor neutralization is measured in vitro by following tumor growth in culture over several days, in the presence or absence of lymphocytes targeted with bispecific antibodies. We have found that this activity is different from that measured in a standared 4 hr $^{51}Cr$ release assay by several criteria:

1. Subsets of cells that do not mediate cytotoxicity, do block tumor growth. Such cells include high buoyant density PBL and CD4+ PBL. Cells that are cytotoxic also mediate this activity.
2. Bystander cells are not lysed in cytotoxicity assays, but the growth of bystander tumor cells is blocked in tumor growth inhibition assays. In the cytotoxicity assay, the target cell must be bound directly to the effector cell in order for lysis to occur. In the growth inhibition assay, crosslinking of cell surface receptors is sufficient to elicit the effect. We have found that anti-CD3 absorbed to tissue culture wells will promote tumor growth inhibition by PBL, as will anti-CD3 in the presence of monocytes. Anti-CD3 $F(ab')_2$ did not induce tumor growth inhibition in either the presence or absence of monocytes, suggesting that anti-CD3 in the presence of monocytes induces tumor growth inhibitory activity by crosslinking CD3 on the T cells, using the Fc$\gamma$R on the monocytes to crosslink the anti-CD3.
3. Tumor growth inhibition can be mediated by factors secreted into the medium from PBL cultured on anti-CD3-coated flasks. These factors appear at 2-3 days of culture, and then decrease. None is seen within 4 hr of culture, the time used in cytotoxic assays.

We have not isolated the factors involved in tumor growth inhibition, but they probably consist of a mixture known cytokines and lymphokines, because we have found that antibodies to $\gamma$-interferon, and probably TNF-$\alpha$ and B partially block tumor growth inhibitory activity. The tumor inhibition activity measured in vitro, appears to be important in at least one in vivo situation,

the Winn type tumor neutralization assay. We have found that targeted effector cells block the growth of bystander tumor cells in Winn assays, and that CD4+ and high buoyant density cells, cells unable to mediate cytolysis, are also effective in Winn assays.

Targeted Cytotoxicity in vivo

We have established a system to test whether targeted cytotoxic cells could eradicate an established tumor. Nude mice with intraperitoneal growth of the human ovarian adenocarcinoma line, OVCAR3, are given intraperitoneal injections of target human PBL. We chose this sytem because both the tumor and the treatment are confined to the peritoneum, thereby minimizing problems of delivering the targeted cytotoxic cells to the tumor.

In short term assays, where mice are sacrificed after 15 days and peritoneal lavages are tested for tumor cells, we found that targeted PBL are highly effective at eradicating tumor growth, and that the type of heteroconjugate and activation state of PBL determine the potency of this effect. In general, hybrid hybridomas were more effective in targeting than the heteroconjugates. Moreover, cells activated overnight with IL-2 alone (low buoyant density T cells) were less effective than high buoyant density cells activated for several days with immobolized anti-CD3 and IL-2.

Long term survival assays have also been done. In these studies, we have seen in some experiments, highly significant effects- over 50% of mice treated with targeted PBL were cured of their tumors, for over 5 months post injection. However, targeted PBL from other donors did not stop tumor growth. When mice that were cured of tumor were rechallenged, they all came up tumor positive. Thus, long term immunity was not induced in this system, as expected. In these long term studies we have not yet optimized the anti-tumor activity of targeted PBL with regard to the bispecific antibody used, the activation condition of the PBL, and the treatment schedule.

Proposed Course of Project

The in vivo targeting studies described here will be continued by Dr. Mezzanzanica in Milan, as a collaboration. These studies will obtain more long term data using various activation conditions, treatment schedules, but concentrating on bispecific antibodies containing the Mov18 anti-ovarian cancer antibody, developed in Milan. The production of bispecific antibodies suitable for in vivo human use is a very cumbersome and expensive process, and we are currently developing genetically engineered single chain bispecific antibodies to circumvent these problems. As they become available, we intend to test them in in vivo and in vitro targeting systems. Clinical studies using Mov18/anti-CD3 bispecific antibodies to treat human ovarian carcinoma are now underway in Europe, and results from our mouse models are being used in the design of these studies. Instead of following human tumor growth in nude mice, we intend to continue the targeting studies by developing a syngeneic all mouse model, using targeted mouse lymphocytes to treat syngeneic murine tumors in normal inbred mice. This will allow us to study targeting in cancers where the tumors are not localized to a particular cavity, which could lead to a wider application of targeted cytotoxicity to cancer immunotherapy.

Publications:

Segal DM, Qian J-H, Garrido MA, Perez P, Winkler DF, Wunderlich JR, Snider DP, Valdayo MJ and Titus JA. Targeting of cytotoxic cells against tumors with heterocrosslinked bispecific antibodies. In: Immune System and Cancer Hamaoka T, Hodes RJ, Klein G, Sugimum T, Takayama S and Yamamura, eds. Japan Scientific Societies Press, Tokyo, 1989; pp. 323-331.

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Segal DM. The use of flow cytometry to measure cell-cell interactions and conjugate formation. Current Protocols in Immunology, in press.

Segal DM. Antibody mediated killing by leukocytes. In: Fc Receptors and the Action of Antibodies. H. Metzger eds., in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09255-16 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 that you want to identify. 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; D. Deluca, Medical Univ. of South Carolina; S. Katz, DB, DCBDC, D. H. Sachs, IB, DCBDC, C. C. Ting, OD, DCBDC, NCI, S. L. White, Howard Univ. College of Medicine, R. Quinones, Children's National Medical Center.

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The Experimental Immunology Branch flow cytometry laboratory currently supports multiple research projects for more than 55 investigators. These investigations involve quantitative single cell analysis of parameters associated with cells freshly prepared from different species/tissues, as well as a spectrum of in vitro cultured cells. Basic research support is provided to members of the EIB as well as to other investigators within DCBDC. Currently supported projects include, but are not limited to, the following areas of study: a) in vivo and in vitro analyses of intra-cellular signalling via T cell surface molecules, b) analyses of cellular defects in animals with genetic or induced immune dysfunction; c) studies of the pathogenesis of graft-versus-host disease; d) analyses of the coordinate cell surface expression of cell adhesion molecules; e) investigations of T cell ontogeny and differentiation; f) studies of mechanisms of T cell repertoire generation; g) analyses of expression of transplantation antigens; h) investigations of mechanisms involved in antigen presentation processes; and i) analyses of the mechanisms involved in marrow graft rejection versus acceptance.





GPO 914-918

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 antibody. 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.

Dr. A. Singer and colleagues have also utilized flow cytometry in studies of the relationships between T cell receptor expression and intra-thymic T cell differentiation and tolerance induction. In one of these studies, they have analyzed thymocytes from C.B-17/scid mice which are unable to productively rearrange antigen receptor genes and thus generally fail to express T cell receptors. It was found that SCID (severe-combined-immunodeficiency disease) thymocytes express low levels of CD5 and are negative for T cell receptor, CD4 and CD8, and thus phenotypically resemble a specific normal thymocyte subpopulation known to contain precursors for mature thymocytes. It was also found that in the rare SCID thymii which contain T cell receptor positive cells, other SCID thymocytes can further differentiate and express CD4 and/or CD8 accessory molecules. This finding suggested the possibility that T cell receptor positive cells, known to occur early in thymic ontogeny, may play a critical role in subsequent T cell development. To test this hypothesis, normal bone marrow lymphocyte precursors were used to reconstitute SCID mice. In these reconstituted animals, thymii contained both normal and SCID thymocytes which could be distinguished by expression of polymorphic lymphocyte antigens. The presence of normal T cell receptor-bearing thymocytes in SCID thymii was associated with expression of CD4 and/or CD8 accessory molecules by SCID thymocytes which themselves did not express T cell receptor. These results suggest that during thymic development, the expression of molecules critically important to T cell differentiation on immature cells is controlled by other T cells which express T cell receptor.

Dr. A. Singer and colleagues have also utilized flow cytometry in studies of the mechanisms of intra-thymic tolerance induction. It was found that T cell functional tolerance can result either from clonal deletion resulting in the

absence of cells expressing autoreactive T cell receptors, or can occur via clonal anergy evidenced by the continuing presence of cells which bear autoreactive but functionally non-reactive T cell receptors. Further it was found that radiation sensitive, bone-marrow derived cells mediated the deletional mechanism of tolerance, but were not required for the induction of clonal anergy.

Dr. Shearer and colleagues have used flow cytometry to study the effects of cyclosporin A (CsA) on murine T cell development in vivo and in vitro. In one of these studies it was found that CsA inhibited development of T cells in fetal thymus organ culture. This inhibition occurred at two distinct stages of T cell development. First, the generation of $CD4^+$,$CD8^+$ double positive thymocytes was markedly inhibited. Further, the development of $\alpha/\beta$ T cell receptor bearing cells which expressed only CD4 or only CD8 was completely abrogated. In contrast, the development of T cell receptor negative CD8+ cells and that of $\gamma/\delta$ T cell receptor bearing cells was unaffected. These results indicate that there are fundamental differences in the developmental requirements of T cells expressing $\gamma\phi/\delta$ versus $\alpha/\beta$ T cell receptors. Further, these studies demonstrate that CsA can exert immunosuppressive effects within an isolated thymic microenvironment.

Dr. Hodes and colleagues employed flow cytometry in analyses of expression of the repertoire of T cell receptor genes. It was found that there was an association between expression of V$\beta$3 T cell receptors and the ability to react with $Mls^c$ products. Conversely, in vivo expression of $Mls^c$ gene products correlated with the absence of T cells expressing V$\beta$3 positive T cell receptors. It was further found that $Mls^c$ expression was under multi-gene control. These results demonstrate the importance of non major histocompatibility antigens in selection of the T cell repertoire. In another study, clonal deletion of T cells expressing potentially autoreactive receptors was compared in athymic versus normal mice. It was found that while clonal deletion of potentially autoreactive cells occurred in animals with normal thymii, these cells were persistent in animals without a functional thymus. These results demonstrate the critical importance of the thymus in negative selection for the purpose of maintaining tolerance to self antigens.

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, and to analyze cellular components which contribute to rejection versus engraftment of stem cell populations used in reconstitution. 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 were sufficient to reject allogeneic bone marrow grafts. These studies are important to our understanding of the mechanisms of bone marrow engraftment and rejection, especially as applied to clinical problems. In a related study, the EIB flow cytometry laboratory has also provided support to Dr. Quinones and colleagues (Children's National Medical Center) for a clinical bone marrow transplantation protocol used in treatment of leukemia.

Dr. Shaw and colleagues 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. Recent studies have focussed on the $CD4^+$ T cell subset and have 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. One of these investigations has characterized the expression and function of the VLA ($\beta 1$) integrin receptor family on T cells. These cell surface proteins are thought to be important in T cell migration. It was found that memory T cells express 3-4 fold more cell surface VLA-4, VLA-5, and VLA-6 than do virgin T cells. This increased expression is associated with increased binding to the extracellular matrix proteins laminin and fibronectin. These studies have implications for the role of regulated cell surface expression of adhesion molecules in the preferential migration of memory T cells into tissues.

Dr. Sachs (IB) and colleagues have used flow cytometry analysis in a series of studies of allogeneic, xenogeneic, and mixed allogeneic/syngeneic bone marrow transplantation. Recent studies have focussed on analysis of cellular components which contribute to engraftment, as well as upon those which modulate the potentially lethal effects of graft-versus-host disease that can prevent the establishment of a functional immune system in allogeneic bone marrow transplants. One study has demonstrated that an allogeneic bone marrow component, which is removed by treatment with rabbit-anti-mouse brain serum and complement, is capable of enhancing allogeneic chimerism during mixed bone marrow reconstitution. A separate study characterized murine natural suppressor activity found in both normal bone marrow and in spleen early after bone marrow transplantation. These cells, which may function to inhibit graft-versus host disease, were found not to express the stem cell antigen, Sca-1, and could be enriched by depletion of Mac-1 positive cells. In a related investigation, it was found that in murine allogeneic bone marrow transplantation, graft-versus-host disease mortality was reduced in the presence of T cell depleted syngeneic bone marrow plus interleukin 2.

S. Sharrow and colleagues have utilized flow cytometric analysis for investigations of Class I major histocompatibility complex (MHC) antigen expression, and for studies of murine T cell differentiation using both normal thymocytes and T cells developing in fetal thymic organ cultures. One of these studies has demonstrated that epidermal Langerhans cells, the antigen-presenting cells in the skin, express high levels of cell surface H-2D, but are deficient in surface expression of another Class I MHC antigen, H-2K. This differential expression has potential consequences for the efficiency of Class I-restricted immune responses in the skin. The role of T cell receptor interactions in T cell development was studied by the addition of anti-T cell receptor antibodies to murine fetal thymic organ culture. It was found that while productive T cell receptor-ligand interaction were not required for development of $CD4^+$, $CD8^+$ immature thymocytes, the development of T cells with the characteristics of mature T cells expressing $\alpha/\beta$ T cell receptors was abrogated by the addition to organ culture of antibody directed against CD3. In contrast, early developing T cells which express $\gamma/\delta$ T cell receptors were unaffected by this treatment.

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 in the EIB site, tested, and modified a stable version of the new Cluster Analysis Program (CAP) which now provides advanced data analysis capabilities to investigators supported by the EIB flow cytometry laboratory; and c) further investigated, using CAP, the application of automated cluster analysis techniques to flow cytometry multi-parameter data. In one of these studies, cluster analysis defined 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. This novel subpopulation of thymocytes may represent cells which as a consequence of differentiation events is in transition between immature and mature thymocytes.

Publications:

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Cole JA, McCarthy SA, Rees MA, Sharrow SO and Singer A. Cell surface co-modulation of CD4 and T cell receptor by anti-CD4 monoclonal antibody. J Immunol 1989;143:397-402.

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Moses RD, Sundeen JT, Orr KS, Roberts RR and Gress RE. Cardiac allograft survival across major histocompatibility complex barriers in the rhesus monkey following T lymphocyte-depleted autologous marrow transplantation. III. Late graft rejection. Transplantation 1989;48:769-773.

Hirsch R, Chatenoud L, Gress RE, Sachs DH, Bach JF and Bluestone J. Suppression of the humoral response to anti-CD3 monoclonal antibody. Transplantation 1989;47:853-857.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09256-03 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

In Vitro Study of MHC-Specific T Cells

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

| | | | |
|---|---|---|---|
| PI: | A. Singer | Senior Investigator | EIB, NCI |
| Others: | P. Stein | BTP Fellow | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER |
|---|---|---|
| 1.15 | 1.15 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

This project has been aimed at identifying and characterizing the T cell subsets that initiate and effect anti-MHC responses in vitro. We have identified and characterized 3 distinct T helper cell populations that function in vitro to initiate anti-MHC responses, and have studied 3 distinct T killer cell populations that function in vitro as anti-MHC effector cells. We have characterized these cell populations with regard to their specificity, phenotype, activation requirements, and differentiation requirements.



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

Project Description

Major Findings:

In contrast to conventional immune responses against foreign protein antigens that involve a single population of Th cells, we have characterized 3 distinct populations of Th cells that function to initiate anti-MHC responses in vitro, based on their recognition of different MHC specificities expressed on the surface of accessory (Acc) cells: (a) one Th cell population recognizes class II MHC allodeterminants, (b) one Th cell population recognizes class I MHC allodeterminants, and (c) one Th cell population recognizes an antigenic complex composed of foreign class I and self-class II MHC determinants. The two class II restricted Th cell populations are phenotypically $CD4^+$, whereas the class I restricted Th cell population is phenotypically $CD8^+$. One common function performed by these phenotypically distinct anti-MHC Th cell populations was the secretion of IL-2. Furthermore, we found that both IL-1 and IL-6 are able to serve as a common co-factor, together with ligand, for triggering both $CD4^+$ and $CD8^+$ primary Th cell populations to secrete IL-2.

To understand the molecular requirements for MHC recognition by class I allospecific Tk cells, we assessed the role of an exposed portion of the alpha-helical region of a class I MHC molecule in T cell recognition. We found that a peptide identical in sequence to amino acids 163-174 of the Kb molecule blocked Tk cell recognition of the Kb molecule regardless of the site on the Kb molecule that the Tk cells were directed against. The blocking of Tk cell function by peptide Kb163-174 occurred at the level of the T effector cell, and not at the level of the target cell. Furthermore, we found that peptide Kb163-174 not only blocked target cell lysis, but also blocked conjugate formation between anti-Kb Tk cells and Kb-bearing target cells. Taken together, these data indicate that Kb163-174 blocks Tk cell engagement of the Kb molecule by binding to the T cells' clonotypic receptor, and consequently, define a docking site on the Kb molecule utilized by most Kb-specific Tk cells.

Publications:

Schneck J, Munitz T, Coligan JE, Maloy WL, Margulies DH and Singer A. Inhibition of allorecognition by an $H\text{-}2K^b$-derived peptide is evidence for a T cell binding region on an MHC molecule. Proc Natl Acad Sci (USA) 1989;86:8516-8520.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09257-15 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Mechanisms of Cellular Immune Responses

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

| | | | |
|---|---|---|---|
| PI: | S. Shaw | Senior Investigator | EIB, NCI |
| Others: | G. Ginther-Luce | Chemist | EIB, NCI |
| | Y. Shimizu | Damon Runyan Fellow | EIB, NCI |
| | K. Horgan | Fogarty Visiting Associate | EIB, NCI |
| | G. van Seventer | Fogarty Visiting Associate | EIB, NCI |
| | J. Hallam | Biologist | EIB, NCI |
| | Y. Tanaka | Fogarty 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: |
|---|---|---|
| 6.9 | 6.9 | |

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

☐ (a1) Minors

☐ (a2) Interviews

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

Our studies of human T cell recognition emphasize two fundamental areas: 1) identifying and characterizing the functions of cell surface molecules which facilitate T cell recognition; and 2) analysis of heterogeneity among subsets of human T cells and of the functional capacities of those subsets. Major new findings relate to T cell interaction with extracellular matrix (ECM) components. Resting T cells express on their surface three members of the VLA ($\beta$1) integrin family and can adhere to ECM via three distinct VLA-mediated pathways: VLA-4/fibronectin, VLA-5/fibronectin, VLA-6/laminin. Binding via these pathways is regulated by two distinct and complementary mechanisms: 1) Expression of the VLA receptors is regulated with T cell differentiation; 2) the functional capacity of the receptors is rapidly regulated. Since we find that these receptors also facilitate activation of the T cells in vitro, they may be important not only in T cell adhesion/migration, but also in T cell activation. The mechanisms for regulation of adhesion are under investigation; one possibility being explored is that rapid activation-induced shedding of cell surface molecules modulates T cell adhesion. Concurrent studies have extended our understanding of the importance of pathways involving other accessory molecules in facilitating T cell activation. Systematic analyses of T cell LFA-1 interaction with biochemically purified ligand ICAM-1 emphasize the potent role of this molecular interaction as a costimulus for T cell activation. Furthermore, analysis of the costimulatory role of monocytes in T-cell receptor-mediated activation demonstrate important contributions not only of LFA-1/ICAM-1 but also CD2/LFA-3 and other molecules currently under investigation. New subsets of T cells have been defined with a mAb specific for the CD45RB antigen. This marker allows discrimination of two subsets of memory T cells, whose functional capacities are under investigation. Systematic quantitative analysis of expression of many molecules on the surface of T cells from normals and AIDS patients continue to reveal new heterogeneity of T cell subsets, whose physiological relevance is now being investigated.

Project Description

Major Findings

Regulated adhesion of T cells to extracellular matrix (ECM) proteins is likely to be essential in T cell migration. On various non-hematopoietic cells, members of the VLA ($\beta$1) integrin subfamily mediate cell interaction primarily with components of the extracellular matrix (ECM). The potential importance of VLA integrins on resting human T cells was analyzed in in vitro systems of binding to purified ECM proteins and of T cell proliferation. Purified CD4+ human T cells express the VLA-4, VLA-5, and VLA-6 integrins and exhibit strong binding to the ECM proteins laminin (LN) and fibronectin (FN). Binding was observed only after acute activation of T cells with any of three different activation protocols: 1) crosslinking of the T cell receptor with a CD3 mAb and goat anti-mouse IgG; 2) a mitogenic pair of CD2 mAb; or 3) 10 ng/ml of the protein kinase activator PMA. Strong binding to LN and FN was observed within 10 minutes of activation without a quantitative change in the cell surface expression of the VLA integrins. Monoclonal antibody (mAb) blocking studies define three VLA/ECM interactions: VLA-4/FN, VLA-5/FN, and VLA-6/LN. Blocking studies with FN peptides demonstrate that VLA-5 interacts with the well-defined "RGD" sequence on FN and VLA-4 interacts with the "CS-1" sequence present in the IIICS region. A second mode of regulation of VLA-mediated T cell adhesion to FN and LN is dependent on T cell differentiation. As an extension of our previous studies elucidating human naive and memory cells (AR09257-13 and 14), we now find that "memory" T cells express 3-4 fold greater amounts of VLA-4, VLA-5, and VLA-6 than do "naive" T cells. This differential expression of VLA integrins is associated with more efficient binding of memory cells to LN and FN. The potential functional significance of VLA/ECM interactions is suggested by the finding that LN or FN co-immobilized with a CD3 mAb on plastic enables vigorous T cell proliferation. The same VLA integrins which mediate binding also mediate this costimulatory effect. The "CS-1" peptide is particularly effective in inhibiting VLA-4 dependent effects of FN in this proliferation system. The ability of LN and FN to costimulate CD3-mediated T cell activation suggests that T cell interactions with ECM may have relevance not only to T cell migration but also to T cell recognition.

The foregoing studies of T cell interaction with ECM have converged in many ways with ongoing studies of T cell interaction via LFA-1 with its cell surface ligand ICAM-1 (09257-14). All four of these integrin-mediated interactions are rapidly regulated by activation for the resting T cell. A variety of approaches are being used to explore the molecular basis for such regulation. Our studies demonstrate that such regulation may be of broader relevance, since adhesion can be induced not only by crosslinking of CD3, but also by cross-linking of 2 out of 10 other cell surface molecules or by Ca++ ionophore. Studies are in progress to explore whether each of the stimuli activate via the same mechanism. Investigations with inhibitors of various protein kinases suggests that multiple signalling pathways may be involved. Although conventional hypotheses implicating receptor phosphorylation or cytoskeletal association are being evaluated, the less conventional concept that it may be regulated in part by rapid shedding of other cell surface molecules is also being explored. Attempts are in progress to raise mAb against such putative regulatory molecules.

It is now widely accepted that T cell activation often requires not only occupancy of the T cell receptor but costimulatory interactions of other molecules. These molecules have remained largely undefined. Our studies (initiated in 09257-14) have demonstrated that LFA-1 interaction with its ligand ICAM-1 is such a costimulatory interaction. We have used a simple model system designed to be as relevant as possible to antigen-specific recognition: biochemically purified ICAM-1 and T cell receptor cross-linking by anti-CD3 mAb OKT3 immobilized on plastic. Resting T cells adhere minimally to ICAM-1 immobilized on plastic. Nevertheless, ICAM-1 deposited on plastic together with non-mitogenic immobilized OKT3 results in a potent activating stimulus. This costimulation cannot be readily accounted for by ICAM-1-mediated adhesion but is consistent with a role in signalling, which can be directly demonstrated in ICAM-1-mediated augmentation of activation induced by PMA/ionomycin. The proliferative response to co-immobilized OKT3 and ICAM-1 is dependent on the IL-2R, which is induced only in the presence of both OKT3 and P-ICAM-1.

Two related model systems confirm and extend the understanding of LFA-1/ICAM-1 mediated co-stimulation derived from our studies of co-immobilized ICAM-1 and OKT3. First, costimulation by ICAM-1 occurs (albeit more weakly) even when the ICAM-1 is immobilized on one surface and the OKT3 mAb is immobilized on another. Such "remote" costimulation by ICAM-1: 1) indicates a signalling role for ICAM-1 as well as its established role of adhesion; and 2) makes ICAM-1 a candidate to contribute to costimulation in classical three cell experiments. Confirmation of this prediction was provided by studies in such a system: resting T cells stimulated by OKT3 on beads in the presence of costimulation from activated fixed monocytes (plus IL1/IL6). A critical role for ICAM-1 was demonstrated by mAb inhibition. In addition, however, an important role for CD2/LFA-3 interaction was suggested by results of mAb blocking. Studies are in progress to compare and contrast the costimulatory roles of these two pathways which we have previously characterized in cell adhesion (09257-13, 05067-12). Thus, the present data demonstrate that LFA-1/ICAM-1 interaction is a potent costimulus for T cell receptor-mediated activation; this observation, interpreted in light of previous reports, suggests that LFA-1/ICAM-1 is of major physiologic importance as a costimulatory signal.

Our previous studies of T cell subsets (09257-13, -14), demonstrate the importance of the CD45RA and CD45RO isoforms as markers of T cell differentiation and define important differences between the subsets which they distinguish. Purification of these subsets of CD4+ cells by immunomagnetic selection has been further improved and these preparations of cells are the starting point for further analysis. The CD45RB isoform allows us to distinguish two phenotypically distinct subsets within the CD45RO T cells. Functional analysis of these subsets following fluorescence activated cell sorting establishes that both contain "memory" cells capable of responding to recall antigen, and have vigorous responses to TCR-receptor-mediated activation. Characterization of these subsets for cytokine production are in progress, as are novel strategies for raising mAb specific for new informative epitopes on CD45.

Studies of quantitative differences of many surface molecules on CD4+ T cells (09257-14) continues. The complexity of results, the desire to integrate as much information as possible and the process of developing better analytic tools

have made progress slow even on normal donors. Nevertheless, the conclusion that there is much additional unexplained heterogeneity among CD4+ cells is even stronger and raises exciting prospects regarding the insights which will come from better understanding of this heterogeneity. Initial studies of a subset of about 15 mAbs on 6 HIV-infected patients show marked differences between individuals. The significance of such differences can be understood only by careful future comparison to large normal populations and to other clinical and laboratory parameters regarding these patients.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09258-12 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Immune Response Gene Regulation of the Immune Response In Vitro

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

| | | | |
|---|---|---|---|
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| Others: | M. Vacchio | Bio. Lab. Tech | EIB, NCI |
| | J. Berzofsky | Senior Investigator | MB, NCI |

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LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.2 | 0.1 | 0.1 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

In order to analyze the genetic regulation of T cell responses to NASE, a series of cloned lines were generated in BALB/c ($H\text{-}2^d$) as well as ($H\text{-}2^b$ x $H\text{-}2^a$)$F_1$ T cells. Individual clones were restricted to recognizing NASE in the context of either $A_\alpha A_\beta$ or $E_\alpha E_\beta$ products. The antigen fine specificity of cloned NASE-specific T cells was also probed through the use of mutant NASE molecules and synthetic peptides corresponding to segments of NASE. A consistent correlation was found between the fine specificity of a given clone and its MHC restriction specificity. $A^b_\alpha A^b_\beta$ restricted clones were selectively responsive to peptide 91-110; $E^k_\alpha E^k_\beta$ restricted clones were responsive to peptide 81-100.

The nature of the immunogenic peptides which are processed and presented to T cells was further evaluated using a panel of variant NASE peptides. It was observed that negative (inhibitory) interactions appear to occur between amino acids in peptides which interfere with T cell responses. Single amino acid changes, by eliminating such apparent negative interactions, result in the restoration of T cell stimulatory ability in these peptides.

Project Description

Major Findings:

Monoclonal $T_H$ cell populations specific for NASE have been generated in BALB/c ($\underline{H\text{-}2}^d$) and (B10xB10.A)$F_1$ ($\underline{H\text{-}2}^b x \underline{H\text{-}2}^a$) 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)$F_1$ clones respond to native NASE in association with either $A^b_\alpha A^b_\beta$ or $E^k_\alpha E^k_\beta$. When the fine specificity of these clones was analyzed employing NASE fragments and a series of overlapping 20 amino acid synthetic peptides corresponding to the NASE sequence, it was found that $A^b_\alpha A^b_\beta$ restricted clones were highly responsive to peptide 91-110; and not to other synthetic NASE peptide. In contrast, $E^k_\alpha E^k_\beta$ restricted clones were consistently responsive to peptide 81-100 and not to 91-110; certain of these $E^k_\alpha E^k_\beta$ restricted T cells expressed a cross reactivity with peptide 51-70. A BALB/c clone was responsive only to peptide 61-80. No other NASE peptides have revealed antigenic activity to date. Collectively, these findings demonstrate that NASE specific T cells are responsive to discrete peptides. Moreover, these findings suggest that in T cell recognition of a complex and highly foreign protein antigen, a limited number of peptide epitopes is preferentially recognized by T cells in association with a given Ia molecule.

More detailed analysis of T cell fine specificity in response to NASE peptides was carried out using variants in which a single amino acid was altered from its native or wild type form. This analysis revealed that clones with specificity for the same native peptide and self MHC determinant may nonetheless vary significantly in their fine specificity as assessed by responses to variant peptides. It was also observed that certain $E^k_\alpha E^k_\beta$ restricted clones responded to peptide 91-100 but not to 91-105. On the basis of this finding, it was suggested that interactions between amino acids in the 101-105 region with amino acids in the stimulatory 91-100 region were responsible for impaired ability to stimulate T cells. Consistent with this hypothesis, it was found that single amino acid changes including those in the 91-100 as well as those in the 101-105 region were capable of restoring stimulatory ability. Such changes appear to have their effect by eliminating inhibitory interactions among amino acid residues in the 91-105 peptide.

Proposed Course of Project:

Cloned T cell populations and synthetic peptides will be employed to further analyze the mechanism for antigen fine specificity and MHC restriction. Variant peptides will be analyzed to more definitively identify antigenic sites on peptides antigens.

The influence of non-MHC genes on anti-NASE responses will be studied. As noted in project Z01 CB 05106-07, expression of non-MHC Mls determinants can result in deletion of expression of certain V$\beta$ genes. The influence of non-MHC genes, including $Mls^a$ and $Mls^c$, will therefore be studied in T cell responses to NASE. Both the peptide fine specificity of these responses and the patterns of T cell receptor V$\alpha$ and V$\beta$ expression will be used to monitor the expressed repertoire by both functional and structural criteria.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09259-12 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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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Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 3.0 | 1.5 | 1.5 |

CHECK APPROPRIATE BOX(ES)

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

B

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

The long-term effects of parent-into-$F_1$ graft-versus-host reaction (GVHR) was investigated by following splenic T cell markers and function for up to 18 months after GVHR induction. Despite the appearance of adequate numbers of T cells which expressed the normal compliment of T lymphocyte markers, these cell still failed to generate MHC self-restricted, CD4-mediated T helper responses. This defect was not due to suppression or a defect in antigen-presenting cell function. Analysis of donor chimerism in parent-into-$F_1$ GVHR indicated three phases of repopulation: an expansion phase of donor T cells; a phase of host cell destruction; and a donor repopulation phase in which extensive donor cell repopulation is observed.

Donor T cells that induce GVHR were studied for T cell receptor repetoire by V$\beta$ analyses. It was observed that donor T cell expressing the V$\beta$ markers of anti-host reactivity were selectively expanded during GVHR. V$\beta$ analysis was also used to investigate the possible synergy between class I GVHR and donor-anti-host mls recognition. It was found that donor-anti-host recognition of class I H-2 and $mls^a$ resulted in acute GVHR, and preferential expansion of the V$\beta$ T cell receptor reactive with $mls^a$. Depletion of these V$\beta$ T cells resulted in reduced donor chimerism, but not in abrogation of GVH-induced immune deficiency.

Project Description

Major Findings:

1. T cell functional recovery in late GVH:

Recovery of normal T cell mediated immune function is often delayed for many months following bone marrow transplantation. Susceptibility to infections during this period of immune deficiency is a major cause of post transplant mortality. Furthermore, graft vs host disease has been shown to aggravate immune dysfunction and susceptibility to infection. Although the immune deficit can be related to a slow rate of T cell repopulation after marrow transplant, some immune functions lag behind the recovery of normal T cell numbers. We therefore studied the effect of graft vs host reaction (GVHR) on the recovery of T cell function.

We have recently demonstrated that induction of GVHR prior to irradiation and marrow transplant in mice precluded recovery of CD4-dependent cellular immunity. This work further suggested that the basis of the dysfunction was GVHR-induced alterations in the thymus, because implantation of a new thymus prior to the marrow graft could prevent the dysfunction. We wished to determine whether GVHR alone (independent of irradiation) was sufficient to affect thymic maturation of T cells. Futhermore we wanted to better characterize the CD4 functional deficits following GVHR. For these reasons we investigated the long-term recovery of T cell function following induction of acute suppressive GVHR in the parent-into-unirradiated F1 host model.

We have determined that most splenic immune functions recovered from a period of acute graft-vs-host reaction (GVHR) associated immune deficiency to near normal levels within 4-5 months after the induction of GVHR by injection of B10 splenocytes into unirradiated (B10xB10.BR)F1 hosts. This restoration of function included Con A and LPS stimulated proliferation and cytotoxic T lymphocyte (CTL) responses to allogeneic stimulators. Yet even after 12 to 18 months, a deficit in L3T4 (CD4)-dependent T helper functions remained, including CTL generation against TNP-modified syngeneic stimulators and IL-2 generation in autologous mixed lymphocyte reactions, in Lyt-2 (CD8) depleted cultures and in response to Class II MHC diparate stimulators. The IL-2 deficit was not due to either suppression or antigen presenting cell defects, as determined by co-culture experiments. Furthermore, the deficit was not merely a quantitative deficit; CD4+ T cells were present in the spleen in normal or near normal levels.

We then addressed the problem of T cell maturation post-GVHR. We demonstrated that maturation of new T cells from marrow derived pre-T cells could occur after GVHR. Induction of GVHR with a combination of B6 Thy 1.1 T-depleted bone marrow and B6 Thy 1.2 lymph node cells (lacking pre-T cells) demonstrated that mature
donor T cells (Thy 1.2) predominated in the host spleens at 8 weeks of GVHR but that donor pre-T cells (Thy 1.1) maturing in the GVHR host pre-dominated after 9 months of GVHR. Thymic populations were relatively normal by FACS analysis, showing only a slight increase in single positives. Splenic T cells were phenotypically normal, as well, expressing normal levels of CD3, $\alpha/\beta$ TcR

and Qa2. Furthermore, Vβ analysis of splenic T cells demonstrated clonal deletion of T cell populations reactive with host antigens, consistent with a normal intrathymic selection process.

We then further characterized splenic CD4 function in late GVHR mice, and determined that while IL-2 production capacity was consistently reduced, IL-4 production was either normal or enhanced. Thus the GVHR induced alteration in the thymus may involve a shift in the relative production of TH2 and TH1 cells, rather than a block in thymic CD4+ T cell maturation.

2. **Development and stability of chimerism in GVHR:**

The graft-vs-host reaction induced by injection of parental lymphocytes into unirradiated, immune competent F1 mice is associated with an attack by donor cells on host populations. FACS analysis of splenic populations over a timecourse of GVHR demonstrated that this attack could be divided into three phases. In the first "proliferative" phase, donor (and host) T cells -- particularly Lyt-2 cells -- increased in number and as a percentage of splenic populations. In the second "cytotoxic" phase, host lymphoid and myeloid populations were destroyed and most mature donor lymphocytes died. In the final "recovery" phase, donor derived cells repopulated the lymphohematopoietic system, following the sequence: myeloid cells (Mac-1+), B cells (IgM+) and finally T cells (CD4+,CD8+). Recovery of normal T cell numbers, particularly CD4+ T cells, was protracted, often requiring 6 months or longer. This repopulation of the spleen by donor lymphocytes was associated with the recovery of immune functions following GVHR induced immune deficiency. Donor repopulation of the host lymphoid system was further demonstrated to be dependent upon the presence of Lyt-2 (CD8) cells in the original donor inoculum. Following injection of CD8 depleted donor cells, only donor CD4 T cells engrafted. Depletion of donor CD4 populations usually precluded development of GVHR or any donor engraftment. Finally, the long-term stability of donor/host chimerism was examined. Donor/host chimerism was dependent on both the dose of donor cells in the original inoculum and the strains of mice involved.

3. **Analysis of T cell receptor Vβ repetoire in GVH:**

Recent analyses of the T cell receptor repetoire have revealed that certain Vβ families are associated with the responses to Class II I-E and mls antigens. For example, the Vβ8.1, Vβ6 and Vβ9 families of α/β T cell receptors have been linked to responses to $mls^a$, the Vβ3 family has been linked to $mls^c$ responses and the Vβ5 and Vβ11 families have been associated with I-E restricted responses. T cells expressing these Vβ markers were found to predominate in the in vitro response to the relevant antigens and to be clonally deleted during thymic maturation in mice expressing these antigens. Because the GVHR is based upon the response of donor lymphocytes to host cells, we investigated the Vβ expression of donor populations.

We have determined that during the GVHR donor T cells expressing Vβ markers linked to responses to host alloantigens dominated the donor population. For example, donor B10.BR lymphocytes and bone marrow ($H\text{-}2^k$, $mls^b$) were transplanted into lethally irradiated CBA/J hosts ($H\text{-}2^k$, $mls^{a,c}$). At 7-9 days

after the marrow transplant, 75-90% of the donor CD4 lymphocytes expressed Vβ 3, 6 or 8.1, compared with less than 20% in normal B10.BR mice or in syngeneic marrow transplants; more than 50% of the CD8 population expressed these Vb families, compared to 25-30% in the controls. When Balb/c bone marrow and lymphocytes ($H\text{-}2^d$, $mls^c$) were transplanted into DBA/2 hosts ($H\text{-}2^d$, $mls^a$), 75% of the donor CD4 and 40% of the donor CD8 lymphocytes expressed Vβ 6, or 8.1, compared to less than 20% of the repetoire in untreated donor or syngeneic transplants.

In order to investigate the role of these T cells in the induction of GVHR, the host-reactive Vβ populations were depleted from the donor inoculum. Following bone marrow transplantation in the Balb/c-->DBA/2 combination, $VB6^+$ cells constituted 55% of CD4 cells from non-depleted donor inocula, but only 1% of CD4 cells from depleted donors. We are continuing these experiments to test the effect of selective Vβ depletion on GVHR-induced immune deficiency and donor marrow engraftment. The longterm goal of these experiments is to reduce or prevent 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.

We are also using the analysis of Vβ expression to investigate the involvement of mls and I-E diparities in inducing GVHR in unirradiated hosts. We have previously observed that an acute suppressive GHVR was induced by injection of parental donor lymphocytes into an unirradiated adult F1 host expressing Class I and Class II MHC alloantigens disparate from the donor. This GVHR was characterized by a profound immune suppression, an attack on host tissues and extensive engraftment of donor lymphocytes. When the parental donor and the F1 host differed only at Class II loci, a chronic stimulatory GVHR developed, characterized by B cell hyperplasia and autoantibody production, suppression of only CD4 function, and engraftment primarily of donor CD4 cells only. When donor and host differed only at Class I loci, the result with most strain combinations was that no GVHR induced immune deficiency was observed. One exception to the last result has been when the F1 host expressed not only Class I antigens but also $mls^a$ antigens, to which the donor could respond; such mice developed an acute suppressive GVHR, as assessed by immune deficiency and donor/host chimerism. We therefore analyzed the involvement of mls determinants in the generation of GVHR induced immune deficiency and donor engraftment. We observed that T cells expressing Vβ T cell receptors reactive with $mls^a$ (Vβ 6 and 8.1) predominated in the donor T cell population. Furthermore depletion of these Vβ expressing populations from the donor inoculum greatly decreased the extent of donor/host chimerism, although significant immune deficiency was nonetheless produced.

4. Regulation of T cell Vb repetoire in gut associated lymphoid tissue:

Vβ3 and 8 expressing T cell populations have also been demonstrated to expand in response to the superantigen Staphyloccoccal enterotoxin B (SEB). Because mice are frequently exposed to Staphyloccus enterically, we investigated the effect of such repeated stimulation on the T cell Vβ repetoire. We compared the expression of Vβ 3, 8, 11 and 6 in T cells in the spleen, peripheral lymph nodes (axillary and inguinal), mesenteric lymph nodes and Peyer's patches,

using mice maintained in a conventional animal room. We observed no differences in the frequency of expression of these four Vβ populations. Furthermore when we compared these populations in mice maintained in specific pathogen free conditions and in conventional conditions, we again observed no significant differences in Vβ repetoire. We then tested the ability of cells from the mesenteric nodes and Peyer's patches to respond to SEB in vitro. Mesenteric nodes simulated with SEB plus rIL-2 showed a 1.5 to 2 fold expansion of Vβ8 (from 22% to 36-50%) and a 4-9 fold expansion of Vβ3 (from 3% to 11-27%). Representation of Vβ 11 and 6 in the populations declined. Stimulation with Concanavalin A plus rIL-2 produced marked proliferation and blast transformation in mesenteric node cells but did not produce an expansion of Vβ8 or Vβ3. In contrast to the mesenteric lymph nodes, when Peyer's patch lymphocytes were stimulated with SEB plus IL-2, Vβ8 populations did not significantly expand and Vβ3 expansion was reduced. This data suggests that T cell repetoire in the gut associated lymphoid tissue does not become skewed by repeated exposure to enteric bacterial superantigens. The Peyer's patch cells may either be anergic to such stimulation or be suppressed. These possibilities are currently being investigated.

25% of this project is AIDS-related research.

Publications:

Hakim FT, Shearer GM. Immunological and hematopoietic deficiencies of graft versus host disease. In J.L.M. Ferrara, and S.J. Burakoff (Eds.): Graft Versus Host Disease Marcel-Dekker:New York 1989;133-159..

Hakim FT, Pluznik DH and Shearer GM. Alterations in T cell-derived colony-stimulating factors associated with GVH-induced immune deficiency. Transplantation, 1990; 49:773-781.

Hakim FT, Pluznik DH and Shearer GM. Effect of graft-vs-host reaction on T cell production of colony stimulating factors. II. FActors contributing to the decrease in concanavalin A-induced CSF-production in acute, suppressive GVH. Transplantation, 1990; 49:781-787.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09260-10 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Synergistic Effects of Murine Cytomegalovirus and Graft-versus-host Reaction

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

PI: G. M. Shearer Senior Investigator EIB, NCI

COOPERATING UNITS *(if any)*

J. D. Shanley, V.A. Hospital, Newington, CT
C. S. Via, Dept. of Medicine, University of Maryland, School of Medicine, Baltimore, MD

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.5 | 0.1 | 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.)*

Injection of $F_1$ hybrid mice with either murine cytomegalovirus (MCMV) or parental spleen cells (graft-versus-host reaction - GVHR) results in rapid and severe immunosuppression. Inoculation of either the virus or parental cells were selected so that they would be below the threshold for severe immunosuppression. However, when these two inocula were combined, severe immunosuppression was observed. Furthermore, injection of $F_1$ mice with parental lymphocytes that recognize only class I MHC determinants does not result in immune suppression. However, a combination of class I recognition and MCMV infection results in profound immune suppression. In contrast, there was no detectable synergy between class II GVH and MCMV.

Infection of mice with MCMV was found to elevate natural killer (NK) cell number and activity. As a consequence MCMV infection was able to augment natural resistance and the rejection of bone marrow grafts.

Project Description

Major Findings:

Mice infected with MCMV exhibited a substantial increase in NK cell number and activity. NK activity peaked three days after infection and then declined to normal levels. $F_1$ hybrid mice infected with MCMV three days prior to irradiation and parental bone marrow grafting exhibited elevated NK activity, as well as augmented hybrid resistance to H-$2^b$ grafts. These results raise the possibility that cytomegalovirus infection in human marrow graft recipients could interfere with engraftment and could be associated with marrow failure.

Our observations that a) MCMV infection is often associated with enhanced T effector cell immunity to non-CMV antigens; and b) MCMV can synergize with low doses of donor leukocytes to induce GVH disease raises the possibility that MCMV infection can initiate a T helper signal with the production of lymphokines. Such a helper signal must be generated by MCMV-specific, MHC self-restricted helpers with the production of IL-2 (and other T helper factors), which could enhance the CD8 component of T cell immunity. A class I + II GVHR results in the loss of both CD4 and CD8 T function, whereas a class II GVH results in a selective loss of CD4 T helper function. A class I GVH that is limited to recognition of H-$2D^b$ or H-$2D^d$ results in no detectable GVH, as determined by host T cell function. We have used the differential effects of these three types of GVH on T cell immune function to determine whether CMV infection is providing a "class I" or "class II" type of signal in its synergistic action with GVH, i.e., with or without CMV the class II GVH abrogated only CD4 function. In contrast, when MCMV was given in conjunction with the H-2D GVH (which had no detectable effect alone), the combination of MCMV and H-2D class I recognition abrogated both CD4 and CD8 T cell function - characteristic of a class I + II GVH effects of MCMV recognition by T cells in vitro may elucidate the immune mechanisms responsible for the augmenting effects of MCMV in synergy with GVH, and in other models of immune enhancement.

In some experiments we have attempted to design protocols in which lethally irradiated mice were transplanted with allogeneic bone marrow with or without T cells and with or without CMV infection. Such experimental designs would more closely approximate the situation encountered in human marrow transplantation. Due to the high incidence of mortality in CMV-infected, GVH, irradiated mice, these complex experiments have not been successful. However, the mice that survived long-term were those that were irradiated and grafted with syngeneic bone marrow, with or without CMV infection. Between 12 and 18 months after irradiation and syngeneic marrow grafting, these mice began to develop sarcomas, probably as a result of exposure to 900R of whole-body irradiation. However, the development of the sarcomas (5/11) appeared to be limited to those mice that received 900R, and syngeneic marrow, but not MCMV. Mice that were exposed to

MCMV, in addition to receiving irradiation and syngeneic marrow did not develop detectable tumors (0/17). Although these results (compilation of two experiments) are incomplete and need to be redesigned and repeated, the preliminary observations raise the possibility that exposure to MCMV has provided some element of immune protection against the development of the sarcomas. One possibility is that the mechanism responsible for enhanced, immune reactivity of MCMV-infected mice noted above could contribute to the prevention or delayed development of these radiation-induced tumors.

0% of this projects is AIDS-related research.

Publications:

Muluk SC, Hakim FT and Shearer GM. Enhancement of natural resistance to parental bone marrow grafts by infection of $F_1$ host mice with murine cytomegalovirus. J. Immunol. 1990, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09263-08 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*


| | | | |
|---|---|---|---|
| PI: | P.A. Henkart | Senior Investigator | EIB, NCI |
| Others: | M. Kubicek | Microbiologist | EIB, NCI |
| | S. Winslow | Microbiologist | EIB, NCI |


COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.0 | 2.5 | 0.5 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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


Resting purified CD8+ T lymphocytes from mouse spleen and thymus were induced to become cytotoxic after stimulation with immobilized MAb and lymphokines in short term in vitro culture. The cytotoxic activity measured was with red blood cell target cells and aCD3xaDNP heteroconjugated antibodies to allow a polyclonal readout of a cytotoxic pathway assumed to require granule exocytosis and the granule cytolysin. It was found that culture of these CD8+ T cells with immobilized anti CD3 or anti Thy in the presence of both rIL2 and rIL4 gave rise to strong proliferation and cytotoxicity after 5 days. Other combinations of MAb stimuli and lymphokines gave rise to strong proliferation but minimal cytotoxicity, while culture in the absence of MAb or lymphokines results in neither. In order to analyze the expression of genes for granule components associated with cytotoxicity as well as other genes expressed by stimulated CD8+ cells, we have established sensitive assays for specific mRNAs using PCR after reverse transcriptase (RT-RCR). We have established assays for mRNA expression of the following genes: cytolysin (perforin), granzyme A, granzyme B, granule proteoglycan, IL2, IL2 receptor, IL4, gamma interferon, TY5, lymphotoxin, and the housekeeping gene aldolase. Expression of these genes using the RT-PCR technique can be detected semi-quantitatively with less than 106 cells; at this stage we have analyzed mouse and rat cloned CTL, LGL tumor, LAK cells and the mast cell tumor RBL. We have found that the granule proteoglycan gene is expressed in cells derived from LGL, but not from CTLs, whereas most of the other genes are expressed in patterns consistent with literature reports.





PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Major Findings:

Resting purified CD8+ T lymphocytes from mouse spleen and thymus were induced to become cytotoxic after stimulation with immobilized MAb and lymphokines in short term in vitro culture. The cytotoxic activity measured was with red blood cell target cells and aCD3xaDNP heteroconjugated antibodies to allow a polyclonal readout of a cytotoxic pathway assumed to require granule exocytosis and the granule cytolysin. It was found that culture of these CD8+ T cells with immobilized anti CD3 or anti Thy in the presence of both rIL2 and rIL4 gave rise to strong proliferation and cytotoxicity after 5 days. Other combinations of MAb stimuli and lymphokines gave rise to strong proliferation but minimal cytotoxicity, while culture in the absence of MAb or lymphokines results in neither. In order to analyze the expression of genes for granule components associated with cytotoxicity as well as other genes expressed by stimulated CD8+ cells, we have established sensitive assays for specific mRNAs using PCR after reverse transcriptase (RT-RCR). We have established assays for mRNA expression of the following genes: cytolysin (perforin), granzyme A, granzyme B, granule proteoglycan, IL2, IL2 receptor, IL4, gamma interferon, TY5, lymphotoxin, and the housekeeping gene aldolase. Expression of these genes using the RT-PCR technique can be detected semi-quantitatively with less than 106 cells; at this stage we have analyzed mouse and rat cloned CTL, LGL tumor, LAK cells and the mast cell tumor RBL. We have found that the granule proteoglycan gene is expressed in cells derived from LGL, but not from CTLs, whereas most of the other genes are expressed in patterns consistent with literature reports.

Proposed course:

We will combine the two components of this project described above in order to correlate the expression of various genes with the expression of the cytotoxic function. Further refinements in the RT-PCR technique are being made in order to enhance the sensitivity so that less than 105 lymphocytes can be used to make RNA preps and analyze for all the genes described. Further work is also planned to refine the quantitation of the RT-PCR technique. With regard to the stimuli required to generate cytotoxicity during culture, other combinations of MAb and recombinant lymphokines will be tested as they become available, and the expression levels of the various genes described will be analyzed.

Publications:

Hayes MP, Berrebi GA and Henkart PA. Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. J Exp Med 1989; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 09264-03 E

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Cyclosporin A on T Lymphocyte Regulation and Development

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


| | | | |
|---|---|---|---|
| PI: | G. M. Shearer | Senior Investigator | EIB, NCI |
| Others: | S. Sharrow | Senior Investigator | EIB, NCI |
| | A. Kosugi | Visiting Fellow | EIB, NCI |
| | M. Clerici | Visiting Fellow | EIB, NCI |
| | A. Weissman | Senior Investigator | EIB, NCI |


COOPERATING UNITS *(if any)*
M. Weir, Department of Surgery, University of Maryland School of Medicine
C.S. Via, Department of Medicine, University of Maryland School of Medicine
P. Kimmel, Renal Unit, George Washington University School of Medicine

LAB/BRANCH
Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.3 | 1.0 | 0.3 |

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.)*


The effects of cyclosporin A (CsA) on T lymphocyte development was investigated in the mouse by exposing fetal thymic organ cultures (FTOC) to CsA, and subsequently assessing T cell developmental markers by flow cytometry. CsA inhibited: a) the maturation of single positive ($CD4^+CD8^-$ and $CD4^-CD8^+$) thymocytes; and b) the development of double positive from double negative thymocytes.

The stimulation of human T helper cells (Th) in vitro with recall antigens, HLA alloantigens, and mitogens in the presence of different doses of CsA resulted the selective dose-dependent loss of recall responses. This pattern of unresponsiveness resembles the loss of Th seen in AIDS development. Such results: a) led to the demonstration of three distinct Th pathways in human peripheral blood leukocytes (PBL) which has been valuable in interpreting results in AIDS patients; b) initiated a study of Th analyses in renal allografted patients, with the result that kidney graft rejection could be predicted, by in vitro testing of Th pathways of PBL from the transplanted patients.

Project Description

Major Findings:

The murine model of CsA-induced inhibition of T cell maturation has been extended to study the effects of CsA on T cell development in FTOC. The results indicate that CsA prevented the maturation of single positive ($CD^+CD8^-$ and $CD4^-CD8^+$) thymocytes, as well as the development of double positive ($CD4^+CD8^+$) T cells from double negative ($CD4^-CD8^-$) thymocytes. These results suggest that CsA affects two distinct stages of T cell maturation in the thymus. B cell development was not affected by CsA. However, natural killer (NK) cell activity was affected in that both NK cell number and activity was enhanced in irradiated mice, reconstituted with syngeneic bone marrow, and subsequently treated with CsA.

Human PBL were stimulated in vitro with recall antigens, HLA alloantigens, or T cell mitogens, in the presence or absence of different doses of CsA or of FK-506 (a new immunosuppressive drug). There was a dose-dependent selective inhibition of Th function such that the response to recall antigens was lost at lower doses than other responses. FK-506 had a different effect in that it: a) inhibited the response to recall antigens at a lower dose than CsA; and b) did not appear to affect the allogeneic and mitogen responses, even at higher doses, in contrast to CsA.

The observations that Th responses were not uniform for different stimuli in our models of AIDS development and selective inhibition of responses by immunosuppressive drugs raised the possibility that different Th pathways exist in the human. Using cell fractionation techniques, it was demonstrated that the response to recall antigens require self antigen-presenting cells (APC) and $CD4^+$ Th. In contrast, the Th response to HLA alloantigens can be mediated by any of three pathways: self APC + $CD4^+$ Th; allogeneic APC + $CD4^+$ Th; and allogeneic APC + $CD8^+$ Th. The demonstration of these multiple Th-APC pathways permits an interpretation of the AIDS data and of the differential sensitivity of responses to immunosuppressive drugs.

Based on the findings that the Th- APC pathways are differentially sensitive to the suppressive effects of CsA, 80 renal allograft patients who had received cadaveric kidney transplants and who were on immunosuppressive drugs were analyzed for Th function to HLA alloantigens. Cultures of fractionated PBL from patients were stimulated with alloantigens, and the presence or absence of Th function by the various pathways were determined and then compared with the clinical status of the renal grafts. The presence of the allo-specific self-restricted (self APC) $CD4^+$ Th pathway was associated with graft rejection. If this pathway was non-functional, no graft rejection could be detected. Absence or presence of the other pathways of Th were not correlated with rejection, but there was a correlation between the absence of the non-self-restricted pathways on an increased incidence of opportumistic infections. These results in renal allograft patients suggest that this approach can be used to assess Th function and adjust drug dosage to minimize both graft rejection and susceptibility to infection.

70% of this project is AIDS-related research.

Publications:

Fukuzawa M, Sharrow SO, Shearer GM. Effect of cyclosporin A on T cell immunity. II. Defective thymic education of CD4 T helper cell function in cyclosporin A-treated mice. Eur. J. Immunol. 1989;19:1147-1152.

Kosugi A, Zuniga-Pflucker JC, Sharrow SO, Kruisbeek AM and Shearer GM. Effect of cyclosporin A on lymphopoiesis. II. Developmental defects of immature and mature thymocytes in fetal thymus organ culture treated with cyclosporin A. J. Immunol. 1989; 143:3134-3140.

Via CS, Tsokos GC, Stocks NI, Clerici M and Shearer GM. Human in vitro allogeneic responses. Demonstration of three pathways of helper cell activation. J. Immunol. 1990; 144:2524-2528.

Clerici M and Shearer GM. Abrogation of different T helper cell pathways by in vitro exposure of human leukocytes to cyclosporin A. J. Immunol. 1990; 144:2480-2485.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09265-09 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Analysis of the T Cell Repertoire

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

| | | | |
|---|---|---|---|
| PI: | R. J. Hodes | Senior Investigator | EIB, NCI |
| Others: | R. Abe | Investigator | EIB, NCI |
| | M. Vacchio | Bio. Lab. Tech. | EIB, NCI |
| | S. Sharrow | Senior Investigator | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

An analysis of T cell receptor (TCR) expression in $Mls^c$ reactive T cells demonstrated a striking association of this reactivity with expression of the V$\beta$3 gene product. In addition, it was demonstrated that mouse strains which express $Mls^c$ delete expression of V$\beta$3 in their mature peripheral T cell populations. This failure to express V$\beta$3 presumably reflects a consequence of the maintenance of tolerance to self $Mls^c$ products. These findings provide a basis for the understanding of high T cell precursor frequency for $Mls^c$ products, reflecting the overall expression of V$\beta$3 in the T cell repertoire. Moreover, they demonstrate the importance of these products in selection of the antigen-specific T cell repertoire.

The range of self antigens that influence V$\beta$ usage was evaluated by studying the expression of 16 V$\beta$ families in inbred mouse strains. Significant decreases in expression occurred in 8 of the 16 V$\beta$ families. The self ligands responsible for these deletions appeared to consist of both MHC encoded and non-MHC encoded components. These results demonstrate that strain specific decrease in V$\beta$ expression occur in a major portion of the T cell repertoire and that a number of self-MHC and non-MHC products induced these deletions in the process of eliminating self reactivate T cells from the mature T cell pool.

Studies employing athymic nude mice demonstrated that V$\beta$ deletions associated with self tolerance failed to occur in athymic mice, demonstrating that the thymus has a critical mediating self tolerance by negative selection.



PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Major Findings:

1) Characterization of the Mls system. It was demonstrated that $Mls^a$ and $Mls^c$ are noncrossreative and that the genes encoding $Mls^a$ and $Mls^c$ determinants are non-allelic and unlinked. Mls is thus composed of the products of at least two non-allelic and unlinked genes, with no definitive evidence at present for polymorphism at either of these loci. An influence of MHC polymorphism upon the stimulatory activity of both $Mls^a$ and $Mls^c$ 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 proces of negative selection was studied by examining expression of each of the 16 commonly expressed T cells V$\beta$ gene families in a series of inbred mouse strains. V$\beta$ expression was studied by measuring mRNA specific for each V$\beta$ family. It was found that significant decreases in expression occur in at least 8 of the 16 V$\beta$ families and that each of these deletions is dominant in $F_1$ mice, consistent with the conclusion that these deletions occur in the process of eliminating T cells with potential reactivity for self determinants. 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. MHC encoded E$\alpha$E$\beta$ products, $Mls^a$ and $Mls^c$ determinants play identified roles in some of these deletions; additional as yet unidentified MHC and non-MHC gene products also mediate specific V$\beta$ deletions.

The role of the thymus in mediating T cell receptor negative selection was analyzed by studying congenitally athymic nude mice. A comparison of T cell receptor V$\beta$ expression in congenic pairs of normal and athymic mice indicated that the normal V$\beta$ deletions associated with tolerance to self $Mls^c$ or self MHC encoded E$\alpha$E$\beta$ products did not occur in athymic mice. These results demonstrates that the thymus has a critical role in mediating self tolerance by negative selection.

Proposed Course of Research:

Antibodies specific for T cell receptor structures will be utilized to determine whether the receptors used in recognition of $Mls^a$ and $Mls^c$ are the same or different from those used in recognition of other specificities. Attempts will be made to establish monoclonal cell lines expressing $Mls^a$ determinants and to identify Mls-congenic strains of mice. These resources together will be employed in an attempt to generate monoclonal antibodies specific for Mls products and ultimately to characterize the Mls structures involved in T cell activation. The observed deletion of V$\beta$3 expressing T cells from $Mls^c$ animals will be employed as a system in which to study the process of self-non-self discrimination. The deletions of V$\beta$3 expressing T cells will be studied in thymic and bone marrow chimeras.

Publications:

Abe R, Foo-Phillips M and Hodes RJ. Analysis of $Mls^c$ genetics: A novel instance of genetic redundancy. J Exp Med. 1989; 170:1059-1073.

Vacchio MS and Hodes RJ. Selective decreases in T cell receptor V$\beta$ expression: Decreased expression of specific V$\beta$ families is associated with expression of multiple MHC and non-MHC gene products. J Exp Med. 170:1335-1346, 1989.

Hodes RJ, Sharrow SO and Solomon A. Failure of T cell receptor $V_\beta$ negative selection in an athymic environment. Science 246:1041-1044, 1989.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09266-08 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

T Cell Regulation of B Cell Activation

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

| | | | |
|---|---|---|---|
| PI: | R. J. Hodes | Senior Investigator | EIB, NCI |
| Others: | K. Hathcock | Chemist | EIB, NCI |
| | S. Ullrich | Investigator | LCB, NCI |
| | E. Appella | Senior Investigator | LCB, NCI |
| | S. Murakami | 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

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 1.5 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Supernatants of activated cloned T helper cells are capable of replacing T cells in the antigen specific and MHC restricted induction of B cell IgG antibody responses. Two distinct and synergizing activities were identified in this supernatant: Interleukin 4 and an antigen-specific and MHC-restricted factor. This latter factor can be affinity purified employing monoclonal antibodies specific for T cell receptor V$\beta$8 determinants. Metabolic labeling and immunoprecipitation with anti-V$\beta$8 antibody allowed detection in cell-free helper clone supernatants of a disulfide-linked dimer indistinguishable from the T cell-surface $\alpha\beta$ heterodimer. Biochemical analysis demonstrated that the cell-free $\alpha\beta$ heterodimer is indistinguishable from the cell-derived form. The cell free $\alpha\beta$ heterodimer is selectively associated with components of the CD3/T cell receptor complex in a detergent sensitive and apparently lipid membrane dependent state.

The supernatants of activated type 2 T helper cell clone are capable of inducing polyclonal proliferation and immunoglobulin (Ig) secretion by heterogenous unprimed B cell populations. Studies employing recombinant lymphokines have demonstrated that IL5 is sufficient to induce these responses. Recombinant IL5 stimulation 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 demonstrate 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 Pgp-1 expressed by these cells is capable of mediating binding to the extracellular matrix material hyaluronic acid, indicating a potential role for Pgp-1 in regulating the trafficking of activated B cells in vivo.



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

Project Description

Major Findings:

1. Mechanisms of T helper function.

The nature of the signals transmitted by T helper cells to responding B cells in the course of MHC restricted and antigen specific pathways of B cell activation was studied. Cloned helper T cells were activated in vitro either through exposure to specific antigen and antigen presenting cells or through stimulation with solid phase immobilized antibody to the T cell receptor T3 complex. The cell free supernatant generated under these conditions was capable of functioning in an antigen specific and MHC restricted fashion to activate B cell IgG antibody responses. The helper activity of these supernatants was inhibited by monoclonal antibodies specific for IL4, demonstrating that this lymphokine is one of the essential and synergizing components of the helper cell supernatant. A second component, with the properties of both antigen specificity and MHC restriction, was affinity purified using monoclonal antibodies specific for the V$\beta$8 determinant of the T cell receptor. These findings demonstrate that both specific and non specific T cell products are capable of synergizing in the activation of B cells. Moreover, they strongly suggest that a cell-free form of the T cell receptor is capable of binding to its specific ligand and mediating T helper cell function.

In order to analyze the cell-free products of cloned T cells at a biochemical level, cells were $^{35}$S-methionine labeled and their supernatant products then evaluated by immunoprecipitation. Monoclonal antibodies specific for constant of variable (V$\beta$8-encoded) determinants on the T cell surface $\alpha\beta$ receptor precipitated material from cell-free supernatants which had a molecular weight of 85-90 kd nonreduced and 40-45 kd under reducing conditions. On reduced-nonreduced 2-dimensional gel analysis, this material was indistinguishable from the cell surface $\alpha\beta$ heterodimer.

2. IL5 mediated B cell activation.

Culture of heterogenous unprimed B cells with the supernatant of activated type 2 helper clones results in B cell proliferation, polyclonal Ig secretion, and phenotypic changes in the B cell population. Exposure to recombinant IL5 is sufficient to induce both proliferation and Ig secretion. In addition, IL5 stimulation results 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 Pgp-1 expression, this population was shown to contain nearly all of the proliferative and Ig secretory activity of IL5 activated B cell population. Since evidence has suggested that Pgp-1 can function as a cell adhesion molecule, with hyaluronic acid as one potential ligand, the ability of resting and activated B cells to bind to hyaluronic acid was assessed. It was found that IL5 activated B cells had a uniquely increased binding to hyaluronic acid, and this binding was inhibited by anti-Pgp-1. These findings suggest that Pgp-1 expression may represent a unique marker for B cells driven to proliferation and differentiation and that Pgp-1 itself may function as an adhesion molecule which is involved B cell trafficking in vivo.

Proposed Course of Project:

The signals involved in T cell-mediated activation of B cell responses will be studied through the characterization of cell free T helper products. The antigen specific T helper factor will be assessed by batch affinity purification. Attempts will then be made to more extensively biochemically characterize this product and in particular to establish its relationship to the cell surface T cell receptor. The ability to activate B cells in a highly specific fashion in the absence of intact T cells will also be employed to study the nature of signal transduction by B cells. Early response parameters such as PI hydrolysis and calcium flux will be studied in B cells activated either through lymphokine, antigen specific soluble helper factor, or combinations of such stimuli.

Publications:

Guy R, Ullrich SJ, Foo-Philips M, Hathcock KS, Appella E, and Hodes RJ. Antigen-specific helper function of cell-free T cell products bearing TCR $V_\beta 8$ determinants. Science 1989; 244:1477-1480.

Guy R, Foo-Philips M, and Hodes RJ. Expression of T cell receptor Vβ8 determinants on antigen-specific T helper factor. In Immune System and Cancer, Japan Scientific Societies Press, Tokyo, 1989; pp. 217-225.

Hodes RJ. T helper-B cell interaction. The roles of direct Th-B cell contact and cell-free mediators. Seminars in Immunology, 1989; 1:33-42.

Hodes RJ. T cell-mediated regulation: help and suppression. In Paul, W.E. (Ed.): Fundamental Immunology, 2nd edition, New York, NY, Raven Press, 1989; pp. 587:620.

Guy R and Hodes RJ. Antigen-specific, MHC restricted B cell activation by cell-free Th2 cell products: Synergy between antigen-specific helper factors and IL4. J. Immunol. 1989; 143:1433-1440.

Guy R, Ullrich SJ, Foo-Phillips M, Hathcock KS, Appella E and Hodes RJ. Antigen specific, MHC restricted help mediated by cell-free T cell receptor. In Gallin J and Fauci A. (Eds.): Advances in host immune defense mechanisms, Vol 7, Raven Press, New York, 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09267-08 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

T Cell Immune Function in Asymptomatic HIV Seropositive Patients

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

| | | | |
|---|---|---|---|
| PI: | G. M. Shearer | Senior Investigator | EIB, NCI |
| | M. Clerici | Visiting Fellow | EIB, NCI |
| Others: | 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 |

COOPERATING UNITS *(if any)* C. O. Tacket, Vaccine Program, University of Maryland School of Medicine, Baltimore, MD; D. Lucey, R. A. Zajac and R. N. Boswell, HIV Unit, Lackland AFB, TX; A. Landay and H. Kessler, Rush Medical Center, Chicago, IL; J. Giorgi, Department of Immunology, UCLA Medical School, Los Angeles, CA

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.2 | 1.4 | 0.8 |

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.)*

Peripheral blood leukocytes (PBL) from adult asymptomatic, HIV-infected patients ($HIV^+$) exhibit a complex pattern of T helper cell (Th) dysregulation that: a) involves a sequential loss of function to recall antigens, then to HLA allo-antigens, and finally to mitogens; b) is predictive for progression toward AIDS; and c) has also been verified in pediatric AIDS development (although progression in children appears to be more rapid).

The earliest Th defect in the $HIV^+$ is associated with a $CD8^+$ T cell that down-regulates self-restricted Th function, and produces a soluble factor that also down-regulates the same Th function.

Studies of antigen-presenting cell (APC) function were performed in $HIV^+$ and AIDS patients. No APC defect was detected in $HIV^+$, but 2/3 of AIDS patients exhibited one of two different types of APC defects.

A sensitive HIV-specific Th assay has been developed that can detect exposure to HIV up to 14 months before anti-HIV antibody is detected. This assay has been used to detect exposure to HIV in high-risk seronegative individuals and to monitor T cell immunity in uninfected volunteers immunized with an AIDS trial vaccine. Strong Th immunity was detected in the vaccinees immunized with a recombinant gp160 subunit vaccine.



PHS 6040 (Rev 1/84) GPO 904-917

Project Description

Major Findings:

This laboratory has reported that the Th function of asymptomatic HIV seropositive ($HIV^+$) patients exhibit a complex spectrum of progressive changes, such that patients: a) initially respond to all types of T cell stimuli; b) then selectively lose the ability to respond to MHC self-restricted antigens such as (but not exclusive to) recall antigens; c) next lose the ability to respond to alloantigens; and d) finally fail to respond even to mitogens. These changes can occur 2-4 years before $CD4^+$ cell numbers fall below $400/mm^3$, and are predicitve for progression toward AIDS. The above study is based on Th functional changes of >450 asymptomatic, $HIV^+$ individuals and on >50 AIDS patients from the U.S. Air Force. The above findings have been verified and extended in >40 pediatric, $HIV^+$ patients. (collaboration with Dr. P. Pizzo). Noteworthy is the finding that progression through the various Th functional stages appears to occur more rapidly in children than in adults. This laboratoary has also found that Th functional changes can be reversed in some but not all adult and pediatric patients who are on therapy with AZT, ddI, or soluble CD4. (collaborations with Drs. R. Yarchoan and S. Broder; Dr. P. Pizzo).

Using leukocytes from pairs of monozygotic twins, one of whom is $HIV^+$ and the other of whom is $HIV^-$, the laboratory has recently found that the early selective defect in Th function to recall antigens is due to a negative-regulatory cell, which releases a factor in vitro that can selectively down-regulate Th function of T cells from HIV-donors (manuscript in preparation). Analysis and characterization of this factor may be important for elucidating the early immunological changes that occur after HIV infection, and could be valuable in designing therapeutic protocols of intervention before AIDS symptoms start to appear.

Using an approach in which PBL from AIDS patients or from asymptomatic, $HIV^+$ patients are used as allogeneic stimulators of responses generated by PBL from $HIV^-$ donors, it was found that the $HIV^+$ individuals do not exhibit a detectable defect in antigen presenting/stimulating cell (APC/SC) capacity.

By using synthetic peptides if HIV (collaboration with Dr. J. Berzofsky), the laboratory has also assessed HIV-specific Th and T effector (CTL) function of HIV-infected patients and uninfected volunteers immunized with a recombinant gp160 candidate vaccine. It was found that HIV+ patients whose self-restricted CD4+ Th function was intact also exhibited HIV-specific Th and CTL function, whereas patients who failed to respond to other recall antigens via this pathway also failed to respond to HIV antigens by Th and CTL. The vaccinees elicited only marginal and transient HIV-specific antibody responses, but generated strong and persistent Th and CTL responses to HIV synthetic peptides, even 12 months after the last boost. The T helper cell responses of the vaccinees were up to 50-fold greater than those detected in asymptomatic, HIV-infected patients. These results suggest that an efficient vaccine can be developed, and indicates that human T cells respond to this vaccine.

In pursuit of identifying immune abnormalities in AIDS development, the laboratory has investigated the antigen-presenting cell (APC) function of asymptomatic $HIV^+$ and AIDS patients. The results indicate that no defect was detected in APC function of asymptomatic patients, but approximately 65% of AIDs patients exhibit one or two different types of APC defects (manuscript submitted). These results suggest that immunotherapeutic protocols designed to enhance or restore only T cell function may not be successful in reconstituting the immune system of AIDS patients.

A sensitive in vitro T helper cell assay has been recently developed by stimulating blood lymphocytes in vitro with the envelope protein of HIV. This assay is being employed to advance AIDS research in two ways. First, by using this assay to test blood lymphocytes from high risk individuals studied at intervals during a 3-to-4 year period, demonstrate exposure to HIV was demonstrated up to 14 months before the same high risk donors showed evidence of HIV exposure by antibody production or by PCR. Second, by studying a large population of high risk individuals known to have been multiply exposed over several years to HIV, but who failed to develop AIDS or to produce antibodies to HIV, a subset of individuals was identified whose T lymphocytes nevertheless elicited strong immunity to HIV. Although it is possible that these individuals will eventually develop AIDS, it is also possible that this subpopulation of high risk persons have somehow acquired natural protection against AIDS.

Based on the above findings, the multicenter AIDS Clinical Trials Group (ACTG) of the NIAID has recently adopted a modification of this laboratoy's approach for evaluation of Th function in $HIV^+$ patients that will be used by all of the ACTG Centers for analysis of T cell function before, during, and after AIDS therapeutic protocols.

Part of this research effort has resulted in the submission of an Invention Report entitled: "Method for Detecting Immune Dysfunction in Asymptomatic AIDS Patients and Other Conditions". This Invention Report has been selected by the National Technical Information Service, U.S. Department of Commerce for both U.S. and foreign filing: U.S. Patent Application Serial NO. 07/341,360.

100% of this project is AIDS-related research.

Publications:

Clerici M, Stocks NI, Zajac RA, Boswell RN, Bernstein DC, Mann DL, Shearer GM, Berzofsky JA. Antigenic peptides recognized by T helper lymphocytes from asymptomatic, HIV seropositive individuals. Nature 1989;339:383-385.

Morse HC III, Yetter RA, Via CS, Hardy RR, Cerny A, Hawakawa K, Hugin A, Miller MW and Shearer GM. Functional and phynotypic alterations in T cell subsets during the cause of MAIDS, a murine retrovirus-induced immunodeficiency syndrome. J. Immunol. 1989; 143:844-850.

Clerici M, Stocks NI, Zajac RA, Boswell RN, Lucey DR, Via CS and Shearer GM. Detection of three distinct patterns of T helper cell dysfucntion in asymptomatic, HIV-seropositive patients: Independence of $CD4^+$ cell numbers and clinical staging. J. Clin. Invest. 1989; 84:1892-1899.

Golding H, Shearer GM, Hillman K, Lucas P, Manischewitz J, Zajac RA, Clerici M, Gress RE, Boswell RN, Golding B. Common epitope in HIV I-GP41 and HLA class II elicits immunosuppressive autoantibodies capable of contributing to immune dysfunction in HIV-infected individuals. J. Clin. Invest. 1989;83:1432-1435.

Clerici M, Stocks NI, Zajac RA, Boswell RN and Shearer GM. Accessory cell function in asymptomatic human immunodeficiency virus-infected patients. Clin. Immunol. Immunopathol. 1990; 54:168-173.

Hosmalin A, Clerici M, Houghten R, Pendleton CD, Flexuer C, Earl P, Lucey DR, Moss B, Germain RN, Shearer GM and Berzofsky JA. An epitope in HIV-1 reverse transcriptase recognized by both mouse and human CTL. Proc. Natl. Acad. Sci. USA. 1990; 87:2344-2348.

Berzofsky JA, Hale PM, Clerici M, Cease KB, Houghten RA, Putney SD, Zajac RA, Boswell RN, Margalit H, Cornette JL, Spouge JL, DeLisi C and Shearer GM. Multideterminant regions of the HIV-1 envelope with sites seen by murine and human helper T cells: Circumventing the MHC restriction problem. In F. Brown, R.M. Chanock, H.S. Ginsberg and R.A. Lerner (Eds.): Vaccines 90: Modern Approaches to New Vaccines Including Prevention of AIDS. Cold Spring Harbor Laboratory Press. New York, pp. 307-312, 1990.

Fuchs D, Shearer GM, Boswell RN, Clerici M, Reibnegger G, Zajac RA and Wachter H. Activated cell-mediated immunity and reduced proliferative response of T-cells in vitro in patients with HIV-1 infection. Clin. Immunol Immunopathol. 1990, in press.

Clerici M, Stocks NI, Zajac RA, Boswell RN, Via CS and Shearer GM. Circumvention of defective CD4 T helper cell function in HIV-infected individuals by stimulation with HLA alloantigens. J. Immunol. 1990; 144:3266-3271.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09268-03 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Role of CD4 and CD8 Accessory Molecules in T Cell Function

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

| | | | |
|---|---|---|---|
| PI: | A. Singer | Senior Investigator | EIB, NCI |
| Others: | T. Nakayama | Visiting Fellow | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

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NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.25 | 1.25 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have examined the role of CD4 and CD8 in thymocyte differentiation. In these studies, we have evaluated the ability of CD4 and CD8 molecules to function as signalling molecules for mature and developing T cells. Interestingly, we have found that CD4 function is related to TcR expression and appears to play an important signalling role in T cell differentiation. Indeed, there are a number of indications in these studies that the initial function of CD4 in ontogeny is to inhibit TCR expression and signaling function.



PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Major Findings:

To examine the effect of CD4 cross-linking on developing T cells, we injected neonates with anti-CD4 mAb and examined TcR expression on the developing thymocytes. Remarkably, we found that the mAb-induced crosslinking of CD4 on $CD4^{+}8^{+}$ double positive thymocytes caused a 3-5 fold increase in their surface expression of TcR. This was a rapid effect, occurring within 16h, and was a direct effect on the immature double positive thymocytes themselves. Interestingly, the effect of anti-CD4 engagement on the mature single positive thymocytes within the same thymus was to reduce TcR expression. Thus, the effect of anti-CD4 engagement on TcR expression was a function of the developmental state of the responding T cell. It was possible that anti-CD4 treatment induced a positive signal in immature double positive thymocytes to increase TCR expression, or, alternatively, functioned to block a negative signal that tonically inhibits TCR expression in immature double positive thymocytes. To distinguish between these two possibilities, we developed an in vitro system to study TCR expression in double positive thymocytes. We found that separation of immature thymocytes from the thymic epithelium caused the thymocytes to spontaneously increase their expression of TCR. Furthermore, we found that CD4 signals, induced by multivalent cross-linking of anti-CD4 mAb, mimics the presence of thymic epithelium by inhibiting TCR expression. We found that 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 lack, 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.

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.

Publications:

Zuniga-Pflucker JC, McCarthy SA, Weston M, Longo DL, Singer A and Kruisbeek AM. Role of CD4 in thymocyte selection and maturation. J Exp Med 1989;169:2085-2096.

Cole JA, McCarthy SA, Rees MA, Sharrow SO and Singer A. Cell surface co-modulation of T cell receptor by anti-CD4 monoclonal antibody. J Immunol 1989;143:397-402.

Nakayama T, Singer A, Hsi ED and Samelson LE. Intra-thymic signalling in immature $CD4^{+}8^{+}$ thymocytes results in tyrosine phosphorylation of the T cell receptor zeta chain. Nature 1989;341:651-654.

Bonifacino JS, McCarthy SA, Maguire JE, Nakayama T, Singer DS, Klausner RD and Singer A. Novel post-translational regulation of TCR expression in $CD4^{+}8^{+}$ thymocytes influenced by CD4. Nature 1990;344:247-251.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09270-07 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Regulation of Expression of class I MHC genes

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

| | | | |
|---|---|---|---|
| PI: | Dinah Singer | Senior Investigator | EIB, NCI |
| Others: | Jocelyn Weissman | Chemist | EIB, NCI |
| | Jeffrey Richardson | IRTA | EIB, NCI |
| | Jean Maguire | BTP | EIB, NCI |

COOPERATING UNITS *(if any)*

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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.)*

Class I MHC genes encoding transplantation antigens are ubiquitously expressed, although their level of expression varies among the tissues, reflecting regulated expression of the genes. Analysis of the 5′ flanking DNA sequences of a swine class I gene has demonstrated that in addition to the promoter, this region contains a series of negative and positive regulatory elements. Each of these elements functions through its binding of specific trans acting factors. One of the silencer elements associated with this gene consists of a regulatory DNA complex in which reside overlapping negative and positive elements. Analysis of this complex has 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.





GPO 914-918

Project Description

Major Findings:

Expression of individual class I genes is actively regulated: multiple genes within the family show similar patterns and responses, whereas other class I antigens, such as Qa and TL, have been classified as differentiation antigens because of their limited tissue distribution. We speculate that common mechanisms exist which coordinately regulate expression of classical class I genes and separate mechanisms exist which regulate expression of the differentiation antigens. In earlier studies, we demonstrated that introduction of one of the swine class I genes, PD1, into a transgenic mouse resulted in its regulated expression, in a pattern indistinguishable from that observed in situ in the pig. Furthermore, interferon treatment of transgenic animals increased expression of the PD1 gene, indicating that regulatory sequences were contained within the transfected DNA segment. In order to further define the regulation of PD1, we have constructed a variety of deletion mutants generated from the 5' flanking sequences which have allowed us to identify and define a series of regulatory elements.

To date, our studies have focussed primarily on the 1.1 kb of 5' flanking DNA sequences of PD1. Using a series of 5' deletion mutants, 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 negative regulatory elements. Silencer I maps between -700 and -800 bp, while silencer II is located between -400bp and -500bp . Removal of silencer I increases expression 2-5 fold, while further removal of silencer II augments the increase by about 2-fold. Detailed mapping of the region around silencer I revealed that it consists of two overlapping functional elements: a silencer and an enhancer. The silencer binding site consists of two binding domains, whereas the enhancer is comprised of an inverted repeat. Neither element is homologous to other known mammalian regulatory elements. In vivo competition studies have demonstrated that the activities of the elements are mediated by trans acting factors. Each element associates with distinct trans acting factors. The level of repressor factor binding to silencer I is inversely proportional to the level of class I expression, suggesting that this repressor plays a large role in regulating class I transcription. Enhancer binding activity to the silencer I region is constitutively present in all cells. Silencer II competes with silencer I for binding of trans acting factors, although there are no significant sequence homologies between the two silencer elements. Initial studies aimed at characterizing the trans acting factors reveal that each component of silencer I binds a complex of at least two proteins. None of the proteins which bind silencer I can bind independently, but must bind as a complex. Further studies are in progress to further define these proteins.

Publications:

Singer DS, Frels W and Ehrlich R. Regulation of expression of a class I MHC transgene. In Transgenic Animals, ed. N. First, Butterworth Press, Stoneham, MA. 1989; in press.

Maguire J, Ehrlich R, Frels W and Singer DS. Regulation of class I MHC expression: in vivo function of regulatory DNA sequence elements in transgenic mice. In Transgenic mice and mutants in MHC research. eds. C. David and I. Egorov, 1989; in press.

Singer DS and Maguire J. Regulation of expression of class I MHC genes. CRC Reviews in Immunology, 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09271-05 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Graft-versus-Host Disease in Allogeneic Marrow Transplantation and Tumor Purging

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

| | | | |
|---|---|---|---|
| PI: | Ronald E. Gress | Senior Investigator | EIB, NCI |
| Others: | Robert Moses | Biotechnology Fellow | EIB, NCI |
| | Raphael Hirsch | Biotechnology Fellow | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

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|---|---|---|
| 2.5 | 2.5 | |

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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.)*

Graft-versus-host disease (GvHD) is dependent in its generation on the presence of immune competent T cells in the donor marrow with specificity for allogeneic antigens of the host. The activation of these cells in the host after infusion and the resultant tissue injury is clinically recognized as GvHD. One approach to preventing GvHD is the removal of these T cells from the marrow inoculum. We have pursued T cell depletion of human marrow for the purpose of preventing GvHD in marrow transplantation across MHC differences in man by the use of monoclonal antibodies specific for cell surface molecules unique to T cells in combination with complement. Two such antibodies have been derived, one with specificity for CD2 (sheep red blood cell receptor) and one which defines a novel T cell determinant. The latter antibody precipitates a 92 KD molecule and a predominant 45 KD band under non-reducing conditions and reducing conditions, respectively. These two antibodies, in combination with CD7 and CD5 specific antibodies, have been used to carry out studies in human bone marrow T cell depletion. To quantitate T cells remaining in marrow after such depletions, a limiting dilution assay was developed which detects T cells at a level of one T cell per $10^5$-$10^6$ marrow cells. A bank of cryopreserved marrows depleted of T cells by a combination of monoclonal antibodies and complement, with the extent of that depletion assessed by limiting dilution assay, has been established. The techniques developed for the removal of normal T cells from allogeneic marrow have also been applied to the removal of malignant T cells from autologous marrow. A clinical protocol has been developed to assess the feasibility of utilizing these marrows in the treatment of aggressive T cell malignancies with marrow ablative chemotherapy and radiotherapy.

Project Description

Major Findings:

The primary approach taken in these studies of T cell depletion of human marrow has been by antibody and complement; alternative approaches such as the use of immunotoxins, elutriation, and immune rosetting have also been evaluated. Initial studies with antibody and complement established conditions for optimal complement mediated lysis and showed that individual antibodies differed in their ability to effect lysis in the presence of complement. A combination of antibodies was superior to single agents not in the extent of depletion, but in reproducibility from treatment to treatment. In addition to two antibodies available from other sources (CD 7 and CD 5 specific), neither of which was adequate to mediate pan T cell depletion effectively, we derived monoclonal antibodies with screening by complement mediated lysis. Two antibodies of interest were isolated. One was CD2 specific and has been utilized in these studies for T cell depletion and in investigations of the role of the LFA-2 accessory molecule in T cell function. It detects an epitope region distinct from that detected by OKT11. The second antibody is specific for a novel determinant on T cells. The determinant for which this antibody is specific is expressed by cells of hematopoietic origin, is confined to T cells, and is concordant with the expression of CD5 and CD3. Immunoprecipitation demonstrates a 92 KD molecule under non-reducing conditions and a predominate 45 KD band under reducing conditions. Comparisons of expression on a series of T cell lines, including a line deficient in the expression of T cell receptor, distinguished the determinant defined by this antibody from those detected by known anti-T cell monoclonal antibodies.

The number of donor marrow T cells necessary for the generation of GvHD is on the order of 0.1% in the mouse or 1 x 105/kg in man. Assays commonly used for the quantitation of residual T cells after depletion such as proliferative responses to mitogens (PHA) or alloantigens (MLR) or analysis by FACS detect on the order of 1% T cells and so are insufficient in sensitivity. A limiting dilution assay was therefore developed based on the clonogenic potential of peripheral human T cells. The readout is cell growth; the sensitivity of this assay is sufficient to detect one T cell in $10^5$-$10^6$ marrow cells and the specificity has been confirmed by a variety of techniques. This limiting dilution assay has been essential to the monitoring of T cell depletion from human marrow. With this assay, the depletion regimen was refined and different methods of marrow harvest evaluated with respect to influence on final marrow T cell content. Surgical resection of marrow was superior to conventional aspiration as a method of harvest. One consideration in the clinical use of surgically resected marrow is marrow cell yield. The final yield in a series of harvested marrows (after treatment to remove T cells) was 74% of the Ficoll population for a final mean total cell number of 9.3 x $10^9$ cells per donor. The residual T cell content was less than one T cell per $10^5$ marrow cells which represented, in each case, greater than a three log depletion of T cells. Progenitor cell (CFU-C and BFU-E) recovery was 42-100%. These and similarly prepared marrows have been cryopreserved as a "universal donor bank". In evaluating alternative methods of T cell depletion, the most effective and reproducible was elutriation. The processing of human marrow for clinical use

has now been adapted to a semi-automated system, which includes elutriation followed by treatment with antibody plus complement. 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. Evaluation of the efficacy of this new regimen is in progress.

Publications:

Moses RD, Beschorner WE, Singer D, Orr KS, Bacher JD and Gress RE. Restriction fragment length polymorphism analysis with a crossreactive HLA class II DR-beta gene probe for the detection of engraftment of MHC-mismatched marrow in the rhesus monkey. Bone Marrow Transplantation, 1989;4:475-481.

Gress RE. Bone Marrow Transplantation. Current Opinions in Immunology 1989; 1:1213-1220.

Gress RE, Lucas PJ, Cassel J, Nakamura H, Moses RD and Quinones RR. Efficacy of T cell depletion of surgically resected cadaveric marrow: Considerations in HLA-mismatched marrow transplantation. In: Gross S, Gee A and Worthington-White, DA eds, Bone Marrow Purging and Processing. Wiley-Liss. New York, pp. 471-489, 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09273-03 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

T Cell Differentiation and Repertoire Selection

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

| | | | |
|---|---|---|---|
| PI: | A. Singer | Senior Investigator | EIB, NCI |
| Others: | E.W. Shores | Guest Researcher | EIB, NCI |
| | J. Roberts | Guest Researcher | EIB, NCI |
| | Y. Takahama | Visiting Fellow | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

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| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.35 | 1.35 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have examined thymic influence on the MHC-specificity expressed by functionally and phenotypically distinct T cell subsets. Studies on thymocytes from genetically defective scid mice have suggested that TcR($\gamma\delta$) cells may play a critical role in promoting the entry of thymocytes into the CD4/CD8 differentiation pathway. Studies on $CD4^-CD8^-$ thymocytes that express TCR$\alpha\beta$ have revealed that they are most likely an end stage cell type, rather than a precursor phenotype. In other studies, we have found that tolerance to self antigens can be induced in the thymus by either clonal deletion or clonal inactivation.





GPO 914-918

Project Description

Major Findings:

In order to examine the general relationship between TcR expression and T cell differentiation, we have examined a genetically defective mouse strain. Mice with severe combined immune deficiency (scid), lack both receptor bearing T cells and receptor bearing B cells. It is thought that this genetic defect results from a deficiency in the recombinase enzymes necessary for receptor gene rearrangements, making it very difficult for the lymphocytes in these animals to express any antigen receptors. As a result, these animals represent an excellent model for examining how far T cell differentiation can progress in the absence of TcR expression, and for indicating which steps are in fact dependent on TcR. In normal T cell ontogeny, T cells can be characterized by their expression of CD4 and CD8 accessory molecules. Thus, the kinetics of appearance of thymocyte subsets is: $CD4^-CD8^-$ -> $CD4^+CD8^+$ -> $CD4^+CD8^-$, $CD8^+CD4^-$. The $CD4^+CD8^+$ double positive subset consists of approximately equal numbers of $TcR^+$ and $TcR^-$ cells, suggesting that TcR expression is not necessary for thymocytes to become $CD4^+CD8^+$. Consequently, the expectation was that, in scid mice, thymocyte development would proceed through the generation of double positive cells. To our surprise, we have found that $Thy1^+$ thymocytes from most scid mice contain only $CD4^-CD8^-$ (double negative) $TcR^-$ cells. These cells are $IL\text{-}2R^+$ and Ly1 dull, and so are similar to double negative cells from the thymi of normal mice. What is surprising from these results is that, in the absence of $TcR^+$ cells, T cell differentiation does not proceed beyond double negative cells. We found that in "leaky" scid mice (those few mice that spontaneously express $TcR^+$ cells) the intra-thymic presence of $TcR^+$ cells was invariably accompanied by the intra-thymic presence of $CD4^+/CD8^+$ cells. Surprisingly, however, the $CD4^+/CD8^+$ cells were not necessarily themselves $TcR^+$. We further examined this apparent paradox by reconstituting unirradiated scid mice with bone marrow stem cells from normal AKR mice that would give rise to normal $TcR^+$ AKR thymocytes. In these thymi, the scid thymocytes remained $TcR^-$ but became $CD4^+/CD8^+$. Thus, intra-thymic $TcR^+$ cells were able to promote the differentiation of $TcR^-$ thymocytes into CD4/CD8 expressing cells. When applied to normal T cell differentiation, these results suggest a critical role for early appearing TcR($\gamma\delta$) thymocytes in initiating the entry of $CD4^-8^-$ double negative thymocytes into the CD4/CD8 TcR($\alpha\beta$) differentiation pathway.

We have characterized a rare thymocyte subpopulation characterized as $CD4^-CD8^-$ TCR$\alpha\beta$. We have confirmed that these cells have a markedly skewed TCR repertoire, but that the skewing does not reflect the accumulation of non-deleted T cells expressing autoreactive TCR. Furthermore, these cells are not consistently influenced by self-MHC or self-nominal antigens, as their TCR repertoire is not clearly molded by their self-antigens. Finally, these cells have a mature phenotype in that they are $Qa2^+$ and sensitive to cyclosporin treatment.

Finally, we have examined the mechanism by which T cell tolerance is induced in the thymus. We found that the thymus is able to induce either deletion of inactivation of T cells expressing potentially autoreactive TCR. The alternative outcomes are mediated by two distinct cell types that can be distinguished by their sensitivity to $\gamma$-irradiation.

Publications:

Shores EW, Sharrow SO, Uppenkamp I and Singer A. Developmental arrest of T cell receptor (TcR) negative SCID thymocytes and release in the presence of $TcR^+$ Cells. In Melchers, F., et al (Eds.): Progress in Immunology VII. Springer-Verlag, Berlin, 1989;567-571.

Shores EW, Sharrow SO, Uppenkamp I and Singer A. $TcR^-$ thymocytes from SCID mice can be induced to enter the CD4/CD8 differentiation pathway. Eur J Immunol 1990;20:69-77.

Roberts JL, Sharrow SO and Singer A. Clonal deletion and clonal anergy in the thymus induced by cellular elements with different radiation sensitivities. J Exp Med 1990;171:935-940.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09275-03 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

In Vivo Study of MHC-Specific T Cells

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

| | | | |
|---|---|---|---|
| PI: | A. Singer | Senior Investigator | EIB, NCI |
| Others: | P. Stein | BTP Fellow | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.25 | 1.25 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

This project has attempted to apply our understanding of the cellular mechanisms involved in in vitro anti-MHC responses to in vivo transplantation responses. In studying skin allograft rejection, we have identified the phenotype, specificity, and interaction capabilities of the T cells able to initiate and effect in vivo rejection responses. Using allophenic skin grafts, we found that the effector mechanism of skin graft rejection is itself antigen-specific, and that defects in Th cell function result in longterm retention of skin allografts despite the presence of antigen-specific effector cells. In addition, we have assessed the cellular mechanisms mediating the rejection of fetal pancreas and Islet cell allografts.

Project Description

Major Findings:

We have been studying transplantation immunity as the in vivo analog of the anti-MHC responses we had been examining in vitro. Consequently, our initial focus was on determining the phenotype, function, and specificity of the T cells that initiate and mediate in vivo rejection responses. We chose to first study the rejection of skin allografts because skin allografts trigger the most potent of all in vivo rejection responses, and because they are convenient to assess and follow.

Our studies strongly supported the concept that in vivo rejection responses, once initiated by antigen-specific Th cells, are mediated by antigen-specific Tk cells. However, they did not directly demonstrate that antigen-specific Tk cells actually mediate the rejection process. Indeed, it was conceivable that allograft rejection is effected by lymphokines secreted by antigen-specific Th cells or DTH cells that either nonspecifically recruit inflammatory effector cells or induce intense vasospasm, causing nonspecific ischemic necrosis of the graft. To distinguish between antigen-specific and antigen-nonspecific effector mechanisms of allograft rejection, we constructed B6<->A/J allophenic mice which are genetic mosaics whose individual cells express either H-$2^b$ or H-$2^a$ determinants, but not both. The fur of these B6<->A/J allophenic mice consists of both black and white hairs, the black hair resulting from pigment donated by B6 (H-$2^b$) melanocytes and the white hair resulting from A/J (H-$2^a$) melanocytes that are genetically unable to synthesize pigment. Skin from these allophenic mice were grafted onto H-$2^b$ nude mice and rested for 1 month to permit the grafts to regrow their black and white hairs. Because nude mice are immunoincompetent, the experimental grafts were not rejected. The animals were then reconstituted with H-$2^b$ T cells responsive against the H-$2^a$ cellular elements of the allophenic grafts but tolerant to its H-$2^b$ elements. Within two weeks of the T cell transfer, the grafts were enveloped by an intense non-specific inflammatory response that caused all the hairs of the allophenic grafts to fall out. Nonetheless, the allophenic grafts survived the inflammation, and, most remarkably, regrew hair. The color of the regrown hair was overwhelmingly black, the color of the syngeneic H-$2^b$ parent, providing visual proof that the effector mechanism of skin allograft rejection destroys cells expressing allogeneic histocompatibility antigens but spares cells that do not. Thus, the effector mechanism of skin allograft rejection is MHC-specific and, therefore, mediated by a Tk cell that assesses individual cells in the dermis of the graft for expression of foreign histocompatibility antigens. Most 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-$\gamma$. Indeed, we found that rejection of MHC class II disparate grafts was blocked specifically by antibodies against IFN-$\gamma$, whereas rejection of MHC class I disparate grafts were not.

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:

Gill RG, Rosenberg AS, Lafferty KJ and Singer A. Characterization of primary T cell subsets mediating rejection of pancreatic islet grafts. J Immunol 1989;143:2176-2178.

Rosenberg AS, Katz SI and Singer A. Rejection of skin allografts by $CD4^+$ T cells is antigen specific and requires expression of target alloantigen on $Ia^-$ epidermal cells. J Immunol 1989;143:2452-2456.

Rees MA, Rosenberg AS, Munitz TI and Singer A. In vivo induction of antigen specific transplantation tolerance to $Qal^a$ by exposure to alloantigen in the absence of T cell help. Proc Natl Acad Sci (USA) 1990;87:2765-2769.

Rees MA, Rosenberg AS and Singer A. Characterization of T cell subsets mediating rejection of established fetal pancreas grafts and failure to observe graft adaptation. Transplantation In press.

Rosenberg AS, Rees MA, Munitz TI and Singer A. In vivo model of antigen specific transplantation tolerance. In: Lotze MT and Finn OJ (Eds.): Cellular Immunity and the Immunotherapy of Cancer. Wiley-Liss, New York.

Rosenberg AS, Finbloom DS, Maniero TG, Van der Meide PH and Singer A. Specific prolongation of MHC class II disparate skin allografts by in vivo administration of anti-IFN$\gamma$ monoclonal antibody. J Immunol In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 09276-05 E

PERIOD COVERED: October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*: Expression and function of porcine class I MHC genes in transgenic mice.

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

| | | | |
|---|---|---|---|
| PI: | Dinah Singer | Senior Investigator | EIB,NCI |
| Others: | Carmen Bordallo | Visiting Fellow | EIB, NCI |
| | Jean Maguire | BTP | EIB,NCI |
| | Jeffrey Richardson | IRTA | EIB,NCI |
| | W. Frels | Agricultural Research Service | USDA |

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Experimental Immunology Branch

SECTION:

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

| TOTAL MAN-YEARS | PROFESSIONAL: | 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.)*

Porcine class I major histocompatibility (MHC) genes have been introduced into the genome of C57BL/10 mice in order to study their in vivo patterns of expression and to analyze the ability of their products to function. One transgenic line which contains a classical class I MHC gene (PD1) expresses SLA antigen on its cell surface skin grafts from the B10.PD1 transgenic are rejected by normal C57BL/10 mice, indicating that the foreign SLA antigen is recognized as a functional transplantation antigen Expression of PD1 parallels that observed in the swine, indicating that its expression is regulated. In vivo treatment of the transgenic with $\sigma/\beta$-interferon increases PD1 expression in a number of tissues. To further characterize regulatory DNA sequence elements responsible for tissue specific expression of class I gene, a series of 5′ deletion mutants of PD1 was generated. DNA constructs containing the deletions mutants ligated to a reporter gene, CAT, were introduced into transgenic animals and assessed for their patterns of expression. Within 1.1 kb upstream of transcriptional initiation, negative and positive regulatory elements were identified, some of which function in a tissue specific fashion. In particular, a complex element consisting of an overlapping enhancer and silencer demonstrates marked tissue specificity.

Transgenic lines have been generated containing a swine class I gene PD7, which is closely related to PD1. The two lines differ in the extent of non-coding 5′ flanking sequence which has been introduced with the PD7 gene. Analysis of PD7 expression in the two lines has revealed the presence of tissue specific silencers associated with the gene. The difference in extent of 5′ flanking sequences in the two constructs results not only in differential levels of antigen expression but different rates of graft rejection.





GPO 914-918

Project Description

Major Findings:

Expression of individual class I genes is actively regulated: multiple genes within the family show similar patterns and responses, whereas other class I antigens, such as Qa and TL in the mouse, have been classified as differentiation antigens because of their limited tissue distribution. In order to further define the regulation of these genes, we are constructing transgenic mice, containing individual class I MHC genes from the swine. The swine genome contains seven class I genes. We have constructed transgenic animals for three of these genes: PD1, a classical class I MHC gene; PD7, a classical class I gene with a mutation in enhancer A; and PD6, a non-classical class I gene which is highly divergent from the rest of the family.

Analysis of RNA from swine tissues revealed that both PD1 and PD7 are expressed at highest levels in lymphoid tissues, and at lower but varying levels in a variety of other tissues, including thymus. Within the lymphoid compartment, PD1 expression is differentially regulated, as evidenced by the finding that PD1 is preferentially expressed in B cells. Analysis of RNA from PD1 transgenic mouse tissues reveals that the pattern of PD1 expression, both qualitatively and quantitatively, parallels that observed in situ. Thus, regulatory elements necessary for the normal expression of PD1 are contained within the 9 kb pig DNA segment which was introduced into the transgenic mouse. To further characterize these elements, a series of deletion mutants extending from 1.1 kb upstream to 290 bp upstream of transcriptional initiation was generated. Each deletion mutant was ligated to the reporter gene, chloramphenical acetyl transferase (CAT) and tested by transfection in L cells. Informative deletion mutants were selected and introduced into transgenic mice. For each of the six deletion mutants selected, a number of lines were generated and their tissues analyzed for expression of CAT. The longest 5′ construct, containing nearly the entire 1.1 kb of flanking sequences, displayed patterns of expression parallel to those of the complete PD1 gene. That is, expression is highest in lymphoid tissues and lower in others, notably kidney, liver, and brain. Further deletions revealed the presence of negative and positive regulatory elements which function in vivo in a tissue specific fashion. Studies are underway to more precisely define each of these elements and their tissue specificity.

Lines of transgenic mice containing the PD7 gene were generated from two DNA constructs which differed in the extent of 5′ flanking sequences adjacent to the promoter. Patterns of expression of these two constructs were grossly similar. However, there were marked differences in the relative levels of expression of PD7. In non-lymphoid tissues, such as kidney and liver, both constructs were expressed equally. In lymphoid tissues, such as spleen and skin, the shorter construct was preferentially expressed. The observation that the construct containing additional 5′ flanking sequences is expressed less efficiently than a shorter one suggests the presence of a negative regulatory element associated with the PD7 gene. The fact that equivalent expression occurs in some tissues further indicates that this is a tissue-specific element. Both lines of PD7 transgenic are capable of mediating graft rejection. Thus, the PD7 antigen is expressed and functions as a transplantation antigen. However, such grafts are rejected more slowly than those containing the PD1 antigen. Furthermore, grafts with the longer PD7 constructs are rejected more slowly than those with the

shorter construct, consistent with the observed differences in amount of RNA in the skin of the two lines. Studies are currently underway to identify and characterize DNA sequence elements which regulate PD7 expression.

A highly divergent member of the class I MHC gene family, PD6, has previously been demonstrated to have in vivo patterns of expression distinct from PD1. For example, PD1 is expressed in the thymus whereas no PD6 transcripts are detectable in thymus. Within the lymphocyte population, PD1 is preferentially expressed in B cells, whereas PD6 is preferentially expressed in T cells. To further characterize the patterns of PD6 expression and the DNA sequence elements which mediate the tissue specific differences, lines of transgenic animals containing PD6 have been generated. These lines are currently being analyzed for expression of the PD6 transgene and for expression of PD6 antigen.

Publications:

Ehrlich R, Maguire J and Singer DS. Effects of in vivo inteferon treatment of class I MHC gene expression in transgenic mice. Immunogenetics, 1988;30:18-26.

Singer DS, Frels W and Ehrlich R. Regulation of expression of a class I MHC transgene. In: Transgenic Animals, ed. N. First, Butterworth Press, Stoneham, MA. 1990; in press.

Maguire J, Ehrlich R, Frels W and Singer D.: Regulation of class I MHC Expression: In vivo function of regulatory DNA sequence elements in transgenic animals. In: Transgenic mice and mutants in MHC research. eds. C. David and I. Egorov, 1990; in press.

Bordallo C, Frels W, Lunney J, Rosenberg A and Singer DS. Expression of anon-classical class I gene in transgenic mice. In: Transgenic mice and mutants in MHC research. eds. C. David and I. Egorov, 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09279-04 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Isolation and characterization of a non-classical H-2 class I gene

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

| | | | |
|---|---|---|---|
| PI: | Dinah Singer | Senior Investigator | EIB, NCI |
| Others: | Stuart Rudikoff | Senior Investigator | LG, NCI |
| | Kevin Howcroft | IRTA | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.7 | | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

A highly divergent member of the class I MHC gene family, M1, represents a direct descendant of a primordial class I gene, which antedates speciation. Using a series of recombinant and congenic strains of mice, this gene has been mapped to a region of chromosome 17 telomeric to Qa. In conjunction with studies of other divergent class I genes, such as those encoding HMT, M1 has been used to define a new MHC subregion, M.

Despite the extensive divergence of M1 from the rest of the H-2 class I family, M1 has open reading frames and legitimate splice sites in all exons. M1 appears to contain a functional promoter since introduction of a 3.4 kb genomic DNA fragment containing M1 into mouse L cells results in transcription of the gene. Nevertheless, M1 is not detectably transcribed in a variety of cell lines or adult somatic tissues and no M1 antigen has been identified. Expression of M1 is controlled by a strong silencer element located within a 16 kb genomic fragment containing the M1 gene.

Project Description

Major Findings:

The murine class I MHC 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 non-classical class I gene. Ml is the first gene to define this region; others which map to the same region are HMT and thy19.4.

Although by DNA sequence analysis Ml appears to have the capacity to encode a product, it has not been possible to identify such a product. Ml transcripts are not detected by direct Northern analysis of either lymphoid or non-lymphoid cell lines or any adult somatic tissue. More sensitive detection assays, namely by PCR amplification, have similarly failed to reveal the presence of Ml transcripts in these tissues. The regulaton of Ml expression is currently under investigation. Introduction into L cells of the Ml gene within a 17 kb genomic fragment ofDNA does not result in the expression of the gene. Howeve, introduction into L cells of a 3.5 kb derivative, containing all of the Ml coding sequences does generate Ml transcripts. These observations suggest the presence of a negative regulatory element associated with Ml flanking sequences; the precise location and sequence of this element is currently being determined. Transgenic mice containing the 3.5 kb genomic fragment of Ml are currently being generated and characterized. To characterize the Ml product, Ml coding sequences have been placed under the control of a constitutive promoter; this construct is being introduced into cells to obtain expression of Ml antigen.

Publicatons: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09281-05 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Receptor Mediated T Cell Activation

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

| | | | |
|---|---|---|---|
| PI: | R. J. Hodes | Senior Investigator | EIB, NCI |
| Others: | K. S. Hathcock | Chemist | EIB, NCI |
| | P. Bohjanan | Howard Hughes Fellow | EIB, NCI |
| | M. Okajima | Biotechnology Fellow | EIB, NCI |

COOPERATING UNITS *(if any)*

Food and Drug Administration

LAB/BRANCH

Experimental Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.0 | 1.0 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The role of endogenous lymphokines in T cell activation has been demonstrated. The proliferative response of type 2 T helper clones is inhibited by antibody specific for the lymphokine IL4, which is produced by these clones. This demonstrates that IL4 acts as an autocrine growth factor for these T cells. In addition, IL4 mRNA levels corresponding to a number of activation genes are inhibited by the presence of anti-IL4 antibody during T cell activation. In contrast, the level of mRNA is actually increased in the presence of this antibody, suggesting that an endogenously produced IL4 may normally down regulate its own gene transcription. The regulation of IL3, IL4, IL5, and GM-CSF lymphokine gene expression by type 2 helper clones was studied in more detail, using multiple inducing stimuli and pharmacologic inhibitors. It was found that independent (non-coordinate) regulation of discrete lymphokine genes can occur and is dependent upon the nature of the inducing stimulus. In particular, it was found that expression of IL4 and IL5 genes, but which are generally co-expressed by type 2 T helper clones in response to T cell receptor-mediated stimuli, can be indepedently regulated. The activation of T helper type 1 clone AE7.6 is influenced by the prior activation history of the cells. Sub-cloned T cells were carried in vivo either by stimulation with IL2 alone or by stimulation with specific antigen and antigen presenting cells in addition to IL2. Cell lines carried under both conditions were capable of proliferating in response to stimulation with anti-CD3 antibody. However, this stimulation induced strong phosphatidyl inosotol hydrolysis and increased intracellular calcium concentrations only in cells which had been maintained by stimulation with IL2 alone; cells that had been repeatedly stimulated with specific antigen generated neither PI nor calcium responses to anti-CD3. The signalling pathways utilized by cloned T cells were therefore influenced by prior stimulation through the T cell receptor.

Major Findings:

1. Activation of Naive T Cells.

Vigorous proliferation was induced in unfractionated or $Lyt2^+$ T cells by soluble F23.1 in the presence of IL-2 and accessory cells; whereas no significant proliferative responses were observed in $L3T4^+$ T cells. In contrast, the Fab of F23.1 covalently linked to the Fab on anti-$A^k_\alpha A^k_\beta$ antibody was capable of activating $Lyt2^+$ or $L3T4^+$ T cells even in the absence of IL-2 but in the presence of $A^k_\alpha$ $A^k_\beta$-positive accessory cells. This activation mediated by heteroaggregate antibody required specific targeting by covalently linked antibodies. Proliferation of naive T cells was also induced by antibodies to a component of the T3 complex, in the absence of exogenous lymphokine. Under these conditions, proliferation was substantially inhibited by antibodies to IL-2 or to IL-4, suggesting a possible role of endogenously produced lymphokines in the proliferative response of T cells to this stimulus.

2. Activation of Cloned T Cells.

The type 1 T helper clone AE7.6 is stimulated to proliferate by immobolized 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 antigen presenting cells 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 calcium in response to anti-CD3. In contrast, cells that had been previously stimulated with specific antigen and antigen presenting cells, and then allowed to "rest" gave markedly reduced PI and calcium responses. The signalling pathways activated in these T cells are thus strongly influenced by recent activation history of these cells.

3. Function of Endogenous Lymphokines in T Cell Activation.

Cloned T helper cells produce a number of now well characterized lymphokines and in addition are themselves responsive to a number of lymphokines. One class of T cell clones, termed type 2, produces IL4 and is induced to proliferate in response to exogenous IL4. The fact that IL4 acts as an autocrine growth factor for these cells was demonstrated. Proliferation and T cell activation were induced by immobilized antibodies specific for the T3 complex of the T cell receptor. Under these conditions, proliferation was blocked by the presence of antibody to IL4. Activation of other genes including the proto-oncogenes c-myc and c-myb were also inhibited by the presence of anti-IL4 antibody. T cell activation mediated through the T cell receptor complex is thus dependent upon an autocrine role of IL4. In contrast to these inhibitory influences, it was

observed that the presence of anti-IL4 antibody resulted in a significant increase in steady state IL4 mRNA levels. These latter data suggest that the presence of endogenously produced IL4 normally down regulates the production of further IL4, and that it does so at the level of gene transcription or of message stabilization. It was found that the pattern of lymphokine genes expressed by type 2 T helper clones was dependent upon the stimulus used to activate the T cells. Some lymphokines, such as IL5, were induced in response to anti-CD3 as well as in response to recombinant IL2 stimulation, whereas IL4 gene expression by the same clone was induced by anti-CD3 but not recombinant IL2. 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 type 2 T helper cells, can be independently regulated.

Proposed Course of Project:

The consequences of T cell binding by anti receptor antibodies will be studied at a biochemical level. Influences of receptor binding on early gene activation, ion flux and IL-2 receptor expression will be assessed. The role of exogenous as well as endogenously produced lymphokines on T cell proliferation and specific gene induction will also be evaluated.

Publications:

Bonvini E, DeBell KE, Kolber ME, Hoffman T, Hodes RJ, and Taplits MS. Hydrolysis of inositol phospholipids induced by stimulation of the T cell antigen receptor complex in antigen-specific, murine T cell clones: requirement for exogenous calcium. J Immunol 1989;143:587:595.

Bohjanen P, Okajima, M and 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, 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09282-04 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

T Cell Immune Deficiency in Mice and Humans With Autoimmune Disease

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

| | | | |
|---|---|---|---|
| PI: | G. M. Shearer | Senior Investigator | EIB, NCI |
| Others: | G. C. Tsokos | Senior Investigator | ARB, NIADDKD |

COOPERATING UNITS *(if any)*

C. S. Via Dept. of Medicine, University of Maryland, School of Medicine, Baltimore, MD

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS | PROFESSIONAL: | OTHER |
|---|---|---|
| 0.1 | 0.1 | 0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Sixteen of twenty human patients with systemic lupus erythramatosis (SLE) exhibited a selective loss of HLA self-restricted, CD4-mediated T helper cell function, which suggests a similarity between the immunological changes that occur in humans with SLE and those observed by this laboratory has observed in murine models of lupus. Little progress has been made in this project during the past year due to other priorities.





GPO 914-916

Project Description

Major Findings:

Similar to the observations that we have made in a graft-versus-host-induced model of SLE and in lpr/lpr autoimmune mice, we have found that peripheral blood leukocytes (PBL) from 16/20 patients with SLE are selectively unresponsive to recall antigens, but respond to HLA alloantigens and T cell mitogens. These results suggest that there is a selective defect in HLA self-restricted, $CD4^+$ T helper cell function.

30% of this project is AIDS-related research.

Publications:

Via CS, Morse HC III and Shearer GM. Autoimmune aspects of HIV infection and relevant murine models. Immunol. Today, 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09283-04 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Regulation of Human T Cell Responses by Adherent Cells

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

| | | | |
|---|---|---|---|
| PI: | G. M. Shearer | Senior Investigator | EIB, NCI |
| Others: | S. Muluk | Medical Staff Fellow | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 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.)*

No progress has been made in this project during the past year due to other priorities. One paper was submitted.



PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Major Findings:

0% of this project is AIDS-related research.

Publications:

Muluk SC, Bernstein DC, Shearer GM. A virus-sensitive suppressor cell is involved in the regulation of human allospecific T cell immunity. J Immunol 1989;143:2209-2215.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09285-03 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Induction of Class I MHC Gene Expression by Ethanol

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

| | | | |
|---|---|---|---|
| PI: | Dinah Singer | Senior Investigator | EIB, NCI |
| Others: | Amy Hutson | Biologist | EIB, NCI |
| | Gary Meadows | Guest Researcher | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .75 | | |

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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.)*

Ethanol enhances expression of cell surface class I major histocompatibility complex (MHC) antigens in a variety of cell lines; up to an eightfold increase is observed in an embryonic cell lines. Treatment of mice with ethanol in vivo causes marked changes in both liver and spleen. In the livers of alcohol-treated mice, a significant increase in the level of class I MHC RNA occurs within 4-5 days of treatment. In both thymus and spleen, there is an acute cellular depletion. In the spleen, the T lymphocyte population is selectively spared. The finding of increased class I MHC expression in liver, with concomitant depletion of splenic lymphocytes, supports models in which autoimmune mechanisms may play a role in alcohol related diseases.





GPO 914-918

Project Description

Major Findings:

Ethanol increases cell surface expression of class I MHC antigens in a variety of cell lines, including thymomas, fibroblasts, and embryonic cells. Both intracellular protein synthesis and steady state RNA levels increase following treatment. These observations led to the speculation that in vivo, ethanol may also induce increased levels of class I MHC expression. To further investigate the effects of ethanol, a murine model of alcoholism has been established. Mice fed a liquid diet, supplemented with 5% ethanol, achieve blood alcohol levels of 250-350 mg/dl. Analysis of tissue from treated animals reveals that class I MHC RNA levels in the liver are induced two-fold relative to liquid diet controls. This increase is specific, in that no other tissue analyzed revealed similar increases. Histological examination revealed a significantly greater accumulation of fat in livers of mice maintained on an ethanol diet than in liquid diet controls. Gross morphological changes also were noted in the spleen and thymus, where an acute cellular depletion of 90% of the cells was observed. In the spleen, T lymphocytes were selectively spared. Among the remaining spleen cells in alcohol-treated animals, the CD4+/CD8+ ratio remains unchanged, relative to controls. Initial studies indicate that ethanol-fed mice are more susceptible to viral infections than controls. These data suggest that ethanol directly affects components of the immune response, possibly leading to a paradoxical situation in which it is both an immunosuppressant and a potential immunostimulant.

Ethanol-induction of class I MHC expression has been shown to result from increased steady state levels of RNA. This effect is promoter dependent. To map regulatory DNA sequences responsible for the ethanol-response, a series of class I promoter constructs linked to the CAT reporter gene has been examined for response to ethanol. In stably transfect L cells, constructs containing between 500bp and 1 kb of 5′ flanking sequences respond to ethanol; constructs containing 290bp of less do not. These studies map the responsive element to the 5′ region of the class I gene, specifically to a segment between -290bp and -516bp.

Publications:

Singer DS, Parent LJ and Kolber M. Ethanol: An enhancer of transplantation antigen expression. Alcoholism:Clinical and Experimental Research, 1989;13:480-484.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09287-03 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Marrow Graft Failure in Allogeneic Bone Marrow Transplantation

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

| | | | |
|---|---|---|---|
| PI: | Ronald E. Gress | Senior Investigator | EIB, NCI |
| Others: | Kiyoshi Hiruma | Visiting Fellow | EIB, NCI |
| | Rafael Hirsch | Biotechnology Fellow | EIB, NCI |
| | Paul Berlin | Biotechnology Fellow | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.0 | 1.0 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B,D

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

A number of observations have suggested that host lymphocytes, specifically cytotoxic T cells (CTL) may play a significant role in mediating allogeneic marrow graft rejection. In a murine model system, CTL were cloned from the spleens of sublethally irradiated animals which had rejected MHC disparate marrow grafts. It was found that cloned CTL were sufficient to effect rejection of T cell depleted allogeneic marrow in lethally irradiated animals. The rejection of marrow grafts by CTL was specific for the MHC gene products expressed by the marrow cells and correlated with the cytotoxic specificity of the individual clones. Since CTL can therefore reject marrow grafts, the administration of anti-CD3 monoclonal antibody in vivo has has been studied as a means to enhance engraftment of T cell depleted marrow in sublethally irradiated murine hosts. In addition, cells capable of specifically suppressing precursor CTL have been studied in vitro and in vivo. In order to similarly evaluate human CTL, xenospecific human cytotoxic responses have been studied. The fine specificity of xenoantigen recognition by human T cells suggests that in transplantation of xenogeneic tissue, as in transplantation of MHC mismatched allogeneic tissue, the particular tissue antigens, and therefore tissue typing, may be a relevant consideration. The generation of human CTL has been characterized and certain essential cellular interactions in that generation have been identified.





GPO 914-918

Project Description

Major Findings:

The purpose of these studies was to directly assess the ability of murine CTL from irradiated hosts to reject allogeneic marrow grafts. First, the maximum dose of radiation which still allowed an effective rejection response by the host was determined. By an $^{125}$IUDR uptake assay of splenic marrow cell proliferation, this dose was found to be 650-750 cGy. Lymphocytes were then isolated and cloned from spleens of B6(H-$2^{b}$) mice seven days after they had received 650 cGy radiation and inoculation with either BDF1 (H-$2^{b/d}$) or B6C3F1 (H-$2^{b/k}$) marrow which had been depleted of T cells by rabbit anti-mouse brain antiserum plus complement. The clones which were recovered were Thy 1+, LyT 2+, L3T4- with cytolytic specificity for the allogeneic MHC-encoded determinants of the marrow donor. No NK activity could be identified. It was found that these clones suppressed MHC mismatched marrow graft proliferation (measured by $^{125}$IUdR uptake) when adaptively transferred into a 1025 cGy lethally irradiated B6 host if and only if the grafted marrow cells expressed MHC determinants for which the individual clone had cytotoxic specificity. Two primary questions were then addressed. The first was the effect of these CTL cells on long term engraftment and the second was the mechanism by which they exerted their effect. Results demonstrated that (1) a cloned CTL population is sufficient to reject an allogeneic marrow graft, and (2) the mechanism by which these marrow grafts were rejected was specific for MHC gene products expressed by the donor marrow corresponding to the cytotoxic specificity of the clone.

With the demonstration that CTL are sufficient to effect marrow graft rejection, a study was undertaken to evaluate the possible beneficial effects of anti-CD3 treatment on marrow engraftment in a mouse model (parallel to anti-CD3 monoclonal antibody treatment which is currently available for clinical use in allogeneic organ transplantation). The antibody used is specific for the $\epsilon$ chain of the murine CD3-T cell receptor complex, can suppress skin graft rejection, and can cause both short term and long term in vivo T cell dysfunction. The intact antibody results in T cell activation in vivo while the $F(ab')_2$ from of the antibody does not. T cell immunosuppression is pronounced at one week after administration of the intact antibody. It was found, however, that in vivo treatment with anti-CD3 administered seven days before infusion of bone marrow did not enhance engraftment of allogeneic marrow in sublethally irradiated hosts. Therefore, immunosuppression provided by treatment with anti-CD3 monoclonal antibody was not sufficient to prevent rejection of the marrow graft. Studies also demonstrated that administration of intact anti-CD3 to mice results in T cell activation within hours of administration (manifest by increased IL-2 receptor expression and by enhanced proliferation of spleen cells from treated animals to exogenous IL-2 in vitro). Because this activation also results in secretion of colony stimulating factors (CSF) detectable in the serum and produces extramedullary hematopoiesis in the spleen, it was thought that it might be beneficial in terms of marrow stimulation and engraftment to time the injection of allogeneic marrow so that it was present during the period of T cell activation in order to provide the possible benefit of factors which stimulate hematopoiesis. In fact, the injection of anti-CD3 monoclonal antibody with the donor marrow results in full allogeneic chimerism. Non-activating anti-T cell monoclonal antibodies also facilitate engraftment, but do not result in full chimerism. Identification of specific factors released in vivo as a

result of host T cell activation by anti-CD3 administration which may promote engraftment of MHC-disparate marrow is in progress. In addition, it was found that IL-2 enhances the veto suppressor activity of T cell depleted donor marrow. 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.

Information about human CTL generation, specificity, and in vivo function is not as extensive as in the case of murine CTL. These studies of human anti-mouse CTL responses indicate that primarily a CD4+ helper pathway functions in the generation of human CTL responses and that there exists a dependence on the presence of human antigen presenting cells. Additional experiments were carried out to characterize the human effector CTL subsequent to its generation. CTL lines and clones were established from a secondary mixed lymphocyte response in which the responder cells were human and the stimulators were H-$2^b$ murine splenocytes. These human xenoreactive CTL were able to distinguish wild type $K^b$ molecules from those altered in their amino acid sequence by in vitro mutagenesis. Such amino acid changes were confined to a single domain and so demonstrated a fine specificity of recognition by human CTL comparable to the specificity of recognition of $K^b$ determinants by alloreactive murine CTL. This fine specificity of antigen recognition by human CTL across species predicts that the recognition by host CTL of MHC encoded antigens expressed by a xenograft would be expected to be comparable in specificity to the recognition of MHC antigens expressed by an MHC mismatched allograft. Studies of the generation of these xenospecific CTL demonstrated a dependence on human (responder) antigen presenting cells. This 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.

Publications:

Hirsch R, Chatenoud L, Gress RE, Sachs DH, Bach JF and Bluestone J. Suppression of the humoral response to anti-CD3 monoclonal antibody. Transplantation 1989;47:853-857.

Gress RE, Nathenson SG and Lucas PL. Fine specificity of xenogeneic antigen recognition by human T cells. Transplantation 1989;48:93-98.

Nakamura H and Gress RE. Graft rejection by cytolytic T cells: MHC specificity of the effector mechanism in the rejection of allogeneic marrow. Transplantation 1990;49:453-458.

Hirsch R, Bluestone J, DeNenno L and 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. In press.

Nakamura H and Gress RE. IL-2 enhancement of veto suppressor cell function in T cell depleted bone marrow in vitro and in vivo. Transplantation. In press.

Lucas PJ, Shearer GM, Neudorf S and Gress RE. The human anti-murine xenogeneic cytotoxic response: I. Dependence on responder antigen presenting cells. J Immunol In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09288-03 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

T Cell Responses in Bone Marrow Transplantation

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

| | | | |
|---|---|---|---|
| PI: | Ronald E. Gress | Senior Investigator | EIB, NCI |
| Others: | Robert Moses | Biotechnology Fellow | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.0 | 1.0 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors B,D

☐ (a2) Interviews

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

Concerns that a functionally effective T cell repertoire might never develop in the setting of MHC-mismatched marrow transplantation are based on defective T cell responses in vivo observed in murine bone marrow chimeras constructed between MHC disparate strains. The incompetence is not due to inherent T cell defects, but rather to the restriction specificities of the T cells such that those MHC determinants recognized as self (host type) are not expressed by the antigen presenting cells which are of donor type. The T cell response to antigen is therefore defective. To evaluate whether T cell maturation is comparable in mouse and man, the development of T cell responses in a primate, the rhesus monkey, has been studied. The biology of T cell recovery has first been investigated in animals following T cell depleted autologous marrow transplantation. The time course of CD4+ T cell reconstitution and the time course of the development of in vivo T cell immunocompetence as defined by the ability to respond to an organ allograft correlated with the number of T cells infused in the marrow, and did not correlate with marrow cell dose or with time of hematopoietic reconstitution, raising the possibility that residual T cells in the infused T cell depleted marrow played a central role in the generation of subsequent T cell populations. This would imply that the generation of T cells in marrow grafted primates may not be the same as in the mouse.

Project Description

Major Findings:

As a preliminary step to the study of T cell repertoire generation in MHC-mismatched BMT, we have studied the generation of T cell function in rhesus monkeys which have been hematologically reconstituted with either untreated or extensively T cell depleted autologous bone marrow. The methods for T cell depletion and the quantitation of residual (reinfused) T cells were patterned after methods developed for carrying out bone marrow transplantation in man. By phenotypic analysis, CD2+/CD8+ T cells recovered by 6-8 weeks post grafting. CD16+ NK cells and CD20+ B cells also recovered at 6-8 weeks. All animals receiving T cell depleted marrow recovered CD4+ cells at later time points. In the animal receiving marrow containing the fewest residual T cells (0.00014% by limiting dilution assay), CD4+ cells were less than 30% of the pretransplant value at ten months after transplant. The total number of T cells required to acutely reject a graft was determined on the basis of results in animals receiving marrow poorly depleted of T cells, and was 5 x $10^4$ per kilogram recipient body weight. In vitro functional studies paralleled the phenotypic analysis. CTL activity, which can be mediated by CD8+ cells, was observed in vitro (with added IL-2) at six weeks post transplant; recovery of helper T cell function, predominately mediated by CD4+ cells, was found only at time points beyond four months. The ability to respond to allogeneic stimulation in vivo as assessed by the rejection of an organ allograft temporally paralleled the recovery of CD4+ helper cells, not the recovery of CD8+ cytotoxic cells. The critical level of recovery of peripheral CD4+ cells which correlated with allograft rejection was 25-50% of the pretransplant value. Approaches to evaluate T cell function by direct stimulation of CD3 were also developed and characterized.

The pattern and time course of reconstitution of T cell populations establishes a baseline for comparing T cell reconstitution in allogeneic donor/recipient combinations and in predicting the time required for successful treatment to reverse virally induced T cell deficiencies. Prolonged periods of depressed T cell number and function existed even in the absence of the allogeneic effects which might be expected in allogeneic BMT. The slow rate of recovery of CD4+ cells was comparable to the rate of recovery for CD8+/CD28+ cells. CD8+/CD28- cells recovered quickly, possibly indicating a different mechanism of generation of this subset of cells. Also, even with very extensive T cell depletion, hematopoietic recovery occurred, indicating that hematopoiesis does not absolutely require many, if any, "hematopoietic T helper cells".

The length of time required for reconstitution of CD4+ cells and for recovery of organ allograft rejection varied inversely with the number of residual T cells in the infused marrow, not with stem cell function as assessed by the number of marrow cells infused and by rapidity of overall hematopoietic recovery. This result is consistent with the possibility that the residual T cells in the infused marrow play a central role in the generation of subsequent T cell populations in the recipient. This suggests that the generation of T cells in the primate marrow recipient may in fact be different than in the mouse where the T cell repertoire appears to develop from marrow precursor cells which mature in the irradiated recipient and which consequently express an MHC

restriction which is strongly influenced by the host environment. The possibility that reconstituting T cells in the primate following marrow transplantation are derived from mature donor T cells (with restriction specificity for donor MHC antigens) remaining in the marrow after depletion rather than from early precursors/stem cells is of central importance to considerations of MHC mismatched BMT in man. These findings have been pursued by molecular analysis of the reconstituting T cell populations. Results to date indicate an early oligoclonal reconstitution with a polyclonal pattern developing over time. Studies in patients receiving T cell depleted autologous marrow show a more rapid reconstitution of CD4+ T cell populations than what was observed in the sub-human primates. Collaborative studies have been initiated to evaluate what effect certain cytokines might have in influencing patterns of reconstitution.

Publications:

Moses RD, Sundeen JT, Orr KS, Roberts RR and Gress RE. Cardiac allograft survival across major histocompatibility complex barriers in the rhesus monkey following T lymphocyte-depleted autologous marrow transplantation III. Late graft rejection. Transplantation 1989;48:769-773.

Moses RD, Sharrow SO, Stephany DA, Orr KS and Gress RE. Cardiac allograft survival across major histocompatibility complex barriers in the rhesus monkey following T lymphocyte-depleted autologous marrow transplantation IV. Immune reconstitution. Transplantation 1989;48:774-781.

Oppenheim JO, Neta R, Tiberghein P, Gress R, Kovatch RM and Longo D. Interleukin 1 enhances survival of supralethally irradiated mice treated with either isologous or allogeneic marrow cells. Blood 1989;74:2257-2263.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09289-01 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 | Senior Investigator | EIB, NCI |
| Others: | S. Andrew | Visiting Fellow | EIB, NCI |
| | P. Perez | Guest Researcher | EIB, NCI |
| | A. George | Special Volunteer | EIB, NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.3 | 2.3 | 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 molecular genetics approaches, a single polypeptide chain bispecific antibody. We have produced several cloned plasmids containing inserts encoding single chain Fv molecules. These will be suitable when linked together to target cytotoxic cells against TNP-coated and tumor cells.





GPO 914-918

Project Description

Major Findings

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 sequece from antibody to antibody. A native antibody molecular contains 2 identical antigen binding regions, each consisting of a light chain variable domain (Vl) and a heavy chain variable domain (Vh). The Vh and Vl 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 antibody can be prepared by linking the C terminus of the Vl to the N terminus of the Vh, or vice versa, providing that a spacer of at least 10 residues is inserted between the Vl and Vh chains. This construct is known as a single chain Fv (sFv), and it has been prepared by recombinant DNA technology. In this project, we are preparing 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. The production of SCBAs should provide an improved way of making bispecific antibodies.

We have generated sFv's of OKT3 (anti-CD3), VD2 (anti-CD16) and anti-DNP by PCR using cDNAs made from mRNAs of the hybridoma cells producing these monoclonal antibodies. These have been cloned into the Bluescript cloning vector, the inserts sequenced and tested for ability to express active protein in a rabbit reticulocyte expression system. At this stage, we know that we have the correct sequences and that they encode proteins of the correct molecular weights.

Proposed Course Project

This project will be continued as a CRADA with Creative Biomolecules, Inc., and later, with Centocor. The sFvs will be put into bacterial expression systems and tested to make sure that they can make active binding proteins. The sFvs will then be linked, genetically, using various types of spacers, sequenced and tested for expression. The proteins produced will then be tested in cellular and antigen targeting systems to test biological activities. Expression systems will be developed to produce the largest amounts of active SCBA most economically. Once a prototype SCBA has been produced, we will create several others, suitable for several biological functions.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09290-01 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 | Senior Investigator | EIB, NCI |
| Others: | I. Kurucz | Visiting Fellow | EIB, NCI |
| | C. Jost | Visiting Fellow | |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.6 | 1.6 | 0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

1. 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.

2. A plasmid containing an insert encoding for a single chain T cell receptor has been constructed and cloned.

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. During the past year we have done experiments to determine whether bispecific antibodies could also increase immune responses in vivo. Bispecific antibodides 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. We then immunized mice with HEL in the presence or absence of bispecific antibodies and followed antibody production after the primary challenge and following a secondary boost. We found that bispecific antibodies that bind antigen to MHC class I or II molecules, to $Fc_{\gamma}R$, but not to surface IgD, enhance the immunogenicity of HEL. Bispecific antibodies that bound HEL to MHC II molecules, for example, 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. In fact, bispecific antibodies were as effective as incomplete Freund's adjuvant in generating antibody responses. Since adjuvants cannot be ussed 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.

Recently we have initiated a project to produce a single chain Fv fragment of a T cell receptor (TcR). Constructs have been prepared using the TcR from the 2B4 hybridoma, because the sequence of the TcR from this hybridoma is known and its specificity well determined. We have the single chain construct cloned into Bluescript and sequenced, and have shown tha it can encode for a protein of the correct molecular weight. We next plan to express it in a microorganism, and check for activity by measuring its ability to block specifically antigen presentation to the 2B4 hybridoma.

Proposed course of project

We plan to use the single chain TcR to follow the binding of antigen (pigeon cytochrome C) to $I\text{-}E^k$ both on the cell surface and internally. Some of this work will be done using electon microscopy. We also plan to establish a system for following antigen presentation of influenza infected cells. This study will have the purpose of seeing whether bispecific antibodies can immunize mice against flu, and to follow the virus as it infects cells and gets bound to MHC molecules.

Publications:

Snider DP and Segal DM. Efficiency of antigen presentation following antigen targeting to surface IgD, IgM, MHC, FC RII, and B220 molecules of murine splenic B cells. J Immunol 1989;143:59-65.

Snider DP, Kaubisch A and Segal DM. Enhanced antign immunogenicity induced by bispecific antibodies. J Exp Med. 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09291-01 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Genomic organization and characterization 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: | A. Taylor | Chemist | EIB, NCI |
| | M. Dean | Scientist | PRI, FCRIC |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| | | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The TCR ζ subunit appears to be a limiting component in receptor assembly. In addition, as it is expressed in natural killer cells ζ demonstrates differential expression as compared to the other T cell receptor components. Structural analysis and more recently cDNA cloning of the η subunit of the T cell receptor suggests that the two components are the products of alternative splicing of the same primary transcript. The regulation of the relative expression of ζ and η at a protein level may have important sequealae with regard to T cell function and development. Analysis of the human ζ gene reveals a structure consisting of 8 ζ exons, and a region highly homologous to the η specific region of the murine η cDNA. In addition, a variable number tandem repeat restriction fragment polymorphism exists within the structural ζ gene.





GPO 914-918

Project Description

Major Findings:

Screening of a human leukocyte genomic library with a cDNA encoding the human TCR zeta protein has resulted in the isolation of genomic clones encoding the human zeta. Three independent clones have been isolated that contain all of the exons encoding the protein product, as well as substantial amounts of 5' and 3' untranscribed genomic material. Analysis of these clones reveals 8 exons separated by intervening sequences. The sizes of the intervening sequences are being determined by use of the polymerase chain reaction. The size of the first intron has not been determined with certainty, but is clearly greater than 5kB. All of the other introns are estimated to be between 0.8 and 2.5kB.

Analysis of genomic Southerns with the human zeta cDNA reveals a restriction fragment polymorphism of the variable number tandem repeat (VNTR) type. The VNTR has been localized to a 6kB BamHl fragment, and more precisely to a 1.3kB HinFl fragment. Analysis of the distribution of this VNTR within the population and its linkage with other RFLPs is underway.

It had previously been determined that the $\zeta$ and $\eta$ subunits of the T cell antigen receptor are structurally related to one another. Cloning of the murine $\eta$ subunit suggests $\zeta$ and $\eta$ arise as alternative splice products of the same gene. Human genomic $\zeta$ clones were probed with an oligonucleotide corresponding to unique murine $\eta$ sequence. These results revealed an area of specific hybridization in a fragment of a genomic clone that contains the 3' end of the human $\zeta$ gene. Further characterization of this region is underway.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09292-01 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Signal transduction in permeabilized T cells

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: |
|---|---|---|
| | | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Signal transduction via the T cell antigen receptor results in a complex pattern of events that leads to lymphokine production and cellular proliferation. The proximal events in this pathway include the hydrolysis of phosphoinositides and the activation of a protein tyrosine kinase. In order to understand the coupling mechanisms leading to the proximal events in T cell activation a permeabilized cell system has been established using a murine T cell hybridoma.

Project Description

Major findings:

Through adjustment of cation and anion concentrations conditions have been established whereby cellular events such as tyrosine phosphorylation can be studied. We have been able to reconstitute antibody mediated signalling through the T cell receptor both with anti-CD3 and anti-$\alpha\beta$ reagents. Initial studies indicate that whereas the anti-TCR reagents are able to maintain phosphorylation some other antibodies directed against other cell surface signalling structures fail to reconstitute signalling. The effects of orthovanadate in this system are being explored and studies indicate that treatment of permeabilized cells with sodium orthovanadate substantially increases the amount of tyrosine phosphorylation seen on stimulation. Studies are presently underway to determine the role that various nucleotides and non-hydrolyzable nucleotide analogues have in this system.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09293-01 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Mutational analysis of the TCR ζ subunit

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: | A. Taylor | Chemist | EIB, NCI |
| | C. Cenciarelli | Visiting Fellow | EIB, NCI |
| | J. D. Ashwell | Senior Investigator | BRMP, NCI |
| | R. D. Klausner | Branch Chief | CBMB, NICHD |
| | S. J. Frank | Staff Fellow | CBMB, NICHD |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

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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.)*

The T cell receptor z subunit plays crucial roles in signal transduction and in receptor assembly. Zeta is limiting in receptor assembly in T cell hybridomas, is required for conferring competency for the receptor to be transported from the golgi apparatus to the cell surface and is a substrate for a receptor activated protein tyrosine kinase. The structure of the z subunit deduced from cDNA cloning reveals a potential nucleotide binding domain as well as 6 intracellular tyrosines they may serve as substrates for a tyrosine kinase. A model system for studying the role of ζ exists in the ζ negative hybriodoma variant MA5.8 where transfection with zeta results in the restoration of cell surface receptor expression. In order to further evaluate structure-function relationships in this system we have undertaken site-specific mutagenesis of specific residues contained within zeta. Preliminary results suggest that mutation of intracellular tyrosines results in a marked dimunition of all tyrosine phosphorylation.





GPO 914-918

Project Description

Major Findings:

Site-specific mutagenesis of ζ was undertaken using phosphothiorate based mutagenesis system. Mutation of lysine 150, in the nucleotide binding cleft failed to result in any substantial effect on either tyrosine phosphorylation or on interleukin-2 production. Mutation of tyrosines 142 and 153 each results in a marked loss of cooperative tyrosine phosphorylation. Interleukin-2 production is being evaluated, preliminary results suggest that the two mutations result in different functional phenotypes.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09294-01 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Signal transduction in natural killer cells

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: | J.J. O'Shea | Senior Investigator | NCI |
| | J. Orltaldo | Branch Chief | NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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| | | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The molecular mechanisms of signal transduction in natural killer cells remains poorly understood. Recently, the ζ subunit of the TCR has been found to be present in NK cells. We have been able to demonstrate the presence of both homodimers and heterdimers of zeta on the surface of NK cells. In addition, it is clear that a fraction of ζ exists co-associated with the transmembrane form of CD16. Stimulation of NK cells with an anti-CD16 antibody results in the tyrosine phosphorylation of the zeta chain.

Project Description

Major Findings:

Surface iodination of CD3 negative large granular lymphocytes, revealed the homodimeric form of ζ as well as a lower molecular weight disulfide-linked heterodimeric form. The nature of this heterodimer has not been determined with certainty, but may represent the disulfide linkage of ζ to the γ subunit of the $Fc^e$ receptor. Immunoprecipitation from digitonin solubilized lysates with a monoclonal antibody directed against the Fc γ receptor on NK cells resulted in co-immunoprecipitation of ζ suggesting the physical association of these molecules in vivo. Studies were carried out to determine whether, in a manner analogous to the T lymphocyte, ζ underwent tyrosine phosphorylation on stimulation. Studies were carried out both by loading cells with ortho-phosphate and than stimulating and by anti-phosphotyrosine analysis of western blots. Both methods of analysis demonstrated that zeta undergoes specific phosphorylation in response to stimulation via an anti-Fc γ receptor antibody. No evidence of similar phosphorylation has been detected when stimulation is carried out with either PMA and ionomycin,IL-2 or with anti-CD3.

Publications:

O'Shea JJ, Weissman AM and Orteldo JR. Engagement of the NK Cell IgG Fc Receptor results in tyrosine phosphorylation of the ζ chain. Proc Natl Acad Sci USA, 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09295-01 E

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 | Senior Investigator | EIB,NCI |
| | M. Clerici | Visiting Fellow | EIB NCI |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Experimental Immunology Branch

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NCI, NIH, Bethesda, Maryland 20892

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☐ (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 immunomodulatory effects on uninfected clones and tumor lines. Our initial studies suggest that purified gp 120 may have inhibitory effects on cellular function in PBLs from normal human donors.





GPO 914-918

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. In order to study the molecular mechanism whereby gp120 exerts its immunosuppressive effects we have begun to analyze the effects that gp120 treatment has on the early biochemical events in T cell activation. Specifically we have developed conditions whereby fresh CD4 cells can be analyzed for stimulation specific phosphorylation. Initial studies have suggested 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.

LABORATORY OF CELL BIOLOGY

SUMMARY REPORT

DCBDC, NCI

OCTOBER 1, 1989 TO SEPTEMBER 30, 1990

The Laboratory of Cell Biology was reorganized this year with the retirement of Dr. Lloyd Law, the naming of Dr. Michael M. Gottesman as the new Chief of the Laboratory of Cell Biology, and the addition of the Molecular Cell Genetics Section to the previous existing Chemistry Section in the Laboratory. Work in the Laboratory of Cell Biology now encompasses five project areas. Molecular and biochemical approaches are used to study problems in normal and cancer cell biology including resistance of cancer cells to chemotherapy, mechanisms of negative growth regulation by cAMP and p53, mechanisms of invasiveness and metastasis with an emphasis on surface antigens and proteases, energy-dependent proteolysis in bacterial systems, and the mechanism of antigen presentation.

Resistance of Cancer Cells to Chemotherapy

Resistance of cancer to chemotherapy is the major obstacle to successful treatment of metastatic cancer. Michael M. Gottesman, working in collaboration with Ira Pastan, Chief, Laboratory of Molecular Biology, has shown that one of the major mechanisms responsible for the development of multidrug resistance to natural product drugs such as doxorubicin, *Vinca* alkaloids, and epipodophyllotoxins is the expression of an energy-dependent drug efflux pump. This pump, called P-glycoprotein, or the multidrug transporter, is the product of the *MDR*1 gene in the human. Over 100 kb of this gene, consisting of 28 introns and 29 exons, has been cloned, as has a 4.5 kb cDNA. Expression of the *MDR*1 cDNA in a variety of eukaryotic expression vectors, a retroviral system, and a transgenic mouse system (in collaboration with Glenn Merlino), where expression occurs in bone marrow, has been used to prove that the *MDR*1 gene encodes a multidrug transporter which is functional *in vitro* and *in vivo*. Vectors have been developed which use the *MDR*1 cDNA as a dominant selectable, amplifiable marker for introduction of non-selectable genes into cultured cells and bone marrow.

Structure-function studies on the multidrug transporter have been undertaken using molecular genetic and biochemical approaches. Point mutations, deletions, insertions, and substitutions have been used to demonstrate that both halves of the P-glycoprotein molecule are involved in drug-binding and ATP-utilization. Specific drug interaction sites in the transmembrane regions in both halves of the molecule have been defined using photoaffinity labels. Energy transfer studies using doxorubicin and iodonaphthalene azide indicate that the pump is capable of removing hydrophobic drugs from the membranes of resistant cells.

Clinical studies strongly support a role for P-glycoprotein in both intrinsic and acquired drug resistance of human cancer. The multidrug transporter is found at physiologically significant levels in a wide variety of intrinsically resistant solid tumors including colon, kidney, adrenal, pancreatic and liver cancers. In acute non-lymphocytic leukemias, its expression at the time of presentation predicts poor response to chemotherapy. In ovarian cancer, neuroblastoma, some sarcomas, leukemias, and lymphomas, levels of *MDR*1 RNA increase in tumors which have acquired resistance to

chemotherapy. These results point to a need for clinical trials to determine the ability of agents which inhibit the multidrug transporter to improve efficacy of existing chemotherapy for tumors which express P-glycoprotein.

Mechanisms of Negative Growth Regulation by cAMP and p53

Michael M. Gottesman has isolated Chinese hamster ovary (CHO) cell mutants which are resistant to the growth inhibitory effects of cAMP or its analogs. These mutants have alterations in the regulatory (R1) and catalytic (C) subunits of cAMP-dependent protein kinase (PK), the enzyme that mediates all known cAMP effects in cells by phosphorylating specific protein substrates. Some of these substrates are likely to be transcription factors as demonstrated by failure of PK mutants to carry out cAMP-dependent transcription. Cloned cDNAs for the hamster R1 and C subunits have been obtained. *In vitro* mutagenesis of the R1 subunit results in dominant mutations which block cAMP responses. The mutant R1 subunits have been analyzed after expression in bacteria or in animal cells. Another way to ablate response to cAMP is by expression of a yeast phosphodiesterase gene which breaks down cAMP as it is formed within cells. This gene has been introduced into transgenic mice under control of tissue-specific promoters to study the role of cAMP in development and function of specific organs.

Ettore Appella has studied the role of p53 as an anti-proliferative protein in growing cells. One mutant form of p53 derived from a human osteosarcoma (HOS) cell line has been studied in detail. Wild-type human p53, but not the mutant form cloned from HOS cells, suppresses the colony-forming capacity of SV40 transformed hamster cells. Overexpression of human wild-type p53 in a human glioblastoma cell line blocks entry of these cells into S phase.

Mechanisms of Invasiveness and Metastases: The Role of Surface Antigens and Proteases

Vincent Hearing has been studying malignant melanoma as a model of a highly malignant and metastatic tumor whose growth and metastatic tendencies are nonetheless under control of the host immune system. Together with Lloyd Law, he has demonstrated that a murine melanoma surface antigen, B700, is responsible for tumor rejection by stimulating cytotoxic antibody production, without evidence of a cellular immune response. Metastatic properties of murine melanomas can be blocked with monoclonal antibodies directed against B700, or another melanoma surface antigen, 9B6. Mutant melanoma cells with reduced metastatic potential have been shown to have reduced surface urokinase activity, and preliminary data suggest that transfection of a functional urokinase cDNA into cells enhances their metastatic ability.

Michael M. Gottesman has studied another protease whose expression is closely related to the phenotype of malignant transformation. The acid protease, cathepsin L, is expressed and secreted at extremely high levels by malignantly transformed mouse cells or cells treated with growth factors or tumor promoters. The regulation of expression of cathepsin L has been studied by isolating the mouse cathepsin L gene and demonstrating that regulated expression requires sequences both upstream and downstream of the start site of transcription initiation. Cathepsin L is also expressed at high levels in many human tumors, including renal cell cancers and squamous cell carcinomas of the lung.

Ettore Appella has studied two tumor-specific transplantation antigens (TSTAs) on chemically induced fibrosarcomas in the mouse. One of these TSTAs is an 84/86 kDa protein which has been cloned and sequenced and shown to be

related to the 90 kDa heat shock protein of mammalian cells. The other TSTA, known as p82, is the mouse homolog of the human cytovillus-associated protein, cytovillin.

Vincent Hearing has studied melanogenesis in mammalian melanocytes by using anti-tyrosinase antibodies and DNA probes specific for two genes that determine coat color in the mouse and by isolation of a low molecular weight inhibitor of melanogenesis. These studies indicate the complexity of the genetic loci and the events which regulate melanogenesis in mammals.

Energy-Dependent Proteolysis in Bacteria

ATP-dependent proteases determine the levels of important regulatory proteins in such diverse processes as the response to heat shock, the timing of the cell cycle, repair of DNA damage, and adaptation to nutritional and environmental conditions. Michael Maurizi has been studying two ATP-dependent proteases in *Escherichia coli*: the Lon protease, and the two-component Clp protease. These proteases have been purified to homogeneity and their substrate specificity has been determined. Lon is a single subunit protease, and Clp consists of ClpA, an ATP-binding regulatory subunit, and ClpP, a proteolytic subunit. Mutations in *lon* have pleiotropic effects on cell division, DNA repair, and response to environmental stress, but genetic ablation of *clpA* or *clpP* has no profound effect on cell physiology. ClpA and ClpP can be assembled *in vitro* to form a structure composed of two superimposed hexagonal rings, which resembles the ATP-dependent multicatalytic protease found in eukaryotic cells.

Mechanism of Antigen Presentation

Ettore Appella has been studying the molecular mechanisms that govern the interaction of immunogenic peptides with the major histocompatibility complex (MHC) class II molecules in the mouse. By synthesizing peptides which bind to specific class II molecules, it is possible to define general "motifs" required for peptide binding. In antigen-presenting cells, exogenous protein antigens are digested intracellularly, and structurally immunodominant fragments are found to be associated with class II molecules. However, immunodominant exogenous peptide antigens appear to associate with surface class II molecules during the endocytosis recycling process of these molecules. Expression of both class I and class II molecules is strongly stimulated by gamma interferon (IFN). The cell surface receptor for gamma IFN has been cloned in order to study the details of this mechanism, as well as the immunological significance of gamma IFN and its role in antigen presentation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 03229-20 LCBGY

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

T-Cell Antigen Recognition and Tumor Antigens

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

| | | | |
|---|---|---|---|
| PI: | E. Appella | Medical Officer | LCB, NCI |
| Other: | S.K. Moore | Senior Staff Fellow | LCB, NCI |
| | J.W. Romano | IRTA Fellow | LCB, NCI |
| | K. Sakaguchi | Visiting Fellow | LCB, NCI |
| | S.J. Ullrich | Senior Staff Fellow | LCB, NCI |
| | E. Leonard | Medical Officer | BRMP, NCI |
| | K. Ozato | Research Microbiologist | LDMI, NICHD |

COOPERATING UNITS *(if any)*

Department of Pathology, Temple University School of Medicine; Cytel, La Jolla, CA; Preclinical Research, Sandoz Ltd., Basel, Switzerland; Mikrobiologisk Institute, Copenhagen, Denmark; A.R.C., Villejuif, France

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 5 | 5 | 0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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


Our laboratory is currently investigating several immunological issues. Specifically, our research involves the analysis of the structural and functional relationships between peptide antigens and class II major histocompatibility molecules; requirements for peptide binding to class II molecules; T cell recognition of class II/peptide complexes; MHC transcriptional factors; and the mouse γ-IFN receptor. The lab is also studying the immunological significance of tumor antigens, and the effects of the p53 nuclear protein on the proliferative phenotype of transformed cells.

The proposed model of the class II molecule indicates that in the I-$E^d$ peptide binding site a cluster of negatively charged residues may interact with the positive charged residues of the binding peptides. In antigen presenting cells, exogenous protein antigens, after their cellular processing, appear to associate only with newly synthesized class II molecules, whereas peptide antigens appear to occur on endocytosed cell surface class II molecules. During intractoplasmic recycling of MHC molecules a mechanism for peptide exchange has been identified. A naturally processed peptide derived from hen egg lysozyme has been identified as bound to purified class II molecules. The receptor for mouse interferon γ has been cloned and will be used to analyze the signaling molecules involved in the biological activity of this molecule.

We have structurally and functionally characterized the transformation-related *p53* gene and its protein product. Transfection studies with wild type and mutant forms of the gene indicated the capability for both positive and negative control of proliferation by p53, specifically at the $G_1$/S junction of the cell cycle. Another tumor antigen, previously identified as p82, has been cloned and identified as the murine homolog of cytovillin. Further analysis of the antigenic nature of HSP84/86 is in progress. The secondary and tertiary structure of IL-8 and two zinc peptides have been analyzed.

I. T-Cell Antigen Recognition and Major Histocompatibility Complex Antigens

Over the last several years, evidence has accumulated to indicate that a complex formed between the major histocompatibility complex (MHC) class I or II molecules and an immunogenic peptide fragment is recognized by T-cells. Our studies have focused on the structural characteristics of the peptides which bind to murine class II molecules ($I\text{-}A^d$ and $I\text{-}E^d$) and their peptide ligands. T-cell recognition was very specific whereas the interaction between $I\text{-}A^d$ or $I\text{-}E^d$ and peptide was very permissive; however different peptide sequence "motifs" which correlated with binding to either $I\text{-}A^d$ or $I\text{-}E^d$ could be identified. Hydrophobic amino acid residues were the most critical for peptide binding to $I\text{-}A^d$; multiple basic residues played the major role in $I\text{-}E^d$ binding. In an attempt to provide a molecular basis for the above differences, we compared the amino acid residues from the putative antigen binding site of the $I\text{-}A^d$ and $I\text{-}E^d$ class II molecules. Out of 36 residues in the binding site, 27 are either identical or conservatively substituted in $I\text{-}A^d$ and $I\text{-}E^d$ molecules. Of the 9 non-conservative substitutions observed, three involve creation of negative charge in the predicted $I\text{-}E^d$ binding site. This cluster of negatively charged residues on the $I\text{-}E^d$ molecule may interact with the corresponding positive charges on the $I\text{-}E^d$ binding peptides.

In contrast to the murine system, considerably less is known about the interaction between the human class II DR molecules and peptide fragments. Recently, binding assays for the DR molecules have been developed and the DR binding pattern of a large set of peptides has been analyzed. The results indicate that 15-25% of the peptides tested could bind to a given DR molecule. This is in agreement with a comparable study involving purified murine class II molecules. Similarly, the peptide binding pattern among different DR alleles were indistinguishable, but different binding pattern could be seen between DR molecules expressing products of the $\beta 1$ and $\beta 3$ loci.

Most peptides that bind to a particular MHC class II molecules have been found to share common structural "motifs". The definition of these "motifs" is quite broad since several amino acids at any given position in the peptide are compatible with binding to the MHC molecule. One consequence of this broad range of interaction is that peptides having different sequences may compete for presentation by the same MHC molecules, to T cells. This competition does indeed occur both *in vitro* and *in vivo*. The peptide-MHC complexes are very stable, thus raising the question of how the antigen presenting cells (APC) provide a sufficient number of MHC-binding sites to ensure T-cell immunity and to allow peptide competition for antigen presentation. One view is that there exists a mechanism for peptide exchange that may occur during the intracytoplasmic recycling of MHC molecules. We have obtained results that show that peptides can dissociate from MHC class II molecule and be replaced by a competitor in metabolically active APC. Unlike antigen processing the exchange of the peptides does not require an acidic intracytoplasmic environment, suggesting that these two processes might occur in different cellular compartments. Our results have also shown that treatments known to block endocytosis, such as incubation at 18°C or in the presence of methylamine, also interfere with peptide exchange. These results, therefore, favor an intracytoplasmic location for peptide exchange. A possible candidate would be the early endosomes where sorting and recycling of membrane receptors occurs in a mildly acidic environment.

In order for exogenous antigens to bind to the MHC molecules they are proteolytically degraded or processed down to smaller peptide fragments which in turn bind to the MHC molecule. The site of binding of endogenously generated peptides to the class I molecule appears to be within the endoplasmic reticulum (ER) whereas that of class II molecule is less clear but appears to occur both in a peripheral endocytic compartment and in the ER. We have determined that when the APC are incubated with either cycloheximide or Brefeldin A, the ability of APC to present exogenous protein antigens was inhibited but that peptides corresponding to the immunogenic region of these antigens were not affected. Biochemical analysis of the fate of the class II molecules in these cells indicated that Brefeldin A inhibits the egress of newly synthesized class II molecules from the ER. Whereas, in the case of cycloheximide, the turnover of newly synthesized class II molecules was increased by a factor of 2, resulting in a significant inhibition of the amount of these newly synthesized class II molecules to reach the cell surface. Further, there was no change in the steady state levels of class II molecules in APC incubated with cycloheximide or Brefeldin A. The data indicated that newly synthesized class II molecules appear to be the major target of binding for exogenous protein antigens after their intracellular processing to peptides. Since these drugs do not inhibit endocytosis, endocytosis of cell-surface class II molecules appears not to be involved with presentation of exogenous antigens by class II molecules. However, endocytosis of surface class II molecules may be involved in the binding exogenously added peptide antigens and experiments are in progress to test this possibility.

The above studies have been based on the ability of synthetic peptides to substitute for naturally processed antigens in stimulating T-cells. Naturally processed antigen fragments are thought to arise by limited proteolytic degradation of native antigens, but until now no physiological processed antigen has been directly analyzed. Recently we have reported the characterization of a physiological processed antigen dissociated from purified class II I-$E^d$ molecule after exposure to acid. The antigenic material corresponds to a previously described antigenic determinant of hen egg lysozyme and has a molecular weight of approximately 2000, a value very close to that predicted from analysis with synthetic peptides. Direct amino acid sequencing by tandem mass spectroscopy is in the process of being developed to better define the structure of this peptide and other self peptides which are bound to the class II molecule.

Interferon gamma (IFN-γ) is a potent stimulator of the expression of membrane class I and class II antigens on target cells. Increased MHC antigen expression aids in T-cell recognition of alloantigens, and the processed self or viral peptides carried on the MHC antigens. The first step of IFN-γ action on target cells is the binding to a specific cell surface receptor. The mouse IFN-γ receptor has been purified to homogeneity and the sequence of the N-terminus and several lysine-specific endopeptidase generated peptides was determined. One of the peptides was found to be identical to the portion of the human IFN-γ receptor. A deoxyoligonucleotide probe corresponding to this peptide was used to isolate a full length cDNA clone for the mouse IFN-γ receptor. The mouse IFN-γ receptor cDNA is comprised of 105 base pairs of 5'-untranslated region, an open reading frame coding for a 477-amino acid serine-rich protein having a calculated $M_r$ 52,276, and a 3'-untranslated region of 539 base pairs. The receptor is synthesized as a pre-protein from which a 25-amino acid signal peptide is cleaved. The receptor contains a hydrophobic putative transmembrane portion near the center of the molecule. Northern blot

analysis of various cell lines showed that each contained a single 2.0-kilobase mRNA receptor transcript. A direct correlation between the amount of IFN-γ receptor mRNA and the level of receptor expressed on the cell surface was observed. The mouse and human IFN-γ receptors are structurally similar, showing 51% overall homology in amino acid sequence. Mouse IFN-γ receptor cDNA when inserted in a mammalian shuttle vector and transfected into COS-7 monkey cells was able to direct the expression of specific binding activity for mouse IFN-γ. Currently the intracellular biochemical events following receptor occupation are being investigated. Incubation of mouse interferon-γ with human cells transfected with the mouse IFN-γ receptor fails to induce the expression of HLA antigens. Studies with human-mouse hybrids indicate that HLA expression in this heterologous system requires an additional gene product. Experiments are in progress to identify this gene product.

Interferons induce the transcription of MHC class I genes through interaction at the conserved IFN consensus sequence (ICS). The ICS contains an IFN response motif shared by many IFN-regulated genes. By screening mouse Lambda ZAP libraries with the ICS as a probe, we have isolated a cDNA clone encoding a protein that binds the ICS. This clone encodes 424 amino acids; the N-terminal 115 amino acids correspond to a putative DNA-binding domain and shows significant sequence similarity with other cloned IFN response factors. Northern analysis indicated that the mRNA is expressed predominantly in lymphoid tissues and is inducible preferentially by IFN-γ. This suggests that the ICS binding factor may play a regulatory role in cells of the immune system.

II. Tumor specific transplantation antigens

Individually distinct tumor specific transplantation antigens (TSTA) are expressed in chemically-induced tumors. Previously, our laboratory has purified and identified two TSTAs from the 3-methylcholanthrene-induced murine fibrosarcoma, Meth A. One TSTA was identified as the murine equivalent of the 90 kDa heat shock protein (HSP) consisting of two closely related protein species of 84 and 86 kDa and referred to as HSP84 and 86, respectively.

Although purified HSP84/86 possesses tumor rejection activity, no difference in the sequence of HSP84/86 genes isolated from Meth A tumor have been observed when compared to normal HSP84/86 amino acid sequences. Possible explanations for lack of amino acid differences are: the cDNA clone isolated from Meth A corresponds to the normal non-mutant allele, or that HSP84/86 specifically binds and copurifies with the true TSTA. In order to further analyze the antigenic nature of HSP84/86, subfractions of HSP84/86 from a Mono Q column were analyzed for TSTA activity. TSTA activity was found with a fraction of HSP84/86 which eluted last from the Mono Q column. These results suggest that antigen activity is associated with a portion of the total protein. Experiments are underway to try to separate completely antigen activity from HSP84/86 and to determine if tumor specific peptides are associated with these proteins.

The other TSTA, previously referred to as p82, has now been identified as the murine homolog of the human cytovillus-associated protein, cytovillin. Cytovillin, also known as ezrin, serves as substrate for a protein tyrosine kinase(s). The murine TSTA is an acidic protein of $M_r$ 68,548 consisting of 583 amino acid residues. The mouse and human sequences show 77% homology and an unusually long stretch of proline residues near the

C-terminus is conserved in both species. Comparison of the Meth A cytovillin sequence to that of the normal murine sequence is now in progress to identify any potential mutation in the tumor gene product which may be responsible for its antigenicity.

III. Antiproliferative effect of wild-type *p53* oncogene

The p53 oncogene has recently been found to undergo various genetic alterations, such as mutations, rearrangements, and deletions of one or both alleles. The function of both normal and mutant *p53* are poorly understood. Wild-type *p53* protein is expressed upon mitogenic stimulation of various cell types, and inhibition of its expression results in inhibition of cell growth. Recently, it has been shown that mutant *p53* can cooperate with the *ras* oncogene for oncogenic transformation, whereas, cotransfection of wild-type *p53* will inhibit this process. These properties of *p53* suggested that wild-type *p53* acts as an anti-oncogene or as a suppressor of cellular growth.

Subsequent to our previous structural characterization of the *p53* gene, we initiated a number of studies examining the functional capabilities of this gene product. The SV40-transformed hamster cell line, HR8, was transfected with a construct containing the mutant form of *p53* cloned from the human osteosarcoma cell line, HOS-SL, and with a plasmid construct containing the wild-type form of human *p53*. The expression of these genes was controlled by the mouse mammary tumor virus promoter, which is constitutively active in hamster cells. This transfection study indicated that the wild-type form of p53 had strong antiproliferative activity in a colony forming assay, relative to the mutant form. Clonal cell lines established from the wild-type transformed HR8 cells, were shown to express both p53 transcript and protein. Although these clonal cell lines remained viable despite the expression of the wild-type *p53* gene (confirmed at both the transcript and protein levels), their growth characteristics were different than normal HR8 and mutant p53 transfected cells. Wild-type transfectants grew to lower saturation densities and had significantly longer doubling times relative to the parental- and mutant- transfected cell lines. These studies clearly indicated that wild type p53 had an antiproliferative capacity.

The above analysis involved a heterologous system (i.e., human gene into hamster cells). These studies were extended into a human system by transfecting the human glioblastoma cell line, T98G, with the same wild-type human *p53* construct. In human cells the promoter is only active after induction with dexamethasone. Upon induction of wild-type *p53*, in stable transfectants, the $G_0/G_1$ progression of the cells was inhibited by several criteria, e.g. DNA synthesis, histone H3 mRNA levels, and saturation density. Further, the cells accumulated a DNA content equivalent to cells arrested in $G_0/G_1$ phase of the cell cycle. This effect was reversible by removal of the inducing agent or by infection of the cells with SV40 virus. Thus, our data clearly indicate that wild-type *p53* can act to inhibit cell growth in a homologous cell system.

Biochemical analysis of p53 expression before and after induction revealed that wild-type p53 protein is expressed and that it is phosphorylated to a greater degree compared to the endogenous mutant p53 protein constitutively expressed in these cells. Further, wild-type p53 was found both complexed and uncomplexed with the endogenously expressed mutant

p53. In addition, other proliferation associated gene products, such as PCNA and cdc2 kinase, were found to be modulated in these cells after wild-type p53 expression. At present it is not known if p53 acts directly or indirectly on these proteins, and experiments are underway to determine the cellular target(s) of p53 which are responsible for its antiproliferative effect. These observations are being extended to early passage human cells before and after growth arrest to access their relevance to the cell cycle. Analysis of the pathway(s) whereby p53 exerts its negative growth effect could lead to ways of controlling or inhibiting the growth of transformed cells.

IV. Protein chemistry

Communication between different cells of the immune system is in part achieved by a set of interacting proteins known as cytokines. We have been interested in the structure of a new cytokine, Interleukin 8 (IL-8), also known as neutrophil activation factor. IL-8 is composed of two identical subunits, which are released from several types of cells. A range of *in vitro* and *in vivo* studies have shown that IL-8 possesses two major activities: the first is the capacity to attract neutrophils, basophils and T-cells; and the second involves neutrophil activation. The solution structure of IL-8 dimer has been solved by nuclear magnetic resonance. The three dimensional structure revealed a structural motif in which two-symmetry-related antiparallel $\alpha$-helices lie on top of a six-stranded antiparallel $\beta$-sheet platform; the general architecture of IL-8 is similar to that of the $\alpha 1/\alpha 2$ domains of HLA-A2.

Two zinc finger proteins capable of binding to a specific DNA sequence in the regulatory region of the MHC Class I genes have been described. The protein binding to the DNA sequence, TGGGGATTCCCCAT, contained two zinc finger motifs of the TFIIIA-like sequence. While the other protein which binds the DNA sequence, GGTGAGGTCAGGGTGGG, is member of the steroid hormone superfamily and contained two fingers which chelate zinc through four cysteine residues. Peptides comprising the four single zinc fingers and the two double zinc finger regions have been prepared by solid phase peptide synthesis. Spectral studies with the peptides have shown that the peptides have metal binding capabilities as binding of the metal results in specific conformational changes. Binding studies with $Co^{+2}$ demonstrate that binding is tetrahedral and circular dichroism studies showed the presence of a helical character in all the peptides. NMR results clearly demonstrate that single fingers are capable of forming well-defined three-dimensional structures and the interactions of these peptides with the specific DNA sequences is now being evaluated.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 05597-01 LCBGY

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

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| Co-PI | S. Gottesman | Chief, Biochemical Genetics Section | LMB, NCI |
| Other: | S.H. Kim | Visiting Fellow | LMB, NCI |
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COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

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| TOTAL MAN-YEARS | PROFESSIONAL: | OTHER |
|---|---|---|
| 1.5 | 1.1 | 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.)*

The rapid intracellular degradation of regulatory proteins has been recognized as an important element in the control of vital cellular processes such as the response to heat shock and other stresses, the timing of cell cycle events, the repair of DNA damage, and the adaptation to nutritional and environmental conditions. Research over the last year has focused on the biochemical properties and in vivo functions of a major ATP-dependent protease from *E. coli*, Clp protease. The *clpP* gene, coding for the proteolytic subunit of this two-component protease, has been sequenced and shown to be unique. Although not closely related to any known proteases, Clp P has extensive amino acid homology to a protein of unknown function encoded by plant chloroplasts DNA. Immunochemical screening indicates the presence of proteins closely related to Clp P in a variety of other bacteria, yeast and other lower eukaryotes, and in animal cells. Clp P belongs to the serine protease family and two active site residues, Ser111 and His136, have been identified. Mutational alteration of these residues eliminates proteolytic activity while allowing proper folding of Clp P and binding interaction with the regulatory component, Clp A. *In vitro* studies with purified Clp protease revealed that Clp P has a very weak endoproteolytic activity with low molecular weight substrates and is activated ≥100 fold by Clp A and ATP. Clp cleaves a number of different peptide bonds and the specificity is not affected by the regulatory components. About two molecules of ATP are hydrolyzed per peptide bond cleaved. Electron micrographs of Clp P show a structure composed of two superimposed hexagonal rings, which bears a striking resemblance to the ATP-dependent multicatalytic protease found in eukaryotic cells.

Findings

1. Previous work from this laboratory. Between 80 and 90% of rapid intracellular protein turnover is energy-dependent, yet relatively little is known about the proteases responsible for protein degradation *in vivo*. *E. coli* has provided an invaluable model system for studying this process. Biochemical and genetic studies of the Lon protease, the first ATP-dependent protease to be characterized, allowed the following critical generalizations: (1) Certain proteases are activated by direct interaction with ATP; (2) Depending on the protein substrate, an obligatory hydrolysis of one or more molecules of ATP accompanies catalytic peptide bond cleavage; (3) Different ATP-dependent proteases are responsible for the rate-limiting step in the degradation of specific proteins or groups of proteins *in vivo*; (4) These highly specific proteases also are capable of degrading a broad spectrum of mutant, misfolded, and otherwise abnormal proteins. Mutants lacking Lon protease allowed us to identify unequivocally and eventually purify another ATP-dependent protease from *E. coli*. This protease, Clp, appears to represent a class of ATP-dependent proteases distinct from Lon protease. Moreover, Clp protease has been highly conserved in virtually all organisms and is structurally and biochemically similar to the major ATP-dependent protease identified in eukaryotic cells.

2. Clp P has very weak endoproteolytic activity against small proteins and is activated >100 fold by Clp A and ATP. Clp P cleaves a number of peptide bonds in oxidized insulin B chain; the same sites are cleaved with or without activation by Clp A and ATP. Detectable proteolysis of higher molecular weight proteins does not occur in the absence of Clp A and ATP. With α-casein as a substrate, an average of 1.5-2.0 ATP molecules are hydrolyzed per peptide bond cleaved. Protein degradation occurs at the rate of ≤ 20 bonds cleaved per active site per minute. Protein degradation appears to be highly processive, with multiple cuts within each substrate occurring prior to complete release of products. The mechanism of processivity and the effect of the multimeric state of Clp P on the processivity are being investigated further.

3. Mutational alteration of Ser111 or His136 lead to loss of proteolytic activity of Clp P both *in vivo* and *in vitro*. *In vivo* activity was measured by the Clp-dependent degradation of ß-galactosidase fusion proteins described previously and by a unique self-processing reaction by Clp P. Clp P is synthesized with a 14 amino acid leader peptide which is rapidly removed *in vivo*. In the absence of a wild-type Clp P in the cell, Clp P with altered Ser111 or His136 are not processed; providing active Clp P in addition to the mutants allows processing of both mutant and wild-type Clp P. Clp A is not required for processing of Clp P, indicating that Clp P has at least limited trans-acting proteolytic activity *in vivo* in the absence of Clp A.

4. The amino acid sequence of Clp P derived from the DNA sequence indicates that Clp P belongs to a unique class of serine protease and has no sequence similarity to the ATP-dependent Lon protease of *E. coli*. Homologs of Clp P are encoded in chloroplast genomes, and the regions around the active site serine and histidine are particularly highly conserved. The presence of a chloroplast transit sequence in the Clp A homolog found to be encoded by the tomato nuclear genome strongly suggests that a protease structurally and functionally similar to Clp will be found in plant chloroplasts. Proteins of a similar size to Clp P that cross-react with anti-Clp P antibody are found in other bacteria and lower eukaryotes, and animal cells contain several Clp P-cross reactive proteins. Two subunits of the yeast multicatalytic protease have been shown by others to cross-react with Clp P antibodies, and a cross-reactive protein from

*B. subtilis* is found associated with 50S ribosomes. Thus, structural features of Clp P may be common to proteases that are found as part of very large proteolytic complexes.

The quaternary structure of Clp P resembles two superimposed rings of six subunits and presents an image in the electron microscope that is very similar to that seen for the eukaryotic proteasome. Also, Clp P assembles into a very high molecular weight complex with the ATP-binding regulatory subunit, Clp A, a process that is remarkably analogous to the association of the proteasome with ATP-binding subunits to form the ATP-dependent ubiquitin conjugate-degrading protease. Association between Clp A and Clp P requires the continuous presence of ATP. ADP is an inhibitor of formation of the Clp A/Clp P complex and hydrolysis of ATP leads to dissolution of the complex. The active complex of Clp A and Clp P dissociates under assay conditions with a half-time of about 4 minutes. Interaction between Clp A and Clp P does not affect the ATPase activity of Clp A, but active protein degradation leads to a 50-100% increase in ATP hydrolysis. Non-hydrolyzable analogs of ATP weakly promote association between Clp A and Clp P but do not activate proteolysis; therefore, binding of the two components is not sufficient to allow protein degradation. Mutants of Clp P with altered Ser111 or His136 assemble into the double-ringed structure and also can associate with Clp A in the presence of ATP without stimulating ATPase activity. Taken together, these results indicate that ATP must have a second function in the protein degradative activity of Clp protease.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 05598-01 LCBGY

PERIOD COVERED

October 1, 1989 to September 30, 1990

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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| TOTAL MAN-YEARS: | PROFESSIONAL | OTHER: |
|---|---|---|
| 12.0 | 12.0 | 0.0 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Resistance to multiple drugs is major impediment to the successful chemotherapy of human cancers. One mechanism of multidrug-resistance is the expression of a 170,000 dalton energy-dependent drug efflux pump (P-glycoprotein or the multidrug transporter, the product of the human *MDR*1 gene) which confers resistance to colchicine, adriamycin, vincristine, vinblastine, puromycin, and actinomycin D. Several lines of investigation concerning the multidrug transporter have been pursued: 1) Evidence for ATP-dependent transport activity of P-glycoprotein in vesicles and across epithelial monolayers has been obtained; 2) Novel expression vectors which utilize a full-length *MDR*1 cDNA as a dominant selectable marker are used to introduce and amplify non-selectable genes in cultured cells and *MDR*1 retroviral vectors have been developed; 3) Many human tumors express *MDR*1 RNA, and this expression may predict drug-resistance in some cancers, and correlate with the development of drug-resistance in others; 4) Transgenic mice have been constructed in which the human *MDR*1 mRNA is expressed in the bone marrow at levels comparable to those found in human tumors. This level of *MDR*1 expression is sufficient to confer resistance to leukopenia induced by MDR drugs; 5) Increased expression of *MDR*1 RNA has been demonstrated in regenerating rat liver, in cultured rodent cells after exposure to chemotherapeutic agents, and in kidney cancer cells after heat shock; 6) The intron-exon structure of the human *MDR*1 gene has been determined and supports a model of independent evolution of the two halves of the multidrug transporter. Similarly, a deletion analysis of the *MDR*1 cDNA is consistent with important functions being contributed by both the amino and carboxy-terminal halves of the molecule as is a study demonstrating photoaffinity labeling of both halves of P-glycoprotein by the hydrophobic drug $^{3}$H-azidopine; and 7) Evidence that P-glycoprotein acts by pumping hydrophobic drugs out of the lipid bilayer has been obtained.





GPO 914-918

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Major Findings:

1. Vesicles which transport $^{3}$H-vinblastine have been prepared from human multidrug-resistant KB cells. Analysis of the nucleotide requirement for this transport shows that ATP > GTP and that UTP, CTP, and NAD do not support transport.

2. Epithelial monolayers formed from MDCK cells into which the human *MDR*1 cDNA has been introduced by retroviral infection transport vinblastine, actinomycin D, vincristine, daunorubicin and verapamil. These data support a model in which P-glycoprotein expression in kidney, liver and intestine results in excretion into urine, bile and intestinal lumen of toxic hydrophobic compounds.

3. A unique cloning site downstream from an SV40 promoter has been introduced into a retroviral vector in which the *MDR*1 cDNA is under control of a Harvey sarcoma virus promoter. An IL-2 receptor cDNA has been introduced into this vector. After transfection into NIH 3T3 cells and selection for colchicine-resistance conferred by the *MDR*1 cDNA, IL-2 receptor is expressed in the recipient cells. Selection for increased colchicine-resistance results in high levels of IL-2 receptor expression. Thus, this vector can be used to introduce and amplify expression of non-selectable cDNAs.

4. The human *MDR*1 gene has been introduced via retroviral infection into mouse bone marrow *in vitro*. Infected GM-CSF show up to 30% colchicine and vinblastine-resistant colonies. Expression of the human cDNA persists for several weeks after transplantation of infected marrow into mice, suggesting that this may be a promising approach for gene therapy.

5. A protein chimera between the multidrug transporter and adenosine deaminase (ADA) has been produced by fusing an ADA cDNA to the 3'-end of an *MDR*1 cDNA in a retroviral vector. When packaged as a retrovirus, this can be used to infect cultured NIH and KNIH cells which are colchicine-resistant and express ADA activity. Tumors derived from MDR-ADA infected KNIH cells stably express the functional chimera which can be detected in cell cultures derived from tumors grown in nude mice. These experiments demonstrate the feasibility of using the MDR-ADA retrovirus for gene therapy experiments.

6. We have continued to analyze human cancers for expression of *MDR*1 mRNA with a sensitive and quantitative slot blot technique. The data base of human cancers has been expanded to over 500 different tumors. A high percentage of kidney, adrenal, colon and liver cancers express *MDR*1 mRNA. *MDR*1 RNA is expressed less commonly in leukemias and lymphomas at first presentation, but when expressed in acute non-lymphocytic leukemia it predicts poor response to chemotherapy. High levels of *MDR*1 RNA are found in many chronic lymphocytic leukemias in blast crisis, and in recurrent ovarian cancer and neuroblastoma, which correlates well with failure of these tumors to respond to chemotherapy with MDR drugs.

7. The human *MDR*1 cDNA has been expressed in transgenic mouse bone marrow and spleen under control of a chicken β-actin promoter. RNA levels comparable to those found in human cancers confer resistance to leukopenia induced by many different MDR drugs. Verapamil, an inhibitor of the multidrug transporter, sensitizes the bone marrow of these MDR-transgenic mice to the toxic effects of MDR drugs. This MDR transgenic mouse model will be useful for testing dose intensification chemotherapeutic regiments, the efficacy of inhibition of the multidrug transporter, and therapies based on gene transfer of the *MDR*1 cDNA followed by selection of resistant bone marrow.

8. Model systems in which levels of *mdr* RNA are regulated have been developed. In regenerating rat liver, increases in *mdr* RNA occur without proportionate increases in transcription, suggesting that RNA levels in this system are regulated by mRNA stabilization. In rodent cells in culture, treatment with chemotherapeutic drugs also raises *mdr* RNA levels, but again, increases in transcription by nuclear run-off assays cannot be demonstrated. Most human cells do not show regulation of *MDR*1 RNA levels; however, in response to heat shock, up to 10-fold increases in *MDR*1 RNA are seen in renal adenocarcinoma cells without concomitant increases in transcription. These results suggest that in rodent and human cells *mdr* RNA levels are regulated by different factors, and that mRNA stabilization may play an important role in regulating these levels.

9. The complete *MDR*1 gene has been isolated on a set of overlapping cosmids and lambda phage clones. The positions of all the introns and exons has been determined. Despite the superficial similarities in structure and sequence of the amino and carboxy-terminal halves of P-glycoprotein, the positions of the introns in these two halves are different, suggesting independent evolution, and, possibly, independent function.

10. Structure-function relationships in the *MDR*1 gene have been analyzed by generating deletion, substitution, and insertion mutations in an *MDR*1 cDNA. Only small deletions in the carboxy-terminus are tolerated; all substantial deletions or substitutions result in loss of function in both the amino and carboxy-terminus. These results demonstrate the necessity for two functioning halves of P-glycoprotein, and help define the limits for allowable alterations in this molecule.

11. $^{3}$H-azidopine, an inhibitor of the function of P-glycoprotein which can be used as a photoaffinity label of the transporter, has been used to analyze which regions of P-glycoprotein interact with the drug. Using a combination of proteolytic digestion and antibody precipitation, regions in both the amino and carboxy-termini of P-glycoprotein have been shown to be

labeled by $^{3}$H-azidopine, consistent with an important function for both halves of the molecule.

12. Specific antisera against several different domains of P-glycoprotein have been raised in rabbits. Antigens were prepared after expression of partial *MDR*1 cDNAs in *E.coli* using an inducible T7 expression system.

13. A baculovirus vector expressing P-glycoprotein has been constructed and used to infect insect cells. These cells produce large amounts of P-glycoprotein which binds hydrophobic drugs, but is not glycosylated. This recombinant P-glycoprotein may prove useful for physical or functional studies of the transporter.

14. The non-specific hydrophobic membrane label $^{125}$I-INA (iodinated naphthalene azide) detects P-glycoprotein as a major transmembrane protein in drug-resistant cells. INA can be exploited as a specific label for P-glycoprotein if it is activated by energy transfer from the chromophore (and transport substrate) doxorubicin. By energy transfer from doxorubicin or rhodamine 123, INA labels many proteins in drug-sensitive cells, but predominantly P-glycoprotein in drug-resistant cells. This result suggests that P-glycoprotein is able to extract doxorubicin and rhodamine 123 from the lipid bilayer of resistant cells and supports a model of P-glycoprotein as a "hydrophobic vacuum cleaner."

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08705-14 LCBGY

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Genetic and Biochemical Analysis of Cell Behavior

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

| | | | |
|---|---|---|---|
| PI: | M.M. Gottesman | Chief, Laboratory of Cell Biology | LCB, NCI |
| Other: | S. Goldenberg | Research Biologist | LCB, NCI |
| | R. Fleischmann | Staff Fellow | LCB, NCI |
| | D. Ray | Research Biologist | LCB, NCI |
| | M. Gosse | Guest Researcher | |

COOPERATING UNITS *(if any)*

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| C. Jhappan | Visiting Associate | LMB, NCI |

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL | OTHER |
|---|---|---|
| 2.5 | 2.5 | 0 |

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

☐ (a1) Minors

☐ (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 to study the genetics and biochemistry of some aspects of the behavior of cultured cells. Our recent work has emphasized the manner in which cyclic AMP regulates cell growth and gene expression in CHO cells. The mechanism of cAMP action on CHO cells has been studied by isolating CHO mutants resistant to growth inhibition by cAMP. These mutants have defective cAMP-dependent protein kinases with either altered regulatory (RI) or catalytic (C) subunits. DNA from cells carrying dominant cAMP-resistant defects can be used to transfer the cAMP-resistance phenotype to sensitive cells as can cDNA expression vectors encoding mutant regulatory subunits. 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. A yeast phosphodiesterase (PDE) has also been expressed in CHO cells. Expression of the PDE gene results in insensitivity to cAMP. To test the effect of ablating cAMP responses in pancreatic islet cells in mice, transgenic mice carrying the yeast PDE gene under control of an insulin promoter have been constructed.

Major Findings:

1. Some cAMP-dependent protein kinase mutants are supersensitive to natural product cytotoxic drugs such as colchicine, vinblastine, adriamycin and puromycin. Revertant cells which have recovered normal levels of drug-resistance have lost their protein kinase mutant phenotype. This result suggests that the expression of resistance to multiple drugs can be modulated by cAMP-dependent protein kinase, perhaps through a mechanism involving the multidrug transporter (*mdr*1 gene product). In confirmation of this hypothesis, CHO protein kinase mutants were found to have reduced levels of *mdr* RNA and reduced amounts of the transporter, indicating that in CHO cells, cAMP-dependent protein kinase modulates *mdr* mRNA levels.

2. The full-length cDNA for the CHO RI subunit has been expressed in *E.coli*. The recombinant protein is active as measured by its ability to inhibit C activity. Previously characterized mutations affecting cAMP binding, and novel mutations affecting cAMP binding and association with C have been introduced into the cloned RI cDNA by site-directed mutagenesis. These bacterially expressed RI subunits provide a rapid way to screen for mutations which affect the function of RI. Novel 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.

3. The yeast cyclic nucleotide phosphodiesterase (PDE) gene has been introduced into CHO cells where its expression ablates growth and transcription responses to cAMP. Since the yeast PDE gene has a high turnover number and yeast PDE is expressed at high levels, it effectively degrades endogenous cAMP generated by cholera toxin or prostaglandin E1, and also inactivates cAMP analogs such as 8-Br-cAMP which are resistant to the action of the mammalian PDEs. The use of yeast PDE provides another dominant genetic system for inactivation of cAMP in activated cells and, potentially, in transgenic mice.

4. We have constructed five founder transgenic mice in which the yeast PDE gene is under control of an insulin promoter has been inserted into the mouse genome. Lines of transgenic mice from each of these founders have been bred. Studies on the expression of yeast PDE mRNA and protein in pancreatic islet cells of these transgenic mice are in progress.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08715-12 LCBGY

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Synthesis and Function of a Transformation-Dependent Secreted Lysosomal Protease

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

| | | | |
|---|---|---|---|
| PI: | M.M. Gottesman | Chief, Laboratory of Cell Biology | LCB, NCI |
| Other: | S. Goldenberg | Research Biologist | LCB, NCI |
| | D. Ray | Research Biologist | LCB, NCI |
| | S. Chauhan | Visiting Fellow | LCB, NCI |

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|---|---|---|
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☐ (a1) Minors

☐ (a2) Interviews

B

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

Cultured mouse fibroblasts which are malignantly transformed or treated with TPA or growth factors such as PDGF synthesize and secrete the 39,000 $M_r$ precursor to cathepsin L (also called MEP, for major excreted protein) in large amounts. Cathepsin L is an active acid protease whether or not it is glycosylated. Transformation, TPA and PDGF stimulate transcription of cathepsin L. We have cloned a functional procathepsin L gene from the mouse and have identified and sequenced the 5' flanking region which acts as a promoter in CAT constructions. Regulated transcription requires sequences in the first two exons and introns acting in concert with upstream promoter elements. A human procathepsin L cDNA has also been cloned and sequenced. A study of the activity of recombinant human procathepsin L produced in bacteria indicates that both the "pro" and the carboxy-terminal part of the protein are needed for full activity. The "pro" piece appears to be involved in proper folding of the enzyme, while a carboxy-terminal segment cysteine is also essential for activity, presumably because it allows formation of a disulfide bridge with the large catalytic subunit. Polyclonal and monoclonal antibodies to human cathepsin L have been prepared using the recombinant human antigen. Using the human procathepsin L cDNA probe, we have shown expression of procathepsin L mRNA in all human tissues and cell lines tested, with increased expression in renal cancer and squamous cell carcinoma of the lung.

Major Findings:

1. The mouse MEP (procathepsin L) gene has been cloned and the promoter region has been isolated. Approximately 5 kb of upstream sequence has been obtained which drives CAT expression in MEP promoter-CAT constructions. However, this expression is not regulated by TPA, but is regulated by cAMP. A 300 bp fragment upstream from the transcription initiation site is an adequate promoter in MEP-promoter-CAT constructions, and is also unregulated by TPA. Regulation of transcription by TPA requires sequence in the first two exons and introns, as indicated by constructions containing these sequences in which 2-3-fold increases in RNA can be observed after transfection and TPA treatment.

2. Recombinant human procathepsin L has been produced in large quantities in *E. coli*. Yields of approximately 0.5 g/10L culture have been obtained. The recombinant enzyme is active after solubilization in urea and reduction/oxidation in glutathione above pH 10. A deletion analysis of the recombinant human cathepsin L shows that both amino and carboxy-terminal sequences are needed for activity. Site-specific deletion of a carboxy-terminal cysteine results in loss of activity of the recombinant molecule, presumably by blocking formation of a cystine bridge needed either structurally or catalytically.

3. Using recombinant procathepsin L produced in E. coli, we have prepared both rabbit polyclonal antisera and two mouse monoclonal antibodies to human procathepsin L. The rabbit polclonal antibodies are good precipitating antibodies for research purposes. The monoclonal antibodies are being developed as reagents for ELISA detection of cathepsin L in human tissues and body fluids.

4. We have measured cathepsin L mRNA levels 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 and squamous cell carcinomas of the lung.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09100-5 LCBGY

PERIOD COVERED
October 1, 1989 through September 30, 1990

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)*

| | | | |
|---|---|---|---|
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| Other: | E. Appella | Medical Officer | LCB, NCI |
| | M. Jiménez | Visiting Associate | LCB, NCI |
| | K. Kameyama | Visiting Fellow | LCB, NCI |
| | T. Tsukamoto | Visiting Fellow | LCB, NCI |

COOPERATING UNITS *(if any)*

| | |
|---|---|
| A. Palumbo, G. Prota | Dept. Organic Chemistry, Univesity of Naples, Naples, Italy |
| D. Gersten | Dept. Pathology, Georgetown University, Medical Center, Washington, DC |

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☐ (a2) Interviews

B

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

This project is aimed at characterizing different aspects of the growth and differentiation of transformed melanocytes, a tumor type termed malignant melanoma, including: (1) the host immune responses elicited in tumor-bearing animals and the role those responses play in progressive tumor growth and subsequent metastatic spread; (2) the production of tumor specific proteins, to determine their mechanism of formation and to examine the feasibility of utilizing their specificity for the immunoassay and immunotherapy of melanoma; (3) the role of various cell surface molecules critical to the cascade of events leading to metastatic spread of tumors; and (4) the control mechanisms involved in the regulation of differentiation, i.e. pigment production, in normal and in transformed melanocytes. We have demonstrated that murine melanomas share a common cell surface antigen, termed B700, which elicits tumor rejection; this antigen is related biochemically and immunologically to a normal melanocyte protein, perhaps a differentiation antigen, and is a member of the albuminoid superfamily. We have found that the immune response elicited by B700 is mediated through cytotoxic antibody production, and not through a demonstrable cellular response. We have produced syngeneic murine monoclonal antibodies with specific antimetastatic activity. We have isolated melanoma cell clones which vary in their tumorigenic and metastatic properties, and have employed transfection protocols with the genes for H-2, urokinase, urokinase receptor, and for the H-ras oncogenes, to further elucidate the factors critical to those phenotypes. We have further characterized the molecular controls involved in mammalian pigmentation and its activation by environmental stimuli, employing synthetic oligonucleotide and peptide probes to different pigmentation related gene products. We have shown that mammalian melanin production is regulated primarily through post-translational activation of latent enzyme and the production of a potent melanogenic inhibitor, that multiple gene products are involved in the process of melanin production, and that alternative splicing of mRNA transcripts of those genes are significantly more frequent in transformed melanocytes.

Other Cooperating Units:

| | |
|---|---|
| A. DeLeo, E. Gorelik | Dept Pathology, Pittsburgh Cancer Institute, Pittsburgh, PA |
| J. Muller | Div Biochem Biophys, Food and Drug Admin Center, Bethesda, MD |
| L. Law | Laboratory of Genetics, NIH, Bethesda, MD |
| F. Blasi | Mikrobiologisk Institut, Copenhagen, Denmark |
| T. Walden | Radiation Biochem, Armed Forces Radiobiol Res Inst, Bethesda, MD |
| R. Yetter | Research Service, Veterans Admin Medical Center, Baltimore, MD |
| S. Leong | University of Arizona Health Sciences Center, Tucson, AZ |

Major Findings:

(1) Immunogenicity of Malignant Melanoma

We (with Law) have previously shown that murine melanomas, including the K1735, JB/RH, JB/MS, B16 and Harding-Passey, share tumor specific transplantation antigens (TSTA) which do not cross-react with the S91 melanoma, although the latter produces an S91-specific TSTA. The cross-reactive melanoma TSTA is extremely interesting since the tumors expressing it have arisen via spontaneous events, chemical or ultraviolet induced transformation. B700, a melanoma specific antigen, when isolated from the above tumors, can be used to immunize mice and elicit tumor rejection activity, with the specificity noted above. Studies characterizing the mechanisms involved in these responses show that although there is no significant cellular immune response, there is a dramatic humoral response; we (with DeLeo, Gersten, and Law) have shown that mice immunized with melanoma cells produce high titers of cytotoxic antibodies, which can be quantitatively and specifically absorbed with the B700 antigen; these have now been used to produce monoclonal antibodies which have the requisite specificity and cytotoxicity, and are effective in experimental antimetastatic assays. The specificity of this cytotoxicity is identical to the pattern of cross-reactive TSTA activity noted above, suggesting that the major immune response of mice to challenge with melanoma cells is the production of cytotoxic antibodies and not the generation of cell mediated immune mechanisms. Since these responses are rarely effective in preventing tumor growth in naive animals, our current studies are directed at examining the reasons behind this ineffective response, and at ways to successfully augment it. B700 has been shown to associate with another melanoma specific antigen termed B50, although B50 does not appear to play any role in the expression of cell surface TSTA, or in the sensitivity of melanoma cells to immune response mechanisms. Another melanoma specific antigen, termed 9B6, has been shown to be associated with the expression of a melanoma specific, ecotropic murine retrovirus; the mechanism whereby the expression of this virus is limited to melanomas arising only from C57Bl6 mice is an exciting question now being addressed in collaborative studies (with Gorelik, Leong, Muller and Yetter). Monoclonal antibodies recognizing the 9B6 antigen or the B700 antigen have now been successfully used to decrease the proliferation of established pulmonary metastases of melanoma cells, primarily through their enhancement of endogenous natural killer (NK) cell activity.

(2) Tumor Specific Antigens Produced by Malignant Melanoma

Transformed cells express dramatically different cellular products than do their normal counterparts; the production of novel products termed tumor specific antigens (TSA) by malignant cells and the involvement of TSA in the growth and function of tumor cells has been a subject of widespread interest. We (with Gersten) have examined two such TSA, termed B700 and B50 - both these antigens are analogous to normal melanocyte proteins, but with alterations in their primary structure which give rise to their immunologic specificities. The ability of B700 to also function as a TSTA in cells which express it on the cell surface has been previously shown by our group. Interestingly, while B700 belongs to the albuminoid superfamily by virtue of biochemical, immunological and functional similarity, our studies (with Gersten and Law) have now demonstrated that the B50 antigen is related to the Ro/SS-A antigen and its superfamily, suggesting that the expression of altered molecules related to normal cellular constituents may be an important mechanism for the generation of TSA. We have now produced murine monoclonal antibodies specific to B700, and have collaborated on a project to clone its gene; a putative clone which encodes a product recognized by B700 specific monoclonal antibodies has been isolated, and studies are underway to analyze the full sequence of the B700 antigen. Such information should allow us to characterize the mechanism(s) whereby this TSA is expressed by transformed melanocytes, but not by normal melanocytes. In collaborative efforts (with Farzaneh, Gersten and Walden) we have functionally analyzed the catalytic potential of B700, and have found that it has an altered catalytic activity on prostaglandin E2 compared to serum albumin, although the similarities present suggest far more sequence homology than was heretofore suspected.

(3) Metastatic Events in Dissemination of Malignant Melanoma

We (with Appella, Blasi and Law) have examined the role of cell surface expression of proteases on tumor invasion and metastasis. We previously demonstrated that urokinase, a protease involved in the control of migration and differentiation of normal cells, is critical to the tumor cell's capability to penetrate membranes and other proteinaceous barriers during metastasis of transformed cells. We have addressed the question of whether this parameter is important in vivo by cloning B16 and JB/MS murine melanoma cells, using no selection pressure, and found that there was a correlation between metastatic activity and surface localization of urokinase activity, which suggests that urokinase is an important, although not a sufficient, parameter in the determination of a tumor cell to metastasize. We have succeeded in transfecting the urokinase and the urokinase receptor gene into poorly metastatic cells in order to directly determine the effect of enhanced surface urokinase expression on metastatic behavior. Those transfected cells have shown enhanced metastatic potential in nude mouse experimental metastasis assays, although further study in syngeneic murine systems will be required before an adequate analysis of these results can be determined. We have examined the interrelationships of differentiation with the tumorigenic, i.e. the capacity to grow as primary subcutaneous tumors, and the metastatic phenotypes of melanoma cells. Using melanocyte stimulating hormone (MSH) as a physiologic stimulus to differentiation, we (with Law, Kameyama and Tsukamoto) have characterized the effect of hormonally induced differentiation on those properties of melanoma cells. While differentiation is inversely correlated with tumorigenic abilities, as expected, it surprisingly has varied effects on their metastatic potential, in most cases causing increases in metastatic growth. We have recently initiated studies aimed at examining the potential use of tumor specific antibodies in the treatment of established metastases.

Treatment of animals with syngeneic monoclonal antibodies recognizing either of the TSA noted above (B700 or 9B6) has resulted in significant reduction of the growth of established pulmonary metastases. These effects seem to be modulated by natural killer cells endogenous in the tissues which can more readily identify and kill metastatic tumor cells coated with these specific antibodies. We are attempting to improve these antimetastatic effects through different regimens of treatment, and eventually by linking these antibodies with cytotoxins. Finally, in collaborative studies (with Palumbo and Prota) we are investigating the potential usefulness of thiouracil derivatives in chemotherapeutic approaches to melanoma. Thiouracil has been previously shown to specifically localize in pigment producing cells, although its potential antimelanoma effect has been limited by its solubility and relatively low cytotoxicity. We are identifying the metabolic melanogenic intermediate in order to better design a more soluble and toxic compound which maintains the desired specificity.

(4) Regulation of Melanogenesis

We (with Jiménez, Kameyama and Tsukamoto) have examined the levels of response of mammalian melanocytes to physiologically relevant stimuli, such as UV light and MSH. The melanocyte provides a unique model system to study intracellular control mechanisms since only the enzyme tyrosinase is essential for melanin biosynthesis, although rates of pigmentation can be influenced at various levels. We have produced tyrosinase specific monoclonal antibodies which cross-react among mammalian tyrosinases, and have been valuable probes for the characterization of post-translational enzyme processing in melanocytes. We have used them to examine the metabolic processing of tyrosinase, which includes glycosylation and intracellular transport to melanosomes, where melanization is initiated. Two different pigment related genes have been cloned in other laboratories from murine and human melanocytes; these genes map to the brown and albino loci of mice, dramatically influence pigmentation, and encode two highly homologous proteins. As an approach to unraveling the function of these gene products in the regulation of melanogenesis, we have synthesized oligonucleotides encoded by those genes (for hybridization studies) and peptides (used to produce specific antibodies) and have examined their expression at the transcriptional and translational level in response to environmental stimuli. In agreement with our earlier studies, we have found only slight increases in the synthesis of *de novo* tyrosinase in response to melanogenic stimulation, and that the dramatic increases in pigmentation result from activation of latent tyrosinase in the melanocyte. These studies have shown that both gene products are functional catalytically in melanogenesis, and we are now characterizing their interactions in the regulation of mammalian pigmentation. In other studies, we have cloned a melanoma cell line that produces a large quantity of a melanogenic inhibitor which may be critical to masking latent tyrosinase catalytic activity as noted above, and have now purified and characterized it. This inhibitor is a low molecular weight molecule (~480), an amine, and probably closely related to a melanogenic intermediate, which functions in a competitive but reversible manner to efficiently inhibit pigment production; it must be produced constitutively to be effective. Aberrations in the regulation of this inhibitory activity may be involved in several types of abnormal pigmentary disorders, such as albinism. The emerging picture indicates that the control of mammalian pigmentation is an order of magnitude more complex than originally thought, but recent advances at the molecular level are slowly unraveling these interrelated control mechanisms, many of which may be malfunctioning in various pigmentary disorders.

Publications:

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Annual Report

LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY
FY 1990

The Laboratory of Tumor Immunology and Biology carries out a range of investigations in tumor immunology, tumor cell biology, and molecular biology. Areas of particular emphasis include: (a) the identification and characterization of cellular genes associated with the development of carcinoma, both in rodents and humans; (b) biochemical mechanisms of oncogene expression, and cell growth regulation; and (c) the characterization and utilization of monoclonal antibodies directed against tumor-associated antigens, with emphasis being placed on the study of human carcinoma cell populations, and potential diagnostic and therapeutic applications.

The Laboratory of Tumor Immunology and Biology is composed of five Sections: The Cellular and Molecular Physiology Section (Dr. Herbert Cooper, Chief); The Biochemistry of Oncogenes Section (Dr. Robert Bassin, Chief); The Oncogenetics Section (Dr. Robert Callahan, Chief); The Experimental Oncology Section (Dr. Jeffrey Schlom, Chief); The Cellular Biochemistry Section (Dr. Yoon Sang Cho-Chung, Chief). Although each of the five Sections represents an independent program of scientific research, many collaborative studies are carried out between Sections. Below is a summary of the research activities being carried out in the Laboratory of Tumor Immunology and Biology.

BIOCHEMISTRY OF ONCOGENES SECTION (R. Bassin, Chief)

"Anti-oncogenes": The Analysis of Cellular Resistance to Transformation. We have developed a new technique for isolating cells that resist transformation by specific oncogenes using 4-*cis*-hydroxy-L-proline as a selective agent. We have begun to characterize additional revertants from *ras*-transformed cells. The new revertants are resistant to transformation by members of the *ras* gene family and to certain tyrosine kinase oncogenes, but they are sensitive to retransformation by *mos*, *raf*, and *fos*. Combining the retransformation data observed here with those from our ouabain-derived revertants isolated previously (C-11 and F-2), we find that all oncogenes can be placed into one of four groups, and we suggest that this represents a primitive functional classification. If true, the *ras* gene would require the role of at least two cellular 2 functions (such as attachment to the cell membrane and kinase activity) to successfully transform mouse cells.

Transfection of a cDNA library prepared from one of the revertants, CHP 9CJ, has resulted in the isolation of several DNA sequences that may be associated with the revertant phenotype. One of these sequences may be identical to the K-*rev*-1 gene previously isolated from *ras*-resistant human cells.

The Role of TGFα in the Etiology and Progression of Breast Cancer. Transforming growth factor alpha (TGFα) has been circumstantially implicated in the autocrine growth of a number of different rodent and human tumor cells *in vitro*. However, its distribution and role in the etiology and/or progression of rodent and human breast cancer are relatively unknown. The present studies have demonstrated that biologically active and immunoreactive TGFα can be detected in hormone (estrogen)-dependent, DMBA- or NMU-induced rat mammary adenocarcinomas and that these tumors possess a specific 4.8-kb TGFα mRNA. These tumors also express elevated levels of c-Ha-*ras* protein (DMBA tumors) or possess a point-mutated c-Ha-*ras* gene (NMU tumors). Ovariectomy results in tumor regression and is preceded by a specific decrease in TGFα mRNA and protein. Transformation of nontransformed mouse mammary epithelial cell lines with a point-mutated c-Ha-*ras* protooncogene results in the loss in mitogenic responsiveness of these cells to exogenous epidermal growth factor (EGF) and a reduction in the number of unoccupied EGF receptors on these cells due in part to an enhanced production and secretion of TGFα. Several human breast cancer cell lines also secrete TGFα and possess TGFα mRNA. In the estrogen-responsive breast cancer cell lines, estrogen can induce a 3- to 5-fold increase in TGFα mRNA and protein. Treatment of these cells with a polyclonal anti-TGFα antibody or with a monoclonal anti-EGF receptor

antibody can inhibit the growth of these cells *in vitro*. Elevated levels of TGFα protein or expression of TGFα mRNA can be detected in 50-60% of primary human breast tumors. No evidence was found for any gross amplifications and/or rearrangements of the TGFα gene in these tumors. Additionally, overexpression of a human TGFα gene in a cloned immortalized population of mouse and human mammary epithelial cells can lead to the malignant transformation of these cells *in vitro* and *in vivo*. Collectively, these results suggest that TGFα can function as an autocrine growth factor for a subset of rodent and human breast tumors, that estrogens or activated protooncogenes such as *ras* can enhance the expression of TGFα, and that enhanced production of TGFα can lead to the transformation of mammary epithelial cells that have a sufficient complement of functional EGF receptors.

## CELLULAR BIOCHEMISTRY SECTION (Y. S. Cho-Chung, Chief)

The Cellular Biochemistry Section studies the control mechanism of cell growth and differentiation by cyclic AMP (cAMP). Using new derivatives of cAMP, site-selective cAMP analogs, whose effects far exceed that of parent cAMP or the previously studied cAMP analogs, we anticipate achieving our research goals in the following areas: (1) a better understanding of the regulatory mechanisms of cell growth and differentiation, (2) a clearer definition of the derangement of cAMP-effector function in neoplastic transformation and progression, and (3) improved management of human cancers by providing the non-toxic biological compounds (site-selective cAMP analogs) as antineoplastics and chemopreventives. Our studies being conducted in two projects are summarized below.

Site-selective cAMP Analogs as Antineoplastics and Chemopreventives. Cyclic AMP (cAMP) in mammalian cells functions by binding to cAMP receptor protein, the regulatory subunit of cAMP-dependent protein kinase. The cAMP receptor protein has two different cAMP binding sites, and cAMP analogs that selectively bind to either one of the two binding sites are known as Site 1-selective (C-2 and C-8 analogs) and Site 2-selective (C-6 analogs), respectively. Such site-selective cAMP analogs at micromolar concentrations exerted potent growth inhibition, with no sign of toxic effects, in a spectrum of human cancer cell lines, including those that are resistant to previously tested cAMP analogs, leukemia lines, v-*ras*-H oncogene-transformed NIH/3T3 cells, and v-$ras^{Ki}$ oncogene-or TGFα-transformed normal rat kidney (NRK) cells; the combination of a C-8 with a C-6 analog showed synergistic effects. The growth inhibition was not due to a block in a specific phase of cell cycle but paralleled selective modulation of type I versus type II protein kinase isozymes, suppression of cellular proto-oncogene expression, inhibition of TGFα production, phenotypic change, and differentiation (leukemic cells). It is suggested that the site-selective cAMP analogs, substituting for endogenous cAMP and binding to protein kinase, suppress the cancer cell growth not via directly inhibiting cell division but rather by promoting cell differentiation. Site-selective cAMP analogs thus provide new biological tools for investigating cell proliferation and differentiation and also for the improved management of human cancer.

8-Cl-cAMP is now in preclinical phase I studies at the National Cancer Institute. A U.S. patent for the cAMP analogs, entitled "Derivatives of cyclic AMP as treatment for cancer," was applied for on May 25, 1988.

Mechanism of cAMP Action in Growth Control and Differentiation: Gene Regulation. In prokaryotes, cAMP is known to regulate the transcription of specific genes by a well-defined mechanism. cAMP binds to the prokaryotic cAMP binding protein CRP (cAMP receptor protein)/CAP (catabolite gene activator protein), and this complex interacts with specific DNA sequences at the 5' ends of targeted genes, modulating the ability of RNA polymerase to bind to the promoters. In mammalian cells, gene expression is altered in response to stimulation by hormones. Stimuli whose action is mediated through adenylate cyclase and cAMP-dependent protein kinase influence the transcriptional efficiency of several genes through the binding of specific factors to distinct *cis*-acting DNA sequences, CRE (cAMP responsive element), located in the 5' noncoding (promoter) region. Recently, a

region containing the CRE has been shown to bind specific nuclear proteins from various tissues. Recent evidence suggests that transcriptional regulation of eukaryotic genes by cAMP requires a cAMP-dependent protein kinase. In this study, we examined the role of cAMP-dependent protein kinase in gene transcription. Since cAMP-dependent protein kinase is exclusively present in the cytoplasm, any nuclear function of the protein kinase must be accompanied by its translocation to the nucleus. It was found that the *ras* gene suppression, growth inhibition, differentiation (leukemic cells), and phenotypic reversion of Ha-MuSV-transformed NIH/3T3 cells by site-selective cAMP analogs correlated with the nuclear translocation of the RII cAMP receptor protein, the regulatory subunit of protein kinase type II, which was detected within 10 min after the analog treatment. Thus, the nuclear translocation of the RII cAMP receptor protein is an early event in the cAMP regulation of growth and differentiation. The goal of this study is to provide direct evidence of interaction between the cAMP consensus sequences and cAMP receptor protein (RII) and/or a phosphoprotein substrate of protein kinase.

## ONCOGENETICS SECTION (R. Callahan, Chief)

Mammary Tumorigenesis in Inbred and Feral Mice. The study of experimentally induced mammary tumors has focused primarily on several mouse strains that are infected with mouse mammary tumor virus (MMTV) and have been inbred for a high incidence of mammary tumors. We have expanded this study to include other species of MMTV-infected feral mouse strains that have not been bred for high mammary tumor incidence. MMTV appears to induce mammary tumors by acting as an insertional mutagen that leads to the activation of previously silent cellular genes (*int* genes). We have previously shown that the inbreeding program used to develop the high-incidence mouse strains may also have fixed other cellular mutations that affect the frequency with which *int*-1 and *int*-2 are activated. The *int*-1 and *int*-2 genes are much less frequently activated in feral mouse mammary tumors, raising the possibility that other *int* loci are active in these tumors. We described one such locus, designated *int*-3, in CZECHII $V^+$ feral mice. The organization of *int*-3 differs from that of *int*-1 or *int*-2. MMTV insertions occur within a 500-bp region of the cellular genome on chromosome 17. Viral insertion at this locus leads to the activation of the expression of two cellular RNA species that correspond to host flanking sequences located 5' and 3' of the viral insertion site, respectively. We are currently determining the nucleotide sequence of recombinant cDNA clones corresponding to each of these RNA species. Mice chronically infected with MMTV frequently contain preneoplastic hyperplastic alveolar nodules (HAN), which can be transplanted as hyperplastic outgrowth (HOG) lines. We have found that in three of four CZECHII HOG lines, *int*-1 is occupied by an MMTV genome. This suggests that activation of *int*-1 is an early event in MMTV-induced mammary tumorigenesis. Human breast tumors frequently express tumor-associated antigens. One of these, the carcinoembryonic antigen (CEA) is a member of the family of related glycoproteins that are expressed normally in fetal tissue, the placenta, and other adult tissues. We have found that MMTV-induced CZECHII $V^+$ mammary tumors express a 4.0-kb RNA species that is related to human CEA.

Biological Effect(s) of Oncogenetic Elements on Mammary Cell Growth and Senescence. A number of genes have been implicated in the process of malignant transformation in the mammary glands of experimental animals and humans. The mouse mammary gland provides an excellent experimental model within which to elucidate the function of these putative mammary oncogenes during the growth, development, and differentiation of mammary tissue *in vivo*. Any portion of the adult mouse mammary gland at any age and throughout all stages of differentiation can repopulate the gland-free mammary fat pad of syngeneic mice upon transplantation. Similar results are obtained when dissociated glandular epithelium is injected into the fat pad or alternatively grown in culture under prescribed conditions prior to reintroduction into the mammary fat pad. We have initiated experiments designed to evaluate the feasibility of introducing functional genetic elements into mouse mammary cells with recombinant retroviral expression vectors antecedent to the reintroduction of these cells into a gland-free fat

pad. Paramount to the success of such a project is the maintenance of the "repopulation potential" of the mammary epithelial cells. Our studies and those of others indicate that the number of cells capable of repopulation of the fat pad is small initially and the ability to repopulate is gradually lost through mitotic events. We have morphologically identified a potential candidate for these "mammagenic" cells *in situ* and in explant cultures. These cells seem to differentially express certain cytokeratins during growth and expansion of the mammary gland *in vivo*, suggesting that specific keratin expression could serve as a marker for these "stem" cells. In addition, primary mammary cell cultures are being evaluated for the efficiency of retroviral gene transfer, the level of recombinant *trans*-gene expression in the infected cultures, and the stability of recombinant retroviral gene expression in "mammagenic cell populations" subsequent to repopulation of the gland-free mammary fat pads. We have developed a number of partially transformed preneoplastic mammary outgrowth lines which are carried serially *in vivo* by transplantation into gland-free mammary fat pads. Cells from these lines have been grown *in vitro* and are capable of repopulating mammary fat pads subsequently. These cells are being tested for retroviral gene transfer studies.

The Identification and Characterization of Human Genes Associated with Neoplasia. The etiology of human breast cancer is thought to involve a complex interplay of genetic, hormonal, and dietary factors that are superimposed on the physiological status of the host. Attempts to derive a cohesive picture of how these factors participate in the etiology of breast cancer have been confounded by a lack of information on specific mutations associated with the initiation and progression of the disease. We have undertaken a strategy that is aimed at determining, on a molecular level, those genetic alterations in primary breast tumor DNA that have a statistically significant association of patient history, tumor characteristics, and patient postsurgical prognosis. The *int*-2 gene is frequently activated in mouse mammary tumors by the mouse mammary tumor virus (MMTV) *via* insertional mutagenesis. We previously found that *int*-2 is amplified 2- to 15-fold in 14% of primary human breast tumor DNAs (n = 118) and has a significant association ($p < 2 \times 10^{-6}$) with a poor prognosis for the patient. We have now defined the amplification unit on chromosome 11q13 and found that 17 of 18 tumors amplified for *int*-2 were also amplified for *hst* and *bcl*-1. The *hst* and *int*-2 genes are both related to basic fibroblast growth factor. The *bcl*-1 locus defines the translocation t(11;14) breakpoint in B-cell chronic lymphocytic leukemias. In a lymph node metastasis of a breast cancer patient, *int*-2 but not *hst* was expressed. In 10% of the primary breast tumor DNAs (n = 122) the c-*erb*B-2 gene was amplified. The c-*erb*B-2 gene is related to the gene encoding the epidermal growth factor receptor. However, in contrast to one published report, we found no significant association with patient postsurgical prognosis or other aspects of patient history and tumor characteristics. In other studies, we detected the expression of MMTV-related human RNA sequences in breast tumor-derived cell lines and have obtained recombinant cDNA clones of these sequences for further analysis.

Cloning of Anti-Tumor Antigen Immunoglobulin Genes (The Summary of these studies appears under the Experimental Oncology Section [below] for continuity.)

## CELLULAR AND MOLECULAR PHYSIOLOGY SECTION (H. Cooper, Chief)

Cytoskeletal Proteins in Oncogenic Transformation and Human Neoplasia. We are examining the relationship between neoplastic transformation and biochemical derangements of expression or utilization of tropomyosins (TMs). In NIH/3T3 fibroblasts transformed by a number of related and unrelated retroviral oncogenes, expression of the transformed phenotype was reproducibly correlated with suppression of synthesis of two specific TM isoforms ("muscle type"). Of all the proteins detectable on two-dimensional gel electrophoresis of whole-cell proteins, only the TMs behaved in this way. Treatment of rat fibroblasts with tumor growth factor-α (TGF-α), in addition to inducing the transformed phenotype, specifically suppressed synthesis of muscle-type TMs and inhibited their

utilization in the cytoskeleton, strengthening the connection between suppression of TM and expression of the transformed phenotype and adding support at the biochemical level for an autocrine pathway in oncogene action. The findings point out the close biochemical relationship between epidermal growth factor (EGF) receptor activation and microfilament organization. Tropomyosin expression in relation to oncogene expression and neoplasia is being studied in murine and human epithelial cell systems. In mouse mammary epithelial cells transformed by the Ha-*ras* oncogene, the ratio of TM to actin accumulating in the cytoskeleton was reduced, indicating the production of microfilaments deficient in TM. The TM isoforms expressed by normal diploid human mammary epithelial cells have been compared with the pattern expressed by breast carcinoma cell lines. Defects in expression of particular TM isoforms occurred in all of the breast carcinoma cell lines studied, suggesting a possible role of deranged TM expression in human neoplasia.

Studies on the Nature and Function of the Phosphoprotein, Prosolin. We have extensively characterized prosolin, a major cytosolic protein of proliferating HL-60 promyelocytic leukemia cells that undergoes very rapid phosphorylation in response to treatment of the cells with tumor-promoting phorbol esters like TPA as an early step in a pathway leading to cessation of cell growth and onset of monocytoid differentiation. Prosolin has been identified in proliferating human peripheral blood lymphocytes (PBL) by tryptic peptide mapping of proteins isolated from two-dimensional electrophoretic gels. While virtually absent from resting PBL, prosolin expression is induced by mitogens as a late event, during the onset of S-phase. Its level of synthesis correlates with the prevalent level of DNA synthesis. Prosolin undergoes rapid phosphorylation in proliferating PBL in response to TPA or A23187 treatment, together with an inhibition of DNA synthesis. Phosphorylation of prosolin may be part of the mechanism by which naturally occurring growth inhibitory factors terminate normal lymphocyte proliferation. T-cell leukemia cell lines show a marked reduction in the phosphorylation of prosolin in response to A23187 and other agents. We are cloning and sequencing cDNAs to prosolin. Partial amino acid sequence data were obtained by analysis of tryptic peptides of prosolin isolated from two-dimensional electrophoretic gels. Degenerate oligonucleotide probes were synthesized and used to probe a lymphoblast cDNA library.

## ENDOCRINOLOGY WORKING GROUP (B. Vonderhaar)

Hormones and Growth Factors in Development of Mammary Glands and Tumorigenesis. The mammary gland is a complex organ whose growth and development 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, thyroid hormone, and estrogens with recent work also examining how epidermal growth factor (EGF), and EGF-like growth factors are affected by the interplay of these three classical hormones. In addition we have explored the relationship of membrane associated antiestrogen binding sites to the lactogenic hormone receptor and the action of platelet derived growth factor (PDGF) on human breast cancer cell growth in culture. T47D cells have specific PDGF receptors and respond to its growth promoting signal by inducing phosphorylation of a 65Kd protein in the calelectrin family. Lobulo-alveolar development of the mammary gland requires the priming action of both estrogen and progesterone to induce EGF receptors and production of EGF-like growth factors. In concert with insulin, prolactin and glucocorticoids, EGF or $\alpha$-TGF can promote full lobulo-alveolar development *in vitro*. This effect is inhibited by $\beta$-TGF. The primed mammary gland is more sensitive to $\alpha$-TGF than to EGF. Growth promotion of MCF-7 human breast cancer cells by prolactin can occur in the complete absence of estrogens. Prolactin induced growth of the estrogen receptor negative Nb2 rat lymphoma cell can be blocked by non-steroidal antiestrogens such as tamoxifen. This action is through

the membranous antiestrogen binding site (AEBS) which may be intimately associated with the prolactin receptor. The antiprolactin action of tamoxifen, working through the AEBS, may have important clinical implications.

EXPERIMENTAL ONCOLOGY SECTION (J. Schlom, Chief)

The studies being conducted within the Experimental Oncology Section can be divided into 8 major areas as detailed below. It should be emphasized that there is a great deal of cooperation and interaction among senior scientists involved in many of these projects.

Monoclonal Antibodies Define Carcinoma-Associated and Differentiation Antigens. These studies involve the generation, characterization, and utilization of monoclonal antibodies (MAbs) directed against antigens associated with human carcinoma. These MAbs are being used to better understand the cell biology and pathogenesis of several human carcinomas and to provide reagents that may be useful in several areas of the management of human carcinoma. These include *in vitro* diagnosis via serum assays and/or immunohistopathology, *in vivo* diagnosis such as gamma scanning, and potentially therapy. The MAbs generated thus far can be classified into three groups based on the expression of the detected antigens. These are (a) antigens differentially expressed in human carcinoma *versus* normal adult tissues, such as the pancarcinoma tumor-associated glycoprotein (TAG)-72 which is detected by MAb B72.3, and carcinoembryonic antigen (CEA) which is detected by MAbs COL-1 through COL-15; (b) tissue-associated antigens, such as the breast-associated antigen detected by MAb DF3, and the colon-associated antigens detected by MAbs D612 and CAA; and (c) oncogene- or retroviral-related gene products, such as *int*-2.

Since MAb B72.3 has been shown to selectively target a range of carcinomas in clinical trials involving several hundred patients, studies were undertaken to characterize a series of "second generation" MAbs to the TAG-72 antigen. Initial studies indicate that some of these second generation CC MAbs, such as CC83 and CC49, have a higher affinity constant for TAG-72 than B72.3, and may be better suited than B72.3 for some clinical applications. A serologic map of TAG-72 has been constructed with 19 anti-TAG-72 MAbs. Studies have also been conducted in the analysis of the COL MAb series and MAb D612 to define which MAbs are best suited for *in vivo* clinical applications. Major emphasis is being placed on the generation and analysis of recombinant/chimeric (rec/chi) MAbs to TAG-72 and anti-CEA MAbs and D612 and modified constructs of these rec/chi immunoglobulins. These rec/chi constructs and modified forms may be better suited for diagnostic and therapeutic applications.

Characterization of Human Tumor-Associated Antigens. Monoclonal antibody B72.3, which has been extensively characterized as to reactivity with a variety of carcinomas versus normal tissues, reacts with a mucin-like glycoprotein. The B72.3-reactive antigen, designated TAG (tumor-associated glycoprotein) -72, was partially purified and used as immunogen to produce 18 second generation anti-TAG-72 antibodies. Based on competitive binding studies, a number of the epitopes recognized by the anti-TAG-72 antibodies appear to be structurally or spatially related, but none appear to be identical. All of the anti-TAG-72 antibodies produced immune precipitate lines with purified antigen in double immunodiffusion assays, indicating that each epitope recognized by the antibodies is expressed in multiple copies on the TAG-72 molecule. All of the epitopes are sensitive to mild periodate oxidation but differ in their sensitivities to neuraminidase, and all of the epitopes have been shown to be expressed on the same molecule or population of molecules. One of the second generation antibodies was selected to develop procedures to purify preparative amounts of TAG-72 from xenografted human colon carcinoma cells and from malignant effusions. TAG-72, extracted from xenografted human colon carcinoma cells, was subjected to antibody affinity chromatography, size exclusion chromatography, and ion exchange chromatography. A double determinant radioimmunoassay revealed a greater than 1000-fold purification with a greater than 10% yield. SDS-PAGE analysis of radiolabeled purified TAG-72 revealed a polydisperse, but apparently homogenous,

high molecular weight glycoprotein. The density of purified TAG-72, as determined by equilibrium centrifugation in a CsCl gradient, was 1.45 g/ml, indicating a high degree of glycosylation. Compositional analysis of the purified TAG-72 revealed amino acid and carbohydrate profiles and content highly similar to that reported for other purified mucins. TAG-72 purified from malignant effusions was shown to have properties highly similar to the TAG-72 purified from the colon carcinoma xenografts. The colon carcinoma-associated antigen recognized by monoclonal antibody D612 has been shown to be a 48 kilodalton glycoprotein, consisting of a 42 kilodalton polypeptide and three N-linked oligosaccharides. The D612 antigen has also been shown to be structurally and antigenically unrelated to the colon carcinoma-associated antigens defined by monoclonal antibodies CO17-1A, GA733, KS1/4, etc.

Augmentation of Tumor Antigen Expression. Experimental data have shown that recombinant human interferons can increase the level of expression of human tumor-associated antigens. Using a large number of colorectal carcinoma cell lines, both Type I (i.e., IFN-$\alpha$ and IFN-$\beta$) and Type II (i.e., IFN-$\gamma$) can enhance the cell surface expression level of CEA and related antigens as measured by monoclonal antibody binding. Complementary DNA probes of CEA and related antigens revealed that the increase was a result of alterations at the transcriptional and/or post-transcriptional level, not changes in the methylated state of the CEA gene(s). The level of expression of another human tumor antigen, tumor-associated glycoprotein-72 (TAG-72), was also found to be regulated by interferon. TAG-72, a high molecular weight mucin, is not found constitutively expressed on most adherent human carcinoma cell lines and, furthermore, its expression cannot be *de novo* induced by interferon. Serous effusions isolated from patients diagnosed with adenocarcinomas were found to constitutively express TAG-72. We have isolated carcinoma cells from >100 serous effusions, incubated them in the presence of Type I and II interferon, and found that level of TAG-72 expression could be enhanced (i.e., >50%) in >80% of the samples. As a result of the preclinical studies, a phase I clinical trial was designed to investigate whether IFN-$\gamma$ could augment tumor antigen expression in patients. Eight patients with adenocarcinoma and secondary ascites were given i.p. escalating doses (i.e., 0.1-100 MU) of IFN-$\gamma$ for eight weeks. Tumor cells isolated from the ascites of seven of the eight patients constitutively expressed TAG-72 as measured in immunocytochemistry and flow cytometry using B72.3. In all seven patients, i.p. administration of IFN-$\gamma$ resulted in a discernable increase in the level of B72.3 binding. The findings present the initial observation that recombinant human interferon may serve as an adjunct by enhancing cell surface tumor antigen expression, thereby augmenting the localization of a monoclonal antibody.

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 180,000-kd glycoprotein and TAG-72 is a high-molecular-weight mucin. Recent studies have demonstrated that CEA belongs to a multigene family related to the immunoglobulin supergene family. These include CEA, normal cross-reacting antigen (NCA), biliary glycoprotein (BGP), and human pregnancy-specific beta 1-glycoprotein (SP1). A 550-base pair CEA probe derived from cloned complementary DNA was used to carry out Southern analysis of the DNA isolated from normal and colon tumor cell lines. At high stringency, the CEA probe detected seven *Bam*HI fragments in all DNAs analyzed. Results of the Southern analysis demonstrate no amplification or rearrangement of the CEA genes in tumor cells. Methylation-sensitive restriction endonucleases, *Hpa*II and *Hha*I, were used to compare the degrees of methylation of the CEA family of genes in normal and colon tumor cell lines. The results demonstrate that the CEA family of genes exists in a state of hypermethylation in normal cells. In contrast, the CEA gene(s) are relatively hypomethylated in the tumor cell lines, suggesting a correlation between the state of methylation and degree of expression of the CEA gene(s). Using a CEA cDNA probe, we have investigated the phenomenon of up-regulation of CEA expression in human tumor cell lines by recombinant human

gamma-interferon (Hu-IFN-γ). An increase in MAb binding was shown to be accompanied by a 6- to 11-fold increase in the steady-state levels of three CEA transcripts with sizes of 4.2, 3.5, and 2.8 kb. Studies are also in progress to identify and characterize the gene(s) encoding the TAG-72 mucin antigen. A number of mucin genes representing several tissue types have been cloned. These include the genes encoding the epitopes for breast cancer, HMFG2, $DF_3$ and $H_{23}$ and the pancreatic antigen DuPAN-2. Nucleic acid sequence analysis of these isolated genes has shown them to be identical and that the difference resides in the sugar epitopes detected by the MAb. The dominant feature of the breast and pancreatic mucin clones was an extremely GC-rich, 60-bp tandem repeat sequence that encoded a repeating peptide unit rich in serine, threonine, and proline.

Antibody Directed Cellular Immunotherapy of Colon Cancer. This project is examining the role that monoclonal antibodies (MAb) against human colonic tumor antigens have in directing and augmenting the cytotoxic activity of NK/LAK cells and T cells. A MAb designated D612 (IgG2a) has been generated that uniformly reacted with the cell surface of approximately 80% of primary or metastatic colorectal carcinomas. Normal tissue reactivity of D612 was confined mainly to the small and large intestine, while non-gastrointestinal normal tissues were routinely negative. D612 mediated antibody-dependent cellular cytotoxicity in conjunction with normal human peripheral blood mononuclear cells was increased by effector cell activation with interleukin 2 (IL-2). For retargeting of T cells, heteroantibody conjugates between the anti-colon tumor MAb, B38.1, and anti-CD3 (OKT-3) were chemically prepared, and these were tested along with effectors obtained from PBMNC stimulated with anti-CD3 and IL-2. The heteroantibody-mediated cellular cytotoxicity activity was similar on a per cell basis among effector populations prepared by stimulation with IL-2, anti-CD3, or a combination of these reagents. However, the total lytic activity of anti-CD3 + IL-2-stimulated cultures was much higher due to the greater expansion in cell number achieved by the combination treatment. These studies suggest that MAbs with restricted normal tissue reactivity and ability to direct the cellular cytotoxicity of a broader spectrum of killer lymphocytes activated by lymphokines and other agents might augment the efficacy of colon cancer immunotherapy.

Cloning of Anti-Tumor Antigen Immunoglobulin Genes (Part of Oncogenetics Section, LTIB). The main objective of this research project is to genetically engineer immunoglobulin genes to generate useful reagents for *in vivo* localization and therapy of human tumors. Several hybridoma cell lines have been developed in this laboratory that produce monoclonal antibodies (MAbs) that selectively react with tumor-associated antigens. These include the carcinoembryonic antigen and a tumor-associated glycoprotein, TAG-72, which is present in a variety of carcinomas. The MAbs are currently being used in a number of diagnostic and therapeutic trials on breast, colon, and ovarian cancers. In clinical tests, one such MAb, B72.3, which was raised against a membrane-enriched extract of a human metastatic breast carcinoma and recognizes TAG-72, has shown promise for being developed into a diagnostic and therapeutic agent. The usefulness of mouse MAbs for *in vivo* therapy and diagnosis of human tumors, however, is limited because of their immunogenicity in patients. This problem can be reduced by replacing the constant region of the mouse antibody with the constant region of the human antibody, using recombinant DNA techniques. To that end, we have cloned the rearranged heavy and 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. They were inserted separately into appropriate expression vectors. Stable chimeric antibody-producing cell lines were developed by introducing these constructs into both fibroblast and myeloma cell lines. Chimeric antibody produced either from cDNA clones or clones of the rearranged genes retained specificity and binding properties of the parental antibody.

Immunoassays for Human Carcinoma Associated Antigens and Localization and Therapy Using Labeled Monoclonal Antibodies: Model Systems. Immunoassays for the detection of circulating tumor associated antigens and assays for the support of

clinical trials have been developed. We are using these assays to examine sera and effusions from a variety of sources to study their utility to detect carcinoma. We used a second generation of MAbs against a tumor-associated antigen, TAG-72, in the development of a more efficient assay for TAG-72. This assay, designated CA 72-4 (using MAbs B72.3 and CC49, developed in collaboration with Centocor) was used to examine a panel of sera from patients with adenocarcinomas as well as benign diseases. Only 0.5% of 744 sera primarily obtained from blood donors had TAG-72 levels >10 U/ml. Similarly only 2.2% of 134 samples from patients with benign gastrointestinal diseases had TAG-72 levels >10 U/ml. Approximately 32% of 303 patients with gastrointestinal malignancies had serum TAG-72 >10 U/ml; approximately 44% of the patients with advanced disease had elevated TAG-72 levels. Patients with adenocarcinomas from a variety of other sites also had levels of TAG-72 above the reference value. The utility of the CA 72-4 assay was clearly demonstrated in the case of patients with gastric cancer; CEA was elevated in 37% of patients with Stage 3 and 4 disease, and 48% of the sera had elevated levels of TAG-72. No relationship was observed between the CEA and the CA 72-4 assays. Thus, the use of both assays to analyze these sera is clearly more advantageous than using the CEA or the CA 72-4 assay alone.

The interaction *in vivo* of TAG-72 with radiolabeled MAbs was also studied to aid in understanding the pharmacology of MAbs used as radiopharmaceuticals. We found immune complex formation, even in the earliest sera drawn, as detected both by HPLC and SDS-PAGE analysis. Three different profiles, dependent on the serum TAG-72 level, were seen in the HPLC patterns. The presence of immune complexes did not appear to alter the clearance pattern of the $^{131}$I-MAb.

MAb B72.3, a murine IgG1, has been shown to react with a high molecular weight glycoprotein (designated TAG-72) found in many epithelial-derived cancers, with minimal reactivity to normal adult tissues. B72.3 was therefore chosen as an antibody that could potentially be used for radioimmunodetection (RID) and radioimmunotherapy (RIT) of human carcinomas and is being evaluated in model systems using the LS-174T human colon carcinoma xenograft to determine the feasibility of these studies. A number of new chelate-conjugates have been developed enabling the binding of radiometals to proteins. These new constructs have been covalently linked to B72.3 IgG to determine which resulted in the best biodistribution after labeling with In-111 and Y-88 (used as a substitute for Y-90). The 1-(p-isothiocyanatobenzyl)diethylenetriaminepentaacetic acid (SCN-Bz-DTPA) chelate-conjugate, gave the best biodistribution after labeling with either In-111 or Y-88. The biodistribution of the Y-88 and In-111 labeled MAbs differed significantly, illustrating the difficulties in using In-111-labeled MAbs to predict the distribution and dosimetry of Y-90-labeled MAbs.

New anti-TAG-72 MAbs (designated CC) have been developed and compared to B72.3 in the LS-174T model system. All exhibited higher %ID/gm and tumor:tissue ratios than B72.3; differences in the pharmacokinetics were noted among the CC MAbs.

A recombinant/chimeric form of B72.3 has been developed using the variable regions of the murine B72.3 and human heavy chain ($\gamma$4) and light chain ($\kappa$) constant regions. The cB72.3($\gamma$4) IgG was radiolabeled and the biodistribution was studied in athymic mice bearing LS-174T xenografts. Small differences were observed between the cB72.3($\gamma$4) and the native B72.3 in the %ID/gm that localized in the tumor, this is mostly likely due its more rapid clearance from the blood and body of the mouse of the cB72.3($\gamma$4) as compared to the native B72.3.

Anti-Carcinoma Monoclonal Antibodies Clinical Trials. This project involves the use and the proposed use of several monoclonal antibodies (MAbs) in both diagnostic and therapeutic clinical trials. Thus far, all our collaborative clinical trials have been conducted with MAb B72.3. This MAb has been previously shown to have differential reactivity for a range of human carcinomas as compared to most normal adult tissues. To date, over 1,000 patients have been administered radiolabeled B72.3 in tumor-targeting studies carried out in several

institutions, with similar findings of approximately 70-80% tumor targeting observed.

We first investigated the administration of radiolabeled MAb B72.3 IgG in colorectal cancer patients. The selective localization of $^{131}$I-MAb B72.3 IgG was demonstrated in biodistribution studies in which the percentage of injected dose of MAb per gram of each tumor was compared with that of the normal tissues, thus providing a relative radiolocalization index (RI) for each lesion. Of the tumor lesions, 70% had an RI of at least 3 (i.e., 3 times greater uptake per gram than normal tissues). We have also conducted studies to determine the feasibility of intraperitoneal administration of radiolabeled B72.3 for tumor localization (via both gamma scanning and direct analyses of biopsy specimens). Several patients studied were negative for tumor detection by both CT scan and X-ray but were positive for tumor localization via gamma scanning. Direct analyses of biopsy specimens of carcinoma and normal tissues demonstrated ratios of up to 70:1 for tumor MAb localization versus normal tissues.

A phase I therapy trial involving intraperitoneal administration of $^{131}$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 other MAb-isotope conjugates are planned.

Parameters Involved in the Tumor Targeting of Monoclonal Antibodies. The overall goal of this project is the delineation of parameters involved in *in vivo* targeting of monoclonal antibodies. These studies will involve determination of the optimal route of inoculation of antibody as well as the antibody form. Major emphasis will be placed on the pharmacokinetics of plasma clearance of recombinant/chimeric monoclonal antibodies and their ability to target tumors.

One approach to the treatment of patients with peritoneal carcinoma may be direct infusion into the peritoneal cavity of radiolabeled antibody conjugates. In comparison to intravenous MAb administration, this method may improve the percent of injected MAb dose per gram of tumor and may lead to less radiolabeled antibody in the bone marrow and therefore less toxicity to the patient. The second major goal of this project is to develop an *in vivo* model system to study immunotherapy of human carcinoma in the peritoneal cavity. These studies will include (a) determination of the animal species eliciting the pattern most comparable to that observed in the human of MAb clearance from the blood, (b) development of a human peritoneal carcinoma xenograft in an animal model, and (c) determination of the utility of intraperitoneally (i.p.) administered MAb conjugates for carcinoma therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 05190-10 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Monoclonal Antibodies Define Carcinoma-Associated and Differentiation Antigens

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

| | | |
|---|---|---|
| Jeffrey Schlom | Chief | LTIB, DCBDC, NCI |
| Patricia Horan-Hand | Chemist | LTIB, DCBDC, NCI |
| Alfredo Molinolo | Visiting Fellow | LTIB, DCBDC, NCI |
| David Colcher | Microbiologist | LTIB, DCBDC, NCI |

COOPERATING UNITS *(if any)* D. Kufe, Dana Farber Cancer Center; R. Muraro and P. Natali, Istituto Regina Elena, Rome

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 |
|---|---|---|
| 5.7 | 1.4 | 4.3 |

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.)*

These studies involve the generation, characterization, and utilization of monoclonal antibodies (MAbs) directed against antigens associated with human carcinoma. These MAbs are being used to better understand the cell biology and pathogenesis of several human carcinomas and to provide reagents that may be useful in several areas of the management of human carcinoma. These include *in vitro* diagnosis via serum assays and/or immunohistopathology, *in vivo* diagnosis such as gamma scanning, and potentially therapy. The MAbs generated thus far can be classified into three groups based on the expression of the detected antigens. These are (a) antigens differentially expressed in human carcinoma *versus* normal adult tissues, such as the pancarcinoma tumor-associated glycoprotein (TAG)-72 which is detected by MAb B72.3, and carcinoembryonic antigen (CEA) which is detected by MAbs COL-1 through COL-15; (b) tissue-associated antigens, such as the breast-associated antigen detected by MAb DF3, and the colon-associated antigens detected by MAbs D612 and CAA; and (c) oncogene- or retroviral-related gene products, such as *int*-2.

Since MAb B72.3 has been shown to selectively target a range of carcinomas in clinical trials involving several hundred patients, studies were undertaken to characterize a series of "second generation" MAbs to the TAG-72 antigen. Initial studies indicate that some of these second generation CC MAbs, such as CC83 and CC49, have a higher affinity constant for TAG-72 than B72.3, and may be better suited than B72.3 for some clinical applications. A serologic map of TAG-72 has been constructed with 19 anti-TAG-72 MAbs. Studies have also been conducted in the analysis of the COL MAb series and MAb D612 to define which MAbs are best suited for *in vivo* clinical applications. Major emphasis is being placed on the generation and analysis of recombinant/chimeric (rec/chi) MAbs to TAG-72 and anti-CEA MAbs and D612 and modified constructs of these rec/chi immunoglobulins. These rec/chi constructs and modified forms may be better suited for diagnostic and therapeutic applications.





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*Major Findings*

In view of the potential clinical applications of monoclonal antibody (MAb) B72.3 and our desire to further characterize the tumor-associated glycoprotein (TAG)-72 antigen, the decision was made to prepare "second generation" anti-TAG-72 MAbs. Because the source of purified TAG-72 used as immunogen was from a human colon cancer (CC) xenograft, MAbs were given a CC designation. Mice were immunized with purified TAG-72, and the resultant 2567 fusion cultures were assayed using solid-phase radioimmunoassays (RIAs) for reactivity to breast and colon cancer biopsies and for lack of reactivity to extracts of the following normal tissues: kidney, bone marrow, lung, liver, colon, stomach, thyroid, polymorphonuclear leukocytes, and erythrocytes. Ninety-nine cultures had the desired properties; these were cloned twice and assayed further. This process resulted in the selection of 28 hybridomas given a CC designation. These 28 CC MAbs were further tested in RIAs and shown to fall into several groups on the basis of differential reactivities to several carcinomas. The CC MAbs were also shown to be nonreactive to carcinoembryonic antigen (CEA) and to biopsy extracts of 32 normal human tissues. All 28 CC MAbs were tested using immunohistochemical analyses and shown to be reactive with formalin-fixed tissues (actually a consequence of a previous selection procedure). The 28 CC MAbs were positive to carcinoma sections and negative to the vast majority of cell types of a wide variety of normal tissues. In general, all the CC MAbs had patterns of reactivity for normal tissues very similar or identical to that shown and previously described for B72.3. The normal human adult tissue that showed strong staining with the CC MAbs (CC49 and CC83) and with B72.3 was secretory endometrium; proliferating endometrium and resting endometrium of postmenopausal women, however, were negative.

Nine CC MAbs were chosen for further study. These were CC11, 15, 29, 30, 40, 46, 49, 83, and 92. These MAbs were analyzed by Western blotting for reactivity to purified TAG-72 and showed the same pattern of reactivity as B72.3, recognizing a high-molecular-weight species of $M_r > 10^6$. The nine CC MAbs and B72.3 were analyzed as purified IgG preparations in several liquid competition RIAs to better define distinctions between binding sites of these MAbs. MAbs CC11, 15, 29, 30, 40, 46, 49, 83, and 92 could all be distinguished from one another. The affinity constants of the purified CC IgGs for binding to the TAG-72 antigen were determined; all CC MAbs chosen for further study had higher affinity constants than B72.3.

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.

Studies were conducted to further characterize the range of reactivities of the anti-CEA COL MAbs. Fifteen competition RIAs, including the 14 COL-MAbs and 1 MAb reactive with NCA (a CEA related antigen), were developed and 15 purified competitor Igs were employed in each assay. Consequently, the 14 COL-MAbs were able to be categorized into six groups according to cross-competition patterns. The chemical nature of the epitopes identified by the COL-MAbs was also tested in solid-phase RIAs or Western blotting using chemically or enzymatically treated CEA. The results demonstrated that the epitopes identified by several of the

COL-MAbs are not identical to each other. In an attempt to define which of the COL-MAbs are best suited for clinical applications, titration experiments were performed to define differential reactivities to colorectal carcinoma *versus* NCA-containing granulocyte extracts; these results were compared with results obtained using several anti-CEA MAbs that had been previously employed in clinical trials. MAbs COL-1, COL-4, COL-6, and COL-8 demonstrated a greater than $10^5$-fold differential for binding to a colon cancer *versus* granulocytes. These serological mapping and biochemical studies provide information as to the range and nature of the epitopes on the CEA molecule recognized by the COL-MAbs and provide additional evidence to form the basis for selecting the anti-CEA MAbs for use in potential clinical applications, either alone or in combination with other anti-CEA MAbs and MAbs to other carcinoma-associated antigens.

We have recently generated and characterized a MAb, D612, with selective reactivity for malignant and normal gastrointestinal epithelium. MAb D612, a murine $IgG_{2a}$, was generated using a membrane-enriched fraction of a human colon carcinoma biopsy as immunogen. Employing RIAs of biopsy extracts to a range of normal and neoplastic tissues and both immunofluorescence and immunoperoxidase assays on frozen sections of a range of normal and neoplastic tissues, we have shown that MAb D612 binds to 82% of the colorectal carcinomas tested ($n$ = 67) and to normal gastrointestinal epithelium but does not bind similarly to either neoplastic or normal tissues from a wide range of other sites. Western blotting has shown MAb D612 to react with a high-molecular-weight antigen. Live cell RIAs and fluorescence-activated cell sorter analyses demonstrated that the reactive epitope is present on the surface of 100% of the colon carcinoma cells tested. Immunohistochemical studies have shown intense membrane staining of colon adenocarcinomas; the vast majority of both primary and metastatic colon adenocarcinomas from a variety of sites were positive, with many lesions showing homogeneous staining of virtually all cells present. Using human effector cells, MAb D612 has been shown to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) of human colon carcinoma cells. Moreover, this activity was enhanced in the presence of interleukin (IL)-2. Radiolabeled D612-purified IgG was also shown to target a human colon carcinoma xenograft *in situ*. The pattern of membrane-associated staining, the molecular weight of the reactive antigen, the $IgG_{2a}$ isotype, the ability to mediate ADCC in the presence of IL-2, and the immunohistochemical and RIA studies demonstrating highly restricted reactivity to malignant and normal gastrointestinal tissue distinguish MAb D612 from other MAbs thus far described.

One of our major efforts during the next few years will focus on the construction, expression, characterization, purification, and utilization of recombinant/chimeric (rec/chi) MAbs. Initial focus will be on various constructs of B72.3, but these will be extended to the anti-TAG-72 MAbs CC49 and CC83, the anti-CEA MAb COL-1, and MAb D612. These studies involve (a) methods of optimizing stability of cultures and Ig expression, (b) assays of the various rec/chi constructs, (c) *in vitro* binding properties of the various constructs, and (d) analyses of the rec/chi MAbs for experimental *in vivo* tumor targeting and potential clinical trials. We have recently generated and characterized a rec/chi B72.3 of the human $IgG_4$ subtype. We have characterized this MAb with respect to binding properties to a range of normal human tissues and tumor tissues, and these studies indicate that the rec, rec/chi, and native B72.3 have virtually indistinguishable binding properties. We have also used an anti-idiotype MAb prepared against B72.3 to show identical binding to native and rec/chi B72.3. Studies are in progress to prepare modified rec/chi constructs that may be better suited than native B72.3 for clinical applications.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 05233-09 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Immunoassays for Human Carcinoma-Associated Antigens and Clinical Studies

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

| | | |
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| Patrizia Ferroni | Visiting Fellow | LTIB, DCBDC, NCI |
| Jeffrey Schlom | Chief | LTIB, DCBDC, NCI |

COOPERATING UNITS (if any) Dr. A. Raubitschek, ROB, DCT, NCI; Drs. J. Carrasquillo, J. Reynolds, Div. Nucl. Med., NIH; Drs. C. Szpak, W. Johnston, Dept. Path., Duke U. School of Med.; Dr. N. Ohuchi, Dept. Surg. Tohoku U. School of Med.; Dr. P. Kaplan, Centocor, Inc.

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS | PROFESSIONAL: | OTHER |
|---|---|---|
| 2.5 | 1.6 | .9 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

Immunoassays for the detection of circulating tumor associated antigens and assays for the support of clinical trials have been developed. We are using these assays to examine sera and effusions from a variety of sources to study their utility to detect carcinoma. We used a second generation of MAbs against a tumor-associated antigen, TAG-72, in the development of a more efficient assay for TAG-72. This assay, designated CA 72-4 (using MAbs B72.3 and CC49, developed in collaboration with Centocor) was used to examine a panel of sera from patients with adenocarcinomas as well as benign diseases. Only 0.5% of 744 sera primarily obtained from blood donors had TAG-72 levels >10 U/ml. Similarly only 2.2% of 134 samples from patients with benign gastrointestinal diseases had TAG-72 levels >10 U/ml. Approximately 32% of 303 patients with gastrointestinal malignancies had serum TAG-72 >10 U/ml; approximately 44% of the patients with advanced disease had elevated TAG-72 levels. Patients with adenocarcinomas from a variety of other sites also had levels of TAG-72 above the reference value. The utility of the CA 72-4 assay was clearly demonstrated in the case of patients with gastric cancer; CEA was elevated in 37% of patients with Stage 3 and 4 disease, and 48% of the sera had elevated levels of TAG-72. No relationship was observed between the CEA and the CA 72-4 assays. Thus, the use of both assays to analyze these sera is clearly more advantageous than using the CEA or the CA 72-4 assay alone.

The interaction *in vivo* of TAG-72 with radiolabeled MAbs was also studied to aid in understanding the pharmacology of MAbs used as radiopharmaceuticals. We found immune complex formation, even in the earliest sera drawn, as detected both by HPLC and SDS-PAGE analysis. Three different profiles, dependent on the serum TAG-72 level, were seen in the HPLC patterns. The presence of immune complexes did not appear to alter the clearance pattern of the $^{131}$I-MAb.

*Major Findings*

The development of immunoassays for the detection of circulating tumor associated antigens and the study of the interaction *in vivo* of these antigens with radiolabeled MAbs may help in the diagnosis of cancer, the monitoring of the clinical course of the disease, as well as understanding the pharmacology of the MAbs used as radiopharmaceuticals.

MAb B72.3, a murine $IgG_1$, has been developed in this laboratory and shown to react with a high molecular weight glycoprotein (designated TAG-72) found in many epithelial-derived cancers, including colon, ovarian, breast, lung, pancreatic and gastric malignancies. We have purified the TAG-72 antigen and prepared a second generation of anti-TAG-72 MAbs. One of these MAbs, designated CC49, was shown to react with an epitope on TAG-72 that could be distinguished from that recognized by B72.3. Moreover, while the $K_a$ of B72.3 was shown to be $2.5\times10^9M^{-1}$, that of CC49 was considerably higher at $16.2\times10^9M^{-1}$. These findings led to the development of a more efficient assay for TAG-72 using both MAb CC49 and B72.3; CC49 was used as the "catcher" and $^{125}$I-B72.3 as the detecting reagent. This combination enabled the development of a sequential assay that could be performed in less than one day which also minimized the "hook-back" effect and yielded the most reproducible recovery of TAG-72 in dilution studies. This is especially important in accurately quantitating TAG-72 levels in patient sera containing high levels of TAG-72.

The CA 72-4 assay was used to examine a panel of sera from patients with malignancies from a number of sites as well as benign diseases. Normal sera obtained primarily from blood donors was examined and only 4 of 744 samples (0.5%) tested had TAG-72 levels >10 U/ml. Similarly, only 3 of 134 samples (2.2%) of patients with benign gastrointestinal diseases had TAG-72 levels >10 U/ml. Whether the cut-off level of 10 U/ml is appropriate for use, or a different level such as 6 U/ml giving greater sensitivity or 20 U/ml giving greater specificity, will have to be further examined with much larger panels of sera from "normal individuals" and patients with benign diseases.

Approximately 40% of 303 patients with gastrointestinal malignancies had serum TAG-72 levels of >6 U/ml (approximately 55% of patients with advanced disease). Whether the expression of TAG-72 in the sera of patients correlates with stage of disease needs to be further investigated with a well characterized set of sera. Approximately 36% of patients with lung adenocarcinomas and 24% of patients with ovarian cancer also had elevated serum TAG-72 levels; 53% of patients with Stage IV ovarian cancer had elevated TAG-72 levels. The utility of the CA 72-4 assay to measure TAG-72 levels was clearly demonstrated in the case of patients with gastric cancer. These sera were also evaluated in a RIA for CEA where 37% of patients with Stage 3 and 4 disease had elevated levels of CEA. The RIA for TAG-72 was positive in 48% of the sera tested from patients with gastric carcinomas. No relationship was observed between elevated TAA levels of sera in the CEA assay and the CA 72-4 assay. Thus, the use of both assays to analyze these sera is clearly an advantage to using the CEA or the CA 72-4 assay alone.

Radiolabeled B72.3, intravenously or intraperitoneally administered, has been shown to localize primary and metastatic lesions of colorectal and ovarian patients as well as patients with other carcinomas. Intravenous administration to colorectal carcinoma patients of $^{131}$I-B72.3 IgG has resulted in localization of the MAb in approximately 75% of the metastatic lesions found at various body sites. We analyzed the serum of colorectal carcinoma patients who have received $^{131}$I-B72.3 IgG by intravenous administration. The presence of TAG-72, the pharmacokinetics of the radiolabeled MAb, the amount of circulating immunoreactive MAb B72.3 IgG, the status of the radionuclide and its association with the MAb were studied. The integrity of the radiolabeled B72.3 IgG in the serum and the formation of immune complexes were also analyzed. Plasma clearance of $^{131}$I-B72.3 IgG was determined for all patients and was similar in 25/27 patients; the mass of antibody injected had no effect on the rate of clearance of

the radiolabeled B72.3 IgG. The level of immunoreactive B72.3 IgG in patient sera was determined using a solid-phase radioimmunoassay. When the level of immunoreactive antibody was normalized to the sera drawn 5 min post-injection, it was found that clearance of the immunoreactive antibody was similar to the clearance of the radiolabeled MAb B72.3. This suggests that the radionuclide is remaining in association with MAb B72.3 following administration to patients. This is further supported by HPLC analysis and autoradiography of the SDS-polyacrylamide gels run on the patients' sera which demonstrated that the radioactivity remains in association with the IgG fraction. HPLC analysis of the sera revealed three different profile patterns. The majority of the patients were in the category in which the radiolabel eluted with a retention time consistent with that of IgG. In others, a small percentage of the radioactivity eluted in the void volume, while in a third group, a large proportion of the radioactivity was associated with the void volume. Elution of the $^{131}$I-B72.3 in the void volume suggests that there is the formation of immune complexes or aggregation of the MAb. The formation of this higher molecular weight entity correlates with elevated antigen levels in patient sera, suggesting that antigen-antibody complexes are forming. We found that immune complex formation occurred even in the earliest sera drawn. Furthermore, immune complex levels remained consistent in the sera drawn at various intervals. These complexes were detected both by HPLC and SDS-PAGE analysis. Interestingly, the presence of the immune complexes did not appear to alter the clearance pattern of the monoclonal antibody. It was of interest also that serum TAG-72 levels correlated with obtaining a positive scan. Serum TAG-72 levels may be a useful criteria for considering a patient for treatment with MAb B72.3.

*Publications*

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PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 08226-14 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

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Laboratory of Tumor Immunology and Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER |
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| 4.0 | 3.0 | 1.0 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The 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, thyroid hormone, and estrogens with recent work also examining how epidermal growth factor (EGF), and EGF-like growth factors are affected by the interplay of these three classical hormones. In addition we have explored the relationship of membrane associated antiestrogen binding sites to the lactogenic hormone receptor and the action of platelet derived growth factor (PDGF) on human breast cancer cell growth in culture. T47D cells have specific PDGF receptors and respond to its growth promoting signal by inducing phosphorylation of a 65Kd protein in the calelectrin family. Lobulo-alveolar development of the mammary gland requires the priming action of both estrogen and progesterone to induce EGF receptors and production of EGF-like growth factors. In concert with insulin, prolactin and glucocorticoids, EGF or α-TGF can promote full lobulo-alveolar development *in vitro*. This effect is inhibited by β-TGF. The primed mammary gland is more sensitive to α-TGF than to EGF. Growth promotion of MCF-7 human breast cancer cells by prolactin can occur in the complete absence of estrogens. Prolactin induced growth of the estrogen receptor negative Nb2 rat lymphoma cell can be blocked by non-steroidal antiestrogens such as tamoxifen. This action is through the membranous antiestrogen binding site (AEBS) which may be intimately associated with the prolactin receptor. The antiprolactin action of tamoxifen, working through the AEBS, may have important clinical implications.

*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 is also under the control of growth factors in the epidermal growth factor (EGF) family. Previously we had shown that the *in vitro* induction of lobulo-alveolar development in mouse mammary glands under the influence of the hormones I, Prl, A and H also required the presence of EGF in order to occur. We have now shown that α-transforming growth factor (α-TGF) is fully active in promoting lobulo-alveolar development *in vitro*. Full activity (>90% of the glands responding) was achieved with EGF at 60ng/ml but only 30% of the glands responded with 20ng EGF/ml. In contrast, α-TGF gave complete response at both high and low doses (60 and 6 ng EGF equivalents/ml). This greater sensitivity of the glands to α-TGF *in vitro* is in complete agreement with our previously published observations *in vivo* using growth factors locally implanted into mammary glands of developing mice. The development of glands *in vitro* is completely inhibited by 10 μm β-TGF.

Glandular development occurs *in vitro* only after priming with estrogen and progesterone (E/P). This priming increases the ability of the mammary tissue to bind the growth factor as well as induces the production of a mammary derived EGF-like growth factor. Estrogen (E) alone or progesterone (P) alone is not sufficient during the priming process nor can the addition of E and P to the culture medium overcome the need for priming *in vivo*. Priming E/P cannot be replaced by EGF priming (either using EGF directly or by elevating salivary gland production of EGF with testosterone). Injecting the animals with anti-EGF during the priming with E/P does not prevent the priming process. All of these data suggest that the role of E and P in priming is a complex one possibly involving positive as well as negative actions by these hormones. Future work will examine the role of each of these hormones in the induction of the growth factor receptors as well as the induction of the EGF-like growth factor using ovariectomized mice. The characteristics of binding of both EGF and 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.

Prolactin/thyroid hormone/estrogen interactions were explored at the level of differentiation as well as growth regulation. The action of Prl in milk protein production is at the transcriptional level. Physiological levels of $T_3$, in the presence of Prl, increase transcription and promote differential glycosylation of α-lactalbumin resulting in its increased secretion. Under the proper conditions of serum staging, Prl promotes growth of several human breast cancer cell lines including MCF-7, ZR-75-1 and T47D. The Prl effect occurs in the complete absence of estrogens (i.e., in phenol red free medium). Prl also promotes the growth of the Nb2 cells in the absence of estrogens. Serum free conditions for the growth

of the Nb2 cells have been developed which enhance the sensitivity of the cells to Prl. The Nb2 cell is estrogen receptor negative. The Nb2 cell does contain membrane associated antiestrogen binding sites (AEBS) which bind tamoxifen and related non-steroidal, triphenylethylene antiestrogens with an apparent Kd of 3.1 x $10^{-10}$ M. The AEBS do not bind estrogens. These sites are distinct from estrogen receptors. Antiestrogens act through the AEBS to inhibit the growth promoting effect of Prl on the Nb2 cells. Antiestrogens of the class which bind to the AEBS also inhibit the binding of Prl to its receptor. The order of affinities of the various antiestrogens for the AEBS parallels the order of their potencies as growth and lactogen binding inhibitors. They do not affect the binding of other ligands to their membrane associated receptors. Thus, in addition to their well defined antiestrogenic actions, these agents may be antilactogenic as well. Future work will center on assessing the AEBS in normal mammary glands and in the Prl responsive breast cancer cells (MCF-7, T47D and ZR-75-1) to determine if they (a) are present, (b) inhibit Prl binding to its receptor, and (c) block the action of Prl on milk protein synthesis (normal) and cell growth (breast cancer cells). We will continue the characterization of the Prl receptor isolated by affinity chromatography and immunopurification and determine what, if any, physical relationship exists between this receptor and the AEBS. The relationship of the prolactin receptor and AEBS 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 $\alpha$-IFN, $\beta$-TGF and hormones such as estrogen, progesterone and thyroid hormones.

*Publications*

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PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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Localization and Therapy Using Labeled Monoclonal Antibodies: Model Systems

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☐ (a1) Minors

☐ (a2) Interviews

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

MAb B72.3, a murine IgG1, has been shown to react with a high molecular weight glycoprotein (designated TAG-72) found in many epithelial-derived cancers, with minimal reactivity to normal adult tissues. B72.3 was therefore chosen as an antibody that could potentially be used for radioimmunodetection (RID) and radioimmunotherapy (RIT) of human carcinomas and is being evaluated in model systems using the LS-174T human colon carcinoma xenograft to determine the feasibility of these studies. A number of new chelate-conjugates have been developed enabling the binding of radiometals to proteins. These new constructs have been covalently linked to B72.3 IgG to determine which resulted in the best biodistribution after labeling with In-111 and Y-88 (used as a substitute for Y-90). The 1-(p-isothiocyanatobenzyl)diethylenetriamine-pentaacetic acid (SCN-Bz-DTPA) chelate-conjugate, gave the best biodistribution after labeling with either In-111 or Y-88. The biodistribution of the Y-88 and In-111 labeled MAbs differed significantly, illustrating the difficulties in using In-111-labeled MAbs to predict the distribution and dosimetry of Y-90-labeled MAbs.

New anti-TAG-72 MAbs (designated CC) have been developed and compared to B72.3 in the LS-174T model system. All exhibited higher %ID/gm and tumor:tissue ratios than B72.3; differences in the pharmacokinetics were noted among the CC MAbs.

A recombinant/chimeric form of B72.3 has been developed using the variable regions of the murine B72.3 and human heavy chain (γ4) and light chain (κ) constant regions. The cB72.3(γ4) IgG was radiolabeled and the biodistribution was studied in athymic mice bearing LS-174T xenografts. Small differences were observed between the cB72.3(γ4) and the native B72.3 in the %ID/gm that localized in the tumor, this is mostly likely due its more rapid clearance from the blood and body of the mouse of the cB72.3(γ4) as compared to the native B72.3.





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*Major Findings*

Monoclonal antibody (MAb) B72.3, a murine $IgG_1$, 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.

Recent interest has focused on the application of radiolabeled MAbs directed against tumor-associated antigens as *in vivo* therapeutic reagents. Labeling techniques have been developed that yield covalent linkage of chelates to proteins, thus permitting the attachment to MAbs of many different radiometals that are considered to be useful for radioimmunotherapy. $^{90}$Y has been selected as one of particular interest for therapeutic applications; it can be complexed with several chelate groups such as DTPA and EDTA which may be linked to proteins using a variety of methodologies. The fact that $^{90}$Y is a pure β-emitter makes it difficult to estimate the radiation dose to a given tissue because the radioactivity accumulated in tissues cannot be accurately detected by external imaging nor can it be accurately quantitated using gamma or beta scintillation counters. To overcome this problem, the γ-emitting $^{111}$In has been proposed as a tracer for yttrium in biodistribution studies. We have initiated studies to compare the biodistribution of yttrium and indium labeled MAb B72.3 using 3 different chelate-conjugates 1-(p-isothiocyanatobenzyl)diethylene-triaminepentaacetic acid (SCN-Bz-DTPA), 1-(p-isothiocyanatobenzyl)ethylene-diaminetetraacetic acid (SCN-Bz-EDTA) and the cyclic anhydride derivative of DTPA (CA-DTPA) to determine if the $^{111}$In can be used to accurately predict the $^{90}$Y localization and therefore allow accurate dosimetric evaluations. For these studies the γ-emitting yttrium radionuclide $^{88}$Y was chosen in order to facilitate the biodistribution analysis employing a well-type gamma counter.

Athymic mice bearing LS-174T human colon carcinoma xenografts were injected *via* tail vein with MAb B72.3 IgG modified by the 3 different chelate-conjugates and labeled either with $^{88}$Y or $^{111}$In. The uptake in the tumor was highest with $^{88}$Y-SCN-Bz-DTPA-MAb (40 %ID/gm at 5-7 days post-injection) compared to the 2 other chelate-conjugates, SCN-Bz-EDTA and CA-DTPA (only 6-8 %ID/gm). This is due in part to the rapid drop in the blood levels for the $^{88}$Y-labeled SCN-Bz-EDTA and CA-DTPA conjugated MAbs in contrast to the $^{88}$Y-SCN-Bz-DTPA-B72.3. The $^{88}$Y-SCN-Bz-EDTA and $^{88}$Y-CA-DTPA-B72.3 chelate-conjugate showed higher liver, spleen and kidney:blood ratios than the $^{88}$Y-SCN-Bz-DTPA-B72.3. The differences in tissue uptake of the 3 MAb chelate-conjugates is consistent with the release of the yttrium from the circulating CA-DPTA and SCN-Bz-EDTA MAb chelate-conjugates as evidenced by the high uptake in the bone, liver, spleen and kidney which is similar to the biodistribution seen after injection of yttrium acetate. Yttrium in its uncomplexed form tends to accumulate mainly in the bone, resulting in bone marrow toxicity. Therefore, particular attention was to be paid to the evaluation of the yttrium levels in the bone. The amount of $^{88}$Y in the bone rose as a function of time in mice injected with the SCN-Bz-EDTA and CA-DTPA-B72.3 resulting in over 14 and 11 %ID/gm at 5 days respectively. The levels of yttrium in the bones of these animals were comparable to those observed in animals given $^{88}$Y-acetate. In contrast, the mice injected with $^{88}$Y-SCN-Bz-DTPA-B72.3 showed significantly lower levels of $^{88}$Y in the bone (3 %ID/gm) indicating a better stability of this complex *in vivo*. The bone uptake for $^{111}$In-labeled B72.3 using all 3 chelate-conjugates was only 2-3 %ID/gm, similar to that found with the $^{88}$Y-SCN-Bz-DTPA-B72.3.

The differences between yttrium and indium biodistribution after administration of labeled MAbs illustrates the difficulties in using $^{111}$In-labeled MAbs to predict the biodistribution and dosimetry of $^{90}$Y-labeled MAbs. Although a given

chelate-conjugate may give a favorable biodistribution with $^{111}In$, the biodistribution of yttrium in that chelate-conjugate may be very different. These studies also demonstrated the importance of using the appropriate chelate for attaching yttrium and indium to the MAb. With the use of chelates such as SCN-Bz-DTPA, one can minimize loss of the radiometal from the chelate and obtain similar biodistributions using both indium and yttrium. The low level of bone uptake of the yttrium when the SCN-Bz-DTPA chelate-conjugate is used is especially important in minimizing marrow toxicity. The high tumor to bone ratios suggest the potential utility of $^{90}Y$ for radioimmunotherapy using this type of chelate-conjugate.

One problem that has been encountered with the *in vivo* use of both the polyclonal and monoclonal antibodies is the patients' development of an immune response to the administered antibody. Greater than 50% of the patients that have been administered B72.3 have developed an immunological response to murine IgG after a single injection. The use of fragments of MAbs has led to a decrease in the incidence of an anti-mouse Ig response in the patients. Most IgG fragments, when used *in vivo*, however, have shown a much lower %ID/gm in the tumor as compared to the intact IgG. Another way to minimize the patients' immune response to the injected antibody is to use human or recombinant/chimeric MAbs. We have developed a recombinant form of the murine B72.3 as well as a recombinant/chimeric antibody, using the variable regions of the murine B72.3 and human heavy chain ($\gamma_4$) and light chain ($\kappa$) constant regions. Both the recombinant B72.3 (rB72.3) and the recombinant/chimeric B72.3 (cB72.3($\gamma_4$) IgGs maintain the tissue binding and idiotypic specificity of the native murine IgG. The native B72.3, rB72.3, and cB72.3($\gamma_4$) IgGs were radiolabeled and the biodistribution of these IgGs was studied in athymic mice bearing LS-174T xenografts. Differences were observed between the cB72.3($\gamma_4$) and the native B72.3 in the %ID/gm that localized in the tumor. The somewhat lower absolute amounts of the cB72.3($\gamma_4$) in the tumor are mostly likely due to the more rapid clearance of the cB72.3($\gamma_4$) from the blood and body of the mouse as compared to the native B72.3 and rB72.3. All three forms (native B72.3, rB72.3 and cB72.3($\gamma_4$) of the IgG, however, were able to localize the LS-174T xenografts with similar radiolocalization indices.

The B72.3 reactive antigen, TAG-72, has been purified and a series of second generation MAbs, designated CC (colon cancer), have been characterized by a range of *in vitro* immunological assays. Six CC MAbs (CC11, CC30, CC46, CC49, CC83 and CC92) were chosen for analyses of the *in vivo* binding to the LS-174T human colon carcinoma xenografts. All 6 MAbs were previously shown to be distinct from B72.3 and each other by a series of reciprocal competition RIAs, and all were shown to have higher $K_a$s than B72.3. All 6 CC MAbs evaluated were superior to B72.3 in an *in vivo* tumor targeting model, in terms of both the percentage of the injected dose of radiolabeled MAb delivered per gram of tumor and in the radiolocalization indices (RIs). Differences in the *in vivo* binding patterns and pharmacokinetics among the CC MAbs have also been evaluated.

The CC MAbs demonstrated several different clearance patterns. One fairly unique pattern was displayed by MAb CC49. The plasma clearance of CC49 was much faster than that of B72.3 and the five other CC MAbs analyzed. This resulted in much higher tumor:blood RIs for CC49 than for the other MAbs. There were also differences in the RIs of the other normal tissues examined. For B72.3 and the other CC MAbs the RIs can be ranked as follows: tumor:liver, spleen and kidney showed the highest RIs, then tumor:lung and lastly tumor:blood. For CC49, however, the order of the RIs (from high to low) were tumor:kidney, lung, blood, spleen and liver. One possible explanation for this phenomenon is that CC49 recognizes an epitope of TAG-72 which is highly accessible when TAG-72 is found in circulation, and the resulting MAb-TAG-72 complexes deposit in the liver and spleen, thus lowering the RIs for these organs. The MAb therefore clears the plasma rapidly. Further evidence for this is found in the observation that when CC49 is administered to mice with small tumors the ranking of the RI values (notably tumor:spleen and tumor:liver) of the tissues is similar to that observed

for B72.3 and the other CC MAbs. Regardless of this phenomenon and the resulting ranking of RIs for tumor to various normal organs, the rapid plasma clearance of the CC49 resulted in CC49 demonstating among the highest RI values for the CC MAbs. The potential clinical advantage of using the CC MAbs for *in vivo* tumor targeting for either diagnostic or therapeutic applications is supported by both the higher %ID/gm of tumor and the higher RI values of the second generation CC MAbs as compared to that of B72.3.

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PROJECT NUMBER

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**NOTICE OF INTRAMURAL RESEARCH PROJECT**

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Augmentation of Tumor Antigen Expression

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☐ (a1) Minors

☐ (a2) Interviews

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

Experimental data have shown that recombinant human interferons can increase the level of expression of human tumor-associated antigens. Using a large number of colorectal carcinoma cell lines, both Type I (i.e., IFN-α and IFN-β) and Type II (i.e., IFN-γ) can enhance the cell surface expression level of CEA and related antigens as measured by monoclonal antibody binding. Complementary DNA probes of CEA and related antigens revealed that the increase was a result of alterations at the transcriptional and/or post-transcriptional level, not changes in the methylated state of the CEA gene(s). The level of expression of another human tumor antigen, tumor-associated glycoprotein-72 (TAG-72), was also found to be regulated by interferon. TAG-72, a high molecular weight mucin, is not found constitutively expressed on most adherent human carcinoma cell lines and, furthermore, its expression cannot be *de novo* induced by interferon. Serous effusions isolated from patients diagnosed with adenocarcinomas were found to constitutively express TAG-72. We have isolated carcinoma cells from >100 serous effusions, incubated them in the presence of Type I and II interferon, and found that level of TAG-72 expression could be enhanced (i.e., >50%) in >80% of the samples. As a result of the preclinical studies, a phase I clinical trial was designed to investigate whether IFN-γ could augment tumor antigen expression in patients. Eight patients with adenocarcinoma and secondary ascites were given i.p. escalating doses (i.e., 0.1-100 MU) of IFN-γ for eight weeks. Tumor cells isolated from the ascites of seven of the eight patients constitutively expressed TAG-72 as measured in immunocytochemistry and flow cytometry using B72.3. In all seven patients, i.p. administration of IFN-γ resulted in a discernable increase in the level of B72.3 binding. The findings present the initial observation that recombinant human interferon may serve as an adjunct by enhancing cell surface tumor antigen expression, thereby augmenting the localization of a monoclonal antibody.

*Major Findings*

We have reported that type I (*i.e.*, IFN-αA) and type II (*i.e.*, IFN-γ) human interferons are potent regulators of tumor antigens expressed on the surface of human breast and colorectal carcinoma cells. Initial studies revealed that *in vitro* treatment with IFN-αA or IFN-γ resulted in a 3- to 6-fold increase in TAG-72 and CEA and related gene products (i.e., NCA) as measured by the binding of respective MAbs. Subsequent investigations revealed that the *in vivo* localization of a radiolabeled MAb, B6.2, to human colorectal xenografts in athymic mice could be increased by IFN-αA administration. More recent studies investigated (1) the parameters involved in the interferon-mediated increase in MAb localization in athymic mice bearing human carcinoma xenografts; (2) the ability of IFN-γ to increase the level of CEA-related mRNA transcripts in human colorectal tumor cells; and (3) the regulation of TAG-72 and CEA expression by type I and type II interferons on human carcinoma and noncarcinoma cells isolated from patients' pleural and peritoneal effusions. These preclinical studies have resulted in a phase 1a clinical trial designed to determine whether i.p. administration of IFN-γ could upregulate tumor antigen expression on adenocarcinoma cells isolated from malignant ascites of patients diagnosed with ovarian or colorectal carcinoma.

The initial study was designed to determine (1) whether *in vitro* responsiveness of established human carcinoma cell lines was predictive of the ability of IFN-αA to enhance *in vivo* MAb localization to that cell type grown as xenografts in athymic mice; and (2) whether any correlation exists between the circulating plasma IFN-αA levels and the amount of radiolabeled MAb localized to the tumor site. WiDr, LS174T, A375, and the transplantable human mammary Clouser tumors were inoculated s.c. into athymic mice. IFN-αA was administered in two daily i.m. injections and resulted in an increase in $^{125}$I-B6.2-F(ab')$_2$ localization to the Clouser and WiDr tumors. For example, thymic mice bearing the human mammary Clouser tumor were given daily i.m. injections of 250,000 antiviral units of IFN-αA for 7 days. The mean percent injected dose of $^{125}$I-B6.2 localized to the Clouser tumor rose from 6.9 to 12.8% as a result of IFN-αA treatment. The range for the percent injected dose of $^{125}$I-B6.2-F(ab')$_2$ was 3.9 to 8.0% for the Clouser tumor-bearing untreated mice, and 8.9 to > 18% for the IFN-αA-treated mice. In contrast, the human tumor cell lines (*i.e.*, LS-174T and A375) that were previously shown either to be unresponsive to the ability of IFN-αA to enhance antigen expression or tumor antigen negative *in vitro* remained so in the *in vivo* studies. These results suggest that *in vivo* screening analyses of human tumor cell populations may be possible for selection of patients for subsequent antigen augmentation therapy.

Until recently, the studies of the regulation of tumor antigens by human interferons have relied on the analysis of antigen levels measured by the binding of MAbs. Several laboratories have now reported the nucleotide sequence of several cDNA clones for the family of CEA-related genes. In collaboration with Drs. J. Kantor and P. Robbins, we studied the effect of IFN-γ on the steady-state levels of CEA mRNA and the level of CEA expression as measured by the binding of MAb COL-4 in human colorectal tumor cells. HT-29, WiDr, and LS174T express different amounts of CEA as measured by the binding of MAb COL-4 and other quantitative assays. Treatment of the moderately differentiated colorectal cells, HT-29 and WiDr, with 1,000-2,000 antiviral units of IFN-γ/ml for 48-72 hrs increased COL-4 binding by 2.5- and 6.5-fold, respectively. Using a 550-bp *Pst*I fragment CEA cDNA probe, the increase in COL-4 binding was accompanied by a 6- to 13-fold increase in the steady-state levels of the three CEA-related transcripts of 4.2, 3.5, and 2.8 kb. In contrast, the highly differentiated colorectal human cell line, LS174T, showed no significant change in either COL-4 binding or the expression of the CEA-related transcripts as a result of IFN-γ treatment. Normal diploid human fibroblasts, WI-38 and MRC-5, did not express constitutive CEA or the CEA-related mRNA transcripts either before or after IFN-γ treatment. Previous studies have shown that IFN-γ is a potent inducer of *de novo* class II HLA expression on a variety of human cells. Indeed, IFN-γ treatment did induce

the HT-29 and WiDr cells to express the class II HLA; however, the LS174T cells remained class II HLA negative. The effects of IFN-γ treatment on 2',5'-oligoadenylate synthetase (2'-5'-A) activity was also studied using the human colorectal tumor cell lines. Interestingly, IFN-γ treatment did increase 2'-5'-A activity in the MIP and LS-174T cells, two colorectal tumor cell types that did not show any surface antigen alteration as a result of IFN-γ treatment. The findings indicate that the cellular events involved in the IFN-γ mediated regulation of 2',5'-A are separate from those involved in alteration in cell surface antigen expression. The results indicate that IFN-γ treatment results in an increase in the steady-state levels of CEA mRNA, indicating the regulation of that antigen at transcriptional and/or post-transcriptional levels. The IFN-γ-induced increase in CEA was accompanied by a concomitant increase in the steady-state levels of the three transcripts. It is unclear whether the increase in CEA mRNA levels resulted from an increased rate of transcription of the CEA gene(s) and/or from the stabilization of the CEA message(s). Preliminary results suggest that post-translational stabilization of the CEA mRNA may be an important element in the regulation of CEA by IFN-γ (see Dr. J. Kantor report). Further studies are planned to address these and other issues concerning the biological relevance of the regulation of a human tumor antigen by IFN-γ.

Several human colorectal tumor cell types are unresponsive to the ability of interferon to alter the level of expression of either tumor-associated or normal cellular antigens. We were interested to determine whether other agents previously shown to influence cellular differentiation could alter antigen expression in those tumor cell types. Specifically, certain cAMP analogs (see Dr. Y-S Cho-Chung report) induce dramatic changes in the stage of differentiation of several different human tumor cell types. A collaborative effort was initiated to investigate whether one specific cAMP analog, 8-Cl-cAMP, could alter the expression of surface antigen in human colorectal tumor cells. We found that treatment of human colorectal tumor cells with a site-selective analogue of cyclic AMP (cAMP), 8-Cl-cAMP, results in a selective enhancement of carcinoembryonic antigen (CEA) expression. The increase in CEA expression is accompanied by increases in the steady-state levels of the CEA-related mRNA transcripts as well as the binding of anti-CEA monoclonal antibodies (MAbs). The level of expression of other surface antigens, such as those of the major histocompatibility complex (MHC), cytokeratins, and 2 other tumor-associated antigens, were not altered after treatment of human colorectal tumor cells or normal, diploid fibroblasts with 8-Cl-cAMP. Dose and temporal kinetic studies revealed that the maximal increase in CEA expression occurred after treating the tumor cells 3 times with 10 μM 8-Cl-cAMP during a 5-day period. A second analogue of cAMP, dibutyryl-cAMP, also caused an increase in CEA expression on the surface of the highly differentiated LS174T human colorectal tumor cell line. However, a comparable increase of CEA could be achieved by administering 100-fold less 8-Cl-cAMP. When LS174T cells were grown as xenografts in athymic mice and subsequently treated with 8-Cl-cAMP, a significant decrease in LS174T tumor growth, as well as a substantial increase in CEA expression, was observed. As stated previously, the LS174T colorectal tumor cell line represents a cell type that is unresponsive to the ability of interferon-γ (IFN-γ) to either enhance the expression of CEA or induce class II HLA. Thus, the results identify another cellular pathway (i.e., cAMP-dependent protein kinase) through which the antigen phenotype of a human tumor cell can be altered both *in vitro* and *in vivo*.

Eight patients diagnosed with adenocarcinoma (6 ovarian, 2 gastrointestinal) and secondary malignant ascites were given escalating doses of IFN-γ (i.e., 0.1-100 MU) intraperitoneally for 8 weeks. Ascites samples were removed each week prior to and at 24 h and 48 h post-IFN-γ, the tumor cells isolated, and the level of expression of carcinoma-associated antigens—tumor-associated glycoprotein-72 (TAG-72) and carcinoembryonic antigen (CEA)—was measured using immuno-cytochemistry and flow cytometry. I.p. IFN-γ administration dramatically increased TAG-72 as well as CEA expression on the surface of carcinoma cells isolated from the malignant ascites. For example, of the eight patients treated, tumor cells isolated from 7 ascites constitutively expressed TAG-72 as measured

by the binding of monoclonal antibody (MAb) B72.3 and IFN-γ administration increased B72.3 binding in all seven cases. In some cases, the percentage of tumor cells reactive with MAb B72.3 increased from approximately 10% to >90% following IFN-γ treatment. Two patients diagnosed with adenocarcinoma of the G.I. tract were also found to express higher CEA levels in isolated tumor cells for their malignant ascites after i.p. administration of IFN-γ. Moreover, the significant changes in tumor antigen expression were observed at doses of IFN-γ which were well tolerated (i.e., 0.1-1.0 MU). The findings indicated that i.p. administration of IFN-γ substantially alters the tumor antigen phenotype of carcinoma cells found in malignant ascites. The results also provide additional evidence that recombinant human interferon treatment may serve as effective adjuvants via enhancing the level of expression of tumor antigens and, thereby, improve localization of a conjugated MAb to the human tumor cell population.

To date, the study of the regulation of TAG-72 by the recombinant human interferons has been confined to the human breast tumor cell line MCF-7. TAG-72 is not well expressed on adherent human cell lines, and the other TAG-72 positive cell line, LS174T, is not responsive to the ability of type I or type II interferons to enhance tumor-associated and normal (*i.e.*, HLA) antigens (see above). Studies have shown that TAG-72 can be found in a high percentage of human tumor cells isolated from serous effusions of patients diagnosed with metastatic adenocarcinoma. A total of 61 serous effusions from patients diagnosed with adenocarcinoma (43), malignant nonepithelial tumors (10), and benign diseases (8) were analyzed for the expression of TAG-72, CEA, and MHC antigens as well as the ability of type I and type II interferons to enhance their level(s) of expression. TAG-72 was found on approximately 80% (*i.e.*, 35 of 43) of the serous effusions isolated from patients with metastatic adenocarcinoma. Of the 35 of 43 effusions from patients with metastatic adenocarcinoma that also contained TAG-72-positive tumor cells, treatment with either type I (*i.e.*, IFN-αA or IFN-$\beta_{ser}$) or type II (*i.e.*, IFN-γ) significantly increased B72.3 binding (*i.e.*, 50% above that of untreated cells) in 77% of the cases (*i.e.*, 27 of 35). Constitutive TAG-72 expression was not, however, a *sine qua non* requirement for its subsequent augmentation by human interferons. In at least 4 of 8 cases, the isolated malignant cells expressed high levels of TAG-72, and a further increase in B72.3 binding could not be demonstrated after interferon treatment. We were also interested to determine whether treatment of those TAG-72-negative malignant cell populations might enhance the expression of other tumor antigens. In nine such cases, treatment of the malignant cell population with either IFN-αA or IFN-γ resulted in a significant increase in CEA expression. Thus, 42 of 43 tumor cell populations isolated from patients with metastatic adenocarcinoma constitutively expressed either TAG-72 and/or CEA. Furthermore, *in vitro* incubations of the cells in the presence of type I or type II human interferons substantially increased the expression of one or both of the tumor antigens in 36 of the 42 cases (85.7%). The findings provide important insight into the criteria for the selection of patients for antigen augmentation therapy: (1) cell surface tumor antigen expression and (2) ability of interferon treatment to augment MAb binding *in vitro*. In addition, these and previous findings have provided the framework which was used to design a phase 1 clinical trial to investigate whether the interferons can increase tumor antigen expression *in vivo*.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CB 09018-06 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Anti-Carcinoma Monoclonal Antibodies Clinical Trials

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

| | | |
|---|---|---|
| Jeffrey Schlom | Chief | LTIB, DCBDC, NCI |
| David Colcher | Supr. Microbiologist | LTIB, DCBDC, NCI |
| Mario Roselli | Visiting Fellow | LTIB, DCBDC, NCI |
| Patricia Hand | Chemist | LTIB, DCBDC, NCI |

COOPERATING UNITS *(if any)* Dr. J. Carrasquillo, CC, NM; Drs. A. Raubitschek and O. Gansow, ROB, DCT, NCI; Dr. E. Reed, COP, DCT, NCI; Dr. E. Martin, Ohio State Univ., Dept. Surg.; Dr. M. Salvatore, NCI (Naples); Dr. S. Larson, Memorial Sloan-Kettering, Nucl. Med.; Dr. A. LoBuglio, U. 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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.5 | 1.0 | 1.5 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

This project involves the use and the proposed use of several monoclonal antibodies (MAbs) in both diagnostic and therapeutic clinical trials. Thus far, all our collaborative clinical trials have been conducted with MAb B72.3. This MAb has been previously shown to have differential reactivity for a range of human carcinomas as compared to most normal adult tissues. To date, over 1,000 patients have been administered radiolabeled B72.3 in tumor-targeting studies carried out in several institutions, with similar findings of approximately 70-80% tumor targeting observed.

We first investigated the administration of radiolabeled MAb B72.3 IgG in colorectal cancer patients. The selective localization of $^{131}$I-MAb B72.3 IgG was demonstrated in biodistribution studies in which the percentage of injected dose of MAb per gram of each tumor was compared with that of the normal tissues, thus providing a relative radiolocalization index (RI) for each lesion. Of the tumor lesions, 70% had an RI of at least 3 (i.e., 3 times greater uptake per gram than normal tissues). We have also conducted studies to determine the feasibility of intraperitoneal administration of radiolabeled B72.3 for tumor localization (via both gamma scanning and direct analyses of biopsy specimens). Several patients studied were negative for tumor detection by both CT scan and X-ray but were positive for tumor localization via gamma scanning. Direct analyses of biopsy specimens of carcinoma and normal tissues demonstrated ratios of up to 70:1 for tumor MAb localization versus normal tissues.

A phase I therapy trial involving intraperitoneal administration of $^{131}$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 other MAb-isotope conjugates are planned.

*Major Findings*

We have investigated the administration of radiolabeled MAb B72.3 IgG in colorectal cancer patients. These studies were possible due to a previously ongoing NCI Surgery Branch protocol for the treatment of patients with metastatic colorectal carcinoma, which involved the surgical resection of metastatic colorectal cancer lesions plus adjacent "normal" tissues for staging and therapeutic purposes. Thus, gamma scanning followed by a direct examination of tumor and a variety of normal tissues for MAb localization could be achieved. In our first trial, B72.3 IgG was labeled with $^{131}I$ and injected i.v. into 20 patients with known or suspected colorectal cancer. The selective localization of $^{131}I$-MAb B72.3 IgG was demonstrated in biodistribution studies in which the percentage of injected dose of MAb per gram of each tumor was compared with that of the normal tissues, thus providing a relative radiolocalization index (RI) for each lesion. Of the tumor lesions, 70% (99/142) had an RI of at least 3 (i.e., 3 times greater uptake per gram than normal tissues), and 31% of the tumor lesions had RIs of over 10. Only 12 of 210 (6%) histologically normal tissues had RIs of >3; either these tissues were adjacent to or draining tumor masses or, as in the case of two patients, the high RI values were apparently due to deposition of immune complexes in spleen. Several parameters were studied to determine factors that might influence MAb localization. No differences in MAb uptake were observed among carcinoma lesions for numerous anatomic locations. No toxicity was observed. Additional studies employing $^{111}In$-B72.3 coadministered with $^{125}I$-B72.3 are in progress.

We have also conducted a series of collaborative studies with the Department of Surgery, Ohio State University (Dr. E. Martin) on the use of $^{125}I$-radiolabeled B72.3 and an intraoperative hand-held probe, i.e., a gamma-detecting probe (GDP) to detect occult gastrointestinal, as well as other carcinoma lesions, at surgery. In initial studies using $^{125}I$-B72.3 IgG, MAb was injected 5-42 days preoperatively, and the hand-held GDP was used intraoperatively to help detect the presence of tumor. Positive probe counts were detected in 5 of 6 (83%) patients with primary colon cancer, and in 31 of 39 (79%) patients with recurrent colon cancer.

We have conducted studies at the NIH to (a) determine the feasibility of intraperitoneal administration of radiolabeled B72.3 for tumor localization (via both gamma scanning and direct analyses of biopsy specimens); (b) determine the specificity of tumor localization by the concomitant i.p. administration of $^{131}I$-labeled B72.3 and an isotype matched control $^{125}I$-labeled MAb BL-3 (anti-idiotype) IgG; (c) compare tumor localization of i.v. versus i.p. administered MAb by the simultaneous administration of $^{125}I$-B72.3 IgG i.v. and $^{131}I$-B72.3 IgG i.p.; and (d) define the pharmacokinetics of plasma clearance of both i.p. and i.v. administered radiolabeled MAb B72.3. At the time of entry into the protocol, all patients had confirmed or suspected metastatic colorectal peritoneal lesions. Subsequent studies have also been conducted with ovarian carcinoma patients with similar results. Four to seven days before surgery, all patients were administered $^{131}I$-labeled B72.3 IgG i.p. via a catheter. Three of 10 patients studied were negative for tumor detection by both CT scan and X-ray but were positive for tumor localization via gamma scanning. Direct analyses of biopsy specimens of carcinoma and normal tissues demonstrated ratios of up to 70:1 (based on percentage of injected dose per kilogram) for tumor MAb localization versus normal tissues. Specificity of $^{131}I$-B72.3 tumor targeting was demonstrated by the concomitant administration of $^{125}I$-labeled isotype identical ($IgG_1$) control MAb. Simultaneous i.p. administration of $^{131}I$-B72.3, and i.v. administration of $^{125}I$-B72.3 in individual patients demonstrated: (a) peritoneal implants are targeted more efficiently via i.p. MAb administration, and (b) hematogenously spread and lymph node metastases as well as local recurrences are targeted more efficiently by i.v. administered MAb. No antibody toxicity was observed in any patients. Pharmacokinetics of MAb clearance demonstrated that only 10-30% of the i.p. administered MAb was found in plasma. These studies thus demonstrated the efficacy of intracavitary MAb administration as well as the

advantage of the concomitant use of intracavitary and i.v. administered MAbs for tumor targeting and for potential MAb-guided therapy of metastatic carcinoma.

Monoclonal antibody (MAb) B72.3 IgG was radiolabeled with $^{131}I$ and administered to female athymic NCr-nu 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. 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.

A phase I trial involving i.p. administration of $^{131}I$-B72.3 IgG in patients with ovarian or colorectal carcinoma confined to the peritoneal cavity is in progress at the NIH. These studies were initiated following dosimetry calculations that were based on analyses of pharmacokinetics and tumor and normal tissues sampling of diagnostic doses of i.p. administered $^{131}I$-B72.3. Studies involving the use of recombinant-chimeric MAbs have also begun.

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PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09021-04 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)


| | | |
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| Paul Robbins | Senior Staff Fellow | LTIB, DCBDC, NCI |
| Scott Meissner | Staff Fellow | LTIB, DCBDC, NCI |
| Chrysanthi Paranavitana | Fogarty Fellow | LTIB, DCBDC, NCI |
| Howard Kaufman | Biotechnology Fellow | LTIB, DCBDC, NCI |
| Judy DiPietro | Biologist | LTIB, DCBDC, NCI |
| Jeffrey Schlom | Chief | LTIB, DCBDC, NCI |


COOPERATING UNITS (if any)

LAB/BRANCH

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SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 6.1 | 5.1 | 1 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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


Monoclonal antibodies (MAbs) have defined several antigens associated with human carcinomas. Two of the most widely studied antigens are carcinoembryonic antigen (CEA) and TAG-72. CEA is a 180,000-kd glycoprotein and TAG-72 is a high- molecular-weight mucin. Recent studies have demonstrated that CEA belongs to a multigene family related to the immunoglobulin supergene family. These include CEA, normal cross-reacting antigen (NCA), biliary glycoprotein (BGP), and human pregnancy-specific beta 1-glycoprotein (SP1). A 550-base pair CEA probe derived from cloned complementary DNA was used to carry out Southern analysis of the DNA isolated from normal and colon tumor cell lines. At high stringency, the CEA probe detected seven *Bam*HI fragments in all DNAs analyzed. Results of the Southern analysis demonstrate no amplification or rearrangement of the CEA genes in tumor cells. Methylation-sensitive restriction endonucleases, *Hpa*II and *Hha*I, were used to compare the degrees of methylation of the CEA family of genes in normal and colon tumor cell lines. The results demonstrate that the CEA family of genes exists in a state of hypermethylation in normal cells. In contrast, the CEA gene(s) are relatively hypomethylated in the tumor cell lines, suggesting a correlation between the state of methylation and degree of expression of the CEA gene(s). Using a CEA cDNA probe, we have investigated the phenomenon of up-regulation of CEA expression in human tumor cell lines by recombinant human gamma-interferon (Hu-IFN-γ). An increase in MAb binding was shown to be accompanied by a 6- to 11-fold increase in the steady-state levels of three CEA transcripts with sizes of 4.2, 3.5, and 2.8 kb. Studies are also in progress to identify and characterize the gene(s) encoding the TAG-72 mucin antigen. A number of mucin genes representing several tissue types have been cloned. These include the genes encoding the epitopes for breast cancer, HMFG2, $DF_3$ and $H_{23}$ and the pancreatic antigen DuPAN-2. Nucleic acid sequence analysis of these isolated genes has shown them to be identical and that the difference resides in the sugar epitopes detected by the MAb. The dominant feature of the breast and pancreatic mucin clones was an extremely GC-rich, 60-bp tandem repeat sequence that encoded a repeating peptide unit rich in serine, threonine, and proline.





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

*Major Findings*

Using an assay based on the binding of a CEA-specific monoclonal antibody (MAb), we examined the expression of carcinoembryonic antigen (CEA) genes in human colon tumor and normal fibroblast cell lines. CEA expression was not detectable in the normal fibroblast cell lines, whereas varying levels of high CEA expression were found in the colon tumor cell lines LS-174T, GEO, and WiDr. We then used a 550-base pair CEA cDNA probe to carry out Southern analysis of the DNA isolated from the normal and colon tumor cell lines. At high stringency, the CEA probe detected seven *Bam*HI fragments in all DNAs analyzed. At low stringency, however, 14 *Bam*HI fragments ranging from 1.5 to 2.3 kb were detected. Results of the Southern analysis demonstrate no amplification or rearrangement of the CEA genes in tumor cells. We used methylation-sensitive restriction endonucleases, *Hpa*II and *Hha*I, to compare the degree of methylation of genes in the CEA family in normal and colon tumor cell lines. Our results demonstrate that the CEA family of genes exists in a state of hypermethylation in the normal cell lines. In contrast, the CEA gene(s) are relatively hypomethylated in the tumor cell lines, suggesting a correlation between the state of methylation and degree of expression of the CEA gene(s). A comparison of the state of methylation of the CEA gene(s) in cells before and after treatment with recombinant human gamma-interferon (Hu-IFN-γ) showed no detectable difference in the degree of DNA methylation. The segments of CEA genes that are hypermethylated in normal cells, but are hypomethylated in tumor cells, were also identified. Thus, these studies may help identify the sites of methylation that are crucial for the control of CEA gene regulation.

The effect of Hu-IFN-γ on the expression of human CEA and its related transcripts was then examined in several human colon carcinoma and normal human fibroblast cell lines. The colon tumor cell lines, HT-29, WiDr, and LS-174T, were each shown to express different constitutive levels of CEA glycopeptide as measured by the binding of the CEA-specific MAb COL-4. Treatment with Hu-IFN-γ enhanced the level of binding of COL-4 in total cell extracts of HT-29 and WiDr cells by 2.5- and 6.5-fold, respectively. Using a CEA cDNA probe, this increase in MAb binding was shown to be accompanied by a 6- to 11-fold increase in the steady-state levels of three CEA transcripts with sizes of 4.2, 3.5, and 2.8 kb. On the other hand, Hu-IFN-γ treatment had no effect on the level of COL-4 binding or expression of CEA transcripts in LS-174T colon carcinoma cells, which are high constitutive expressors of CEA glycoprotein. Normal human fibroblast cell lines MRC-5 and WI38 had no detectable cytoplasmic CEA glycopeptide levels nor did they contain detectable levels of CEA mRNA, either before or after treatment with Hu-IFN-γ. In contrast, Hu-IFN-γ induced the *de novo* expression of the normal major histocompatibility complex class II antigen, HLA-DR, on HT-29 and WiDr colon cancer cells as well as the two fibroblast cell lines. Treatment of the LS-174T cell line with Hu-IFN-γ did not result in the induction of class II HLA-DR antigen. Our observations suggest that some common factors may be involved in the regulation of the CEA and class II histocompatibility genes. In addition, the demonstration that Hu-IFN-γ enhances CEA expression in some carcinoma cell lines, but fails to induce *de novo* expression of CEA transcripts in fibroblasts, supports the potential application of Hu-IFN-γ in enhancement of tumor targeting of anti-tumor MAbs and adds to our understanding of the mechanism of IFN-mediated up-regulation of some tumor antigens.

We have raised a polyclonal antisera to a synthetic peptide consisting of the amino acid sequence Pro-Thr-Pro-Thr-Pro-Thr-Pro-Thr-Ile. This sequence was obtained from a purified prepration of TAG-72 antigen prepared from LS-174T xenograft tissue. This affinity purified antisera has been shown to react with deglycosylated TAG-72 antigen but not with native, nondeglycosylated 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-21, 800 bp, contains the PT PT repeat but contains no tandem repeats. Because all mucin genes isolated to date contain tandem repeats, we are

screening our colon library with this nucleic acid probe to determine whether the TAG-21 gene will have tandem repeats.

*Publications*

Tran R, Kashmiri SVS, Kantor J, Greiner JW, Pestka S, Shively JE, Schlom J. Correlation of DNA hypomethylation with expression of carcinoembryonic antigen in human colon carcinoma cells, Cancer Res 1988;48:5674-5679.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

# NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 09024-03 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Parameters Involved in the Tumor Targeting of Monoclonal Antibodies

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

| | | |
|---|---|---|
| Patricia Horan-Hand | Research Chemist | LTIB, DCBDC, NCI |
| Alfredo Molinolo | Visiting Fellow | LTIB, DCBDC, NCI |
| David Colcher | Supervisory Microbiologist | LTIB, DCBDC, NCI |
| Jeffrey Schlom | Chief | LTIB, DCBDC, NCI |

COOPERATING UNITS *(if any)* Dr. Philip Snoy, Center for Drugs and Biologics, Division of Quality Control, NIH

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 1.7 | 0.6 | 1.1 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The overall goal of this project is the delineation of parameters involved in *in vivo* targeting of monoclonal antibodies. These studies will involve determination of the optimal route of inoculation of antibody as well as the antibody form. Major emphasis will be placed on the pharmacokinetics of plasma clearance of recombinant/chimeric monoclonal antibodies and their ability to target tumors.

One approach to the treatment of patients with peritoneal carcinoma may be direct infusion into the peritoneal cavity of radiolabeled antibody conjugates. In comparison to intravenous MAb administration, this method may improve the percent of injected MAb dose per gram of tumor and may lead to less radiolabeled antibody in the bone marrow and therefore less toxicity to the patient. The second major goal of this project is to develop an *in vivo* model system to study immunotherapy of human carcinoma in the peritoneal cavity. These studies will include (a) determination of the animal species eliciting the pattern most comparable to that observed in the human of MAb clearance from the blood, (b) development of a human peritoneal carcinoma xenograft in an animal model, and (c) determination of the utility of intraperitoneally (i.p.) administered MAb conjugates for carcinoma therapy.

*Major Findings*

Our results suggest that consistent augmentation of radiolabeled antibody localization to tumors pretreated with external beam irradiation is not a universal phenomenon. Inherent differences in tumors, such as cell type of origin, size, spatial configuration, extent of vascularization, and volume of interstitial space, may contribute to variability of the effect of preirradiation of tumors on MAb localization. Extensive evaluation of each individual biological model is therefore required to determine the efficacy of novel therapeutic modalities.

Studies are now in progress to develop a reliable animal model system, which will allow assessment of the toxicity of i.p. MAb administration and provide pharmacokinetics data that may be successfully scaled up to man. The athymic (*nu/nu*) mouse bearing tumor xenografts is generally accepted as the animal model of choice for preclinical MAb studies. Our studies have investigated and compared the pharmacokinetics of plasma clearance of intravenous and i.p. administered MAbs in athymic and Balb/c mice, athymic and Buffalo rats, cynomolgus and rhesus monkeys, and colorectal carcinoma patients. No differences were observed in MAb clearance between the two strains of mice and rats. Peak MAb B72.3 plasma levels in monkeys were observed 18-24 hours after MAb administration, with no substantial differences in plasma clearance between adult and juvenile rhesus monkeys, or cynomolgus and rhesus monkeys. Other anti-carcinoma radiolabeled MAbs COL-12 (anti-CEA), CC49 and CC83 (anti-Tumor Associated Glycoprotein-72) showed i.p. clearance profiles in rhesus monkeys similar to those observed with MAb B72.3 suggesting that differences that may occur among these MAbs do not appreciably alter plasma clearance patterns. Body surface areas, which are proportional to peritoneal surface areas, were determined for each patient receiving MAb and the various animal models, and these values were compared with the time of peak MAb plasma levels after i.p. MAb administration; body surface areas were directly proportional to time of highest MAb plasma levels, i.e., smaller body surface areas were indicative of an earlier occurrence of peak MAb levels while larger body surface areas correlated with a longer interval between MAb administration and peak MAb levels. These studies therefore indicate that there is no ideal model for defining the pharmacokinetics of intraperitoneally administered macromolecules, such as MAbs, in humans and that there appears to be a gradation in MAb clearance from the peritoneal cavity into the plasma among mice, rats, monkeys, and man. These studies thus demonstrate the complexity in extrapolation of animal model data (especially in rodents) for i.p. administered MAb to potential human clinical trials.

Anti-human tumor antibodies are unique because they react specifically with human tumor-associated antigens, such as carcinoembryonic antigen or TAG-72. No cross reactivity of these antibodies to rodent antigens is observed. For this reason, the model of choice for tumor targeting and therapy of MAbs vs. human tumor antigens is the athymic mouse bearing human tumor xenografts. Studies are also in progress to develop and use, as an *in vivo* model system for immunotherapy of human peritoneal carcinoma, i.p. xenografts of human ovarian carcinoma in athymic mice and MAb B72.3

*Publications*

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Shrivastav S, Schlom J, Raubitschek A, Molinolo A, Simpson J, Horan-Hand P. Studies concerning the effect of external irradiation on localization of radiolabeled monoclonal antibody B72.3 to human colon carcinoma xenografts, Int J Radiat Oncol Biol Phys 1989;16:721-729.

Horan Hand P, Shrivastav S, Colcher D, Snoy P, Schlom J. Pharmacokinetics of radiolabeled monoclonal antibodies following intraperitoneal and intravenous administration in rodents, monkeys, and humans, Antibody, Immunoconjugates, and Pharmaceuticals 1989;2:241-255.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09025-03 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Antibody Directed Cellular Immunotherapy of Colon Cancer

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

| | | |
|---|---|---|
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| Tribhuvan K. Pendurthi | Guest Researcher | LTIB, DCBDC, NCI |
| Jeffrey Schlom | Chief | LTIB, DCBDC, NCI |
| Margaret Finch | Biotechnology Research Fellow | LTIB, DCBDC, NCI |
| Alfred Tsang | Special Volunteer | LTIB, DCBDC, NCI |
| Carol Nieroda | Biotechnology Research Fellow | LTIB, DCBDC, NCI |

COOPERATING UNITS *(if any)*

Dr. David Segal, Immunology Branch, DCBDC, NCI, NIH; Dr. John Yanelli, Surgery Branch, DCT, NCI, NIH

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

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Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 5.1 | 5.1 | 0 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

This project is examining the role that monoclonal antibodies (MAb) against human colonic tumor antigens have in directing and augmenting the cytotoxic activity of NK/LAK cells and T cells. A MAb designated D612 (IgG2a) has been generated that uniformly reacted with the cell surface of approximately 80% of primary or metastatic colorectal carcinomas. Normal tissue reactivity of D612 was confined mainly to the small and large intestine, while non-gastrointestinal normal tissues were routinely negative. D612 mediated antibody-dependent cellular cytotoxicity in conjunction with normal human peripheral blood mononuclear cells was increased by effector cell activation with interleukin 2 (IL-2). For retargeting of T cells, heteroantibody conjugates between the anti-colon tumor MAb, B38.1, and anti-CD3 (OKT-3) were chemically prepared, and these were tested along with effectors obtained from PBMNC stimulated with anti-CD3 and IL-2. The heteroantibody-mediated cellular cytotoxicity activity was similar on a per cell basis among effector populations prepared by stimulation with IL-2, anti-CD3, or a combination of these reagents. However, the total lytic activity of anti-CD3 + IL-2-stimulated cultures was much higher due to the greater expansion in cell number achieved by the combination treatment. These studies suggest that MAbs with restricted normal tissue reactivity and ability to direct the cellular cytotoxicity of a broader spectrum of killer lymphocytes activated by lymphokines and other agents might augment the efficacy of colon cancer immunotherapy.

*Major Findings*

A monoclonal antibody (MAb), designated D612 (IgG2a), was generated by immunization of mice with a membrane-enriched fraction of a moderately differentiated primary colon adenocarcinoma. Using membrane extracts derived from a variety of cell lines and normal and neoplastic tissues, D612 was found to react specifically with extracts of cells or tissues originating from the large intestine. The D612 antigen was not detected by immunohistochemical staining of conventionally processed tissue specimens, but was revealed when fresh, frozen tissues were used. An immunohistochemical survey of the tissue distribution of the D612 antigen showed that 70 to 80% of primary or metastatic colorectal carcinomas were positive, independent of their differentiation. In over half of these specimens, the staining was homogeneously distributed among the majority of tumor cells where it localized predominantly to the plasma membrane. Flow cytometry of colon tumor cell lines also showed that the D612 antigen occupied a surface location. D612 did not stain noncarcinoma tumors but did react weakly with a small number of stomach, breast, and ovarian carcinomas in a highly focal pattern. Normal tissue reactivity of D612 was confined to the epithelium of the small and large intestine and of the stomach. It was not found in normal specimens covering a wide range of nongastrointestinal tissues including thyroid, kidney, pancreas, liver, bladder, blood cells, and lung. Normal colon goblet and absorptive cells showed a homogeneous pattern of staining, located primarily along the basolateral cytoplasmic membrane. Other studies showed that the D612 antigen is not secreted into the culture media, has a molecular size of $1 \times 10^6$ or greater, and is not CEA, TAG, or the 17-1A antigen.

The D612 MAb was found to mediate antibody-dependent cellular cytotoxicity (ADCC) in conjunction with normal human peripheral blood mononuclear cells (PBMNC) against antigen-positive colon tumor cell lines. Exposure of PBMNC to IL-2 (100 U/ml; 24 hr) resulted in a 2- to 3-fold increase in specific ADCC cytolytic activity. Although the total specific ADCC lytic activity varied among different donors, its potentiation by IL-2 was very similar. The ADCC activity of the 17-1A MAb was also studied for comparison to D612, and it was found that both MAbs were similar in their ability to mediate ADCC. The association and dissociation constants of these two MAbs were measured using radiolabeled MAb and the LS-174T colon tumor cell line. For D612, the $K_a$ and $K_d$ were calculated to be $1.3 \times 10^8$ $M^{-1}$ $s^{-1}$ and $8.5 \times 10^{-4}$ $s^{-1}$, respectively. The corresponding values for the 17-1A MAb equaled $4.4 \times 10^7$ and $9.3 \times 10^{-4}$, respectively. The number of sites per LS-174T cell was found to be $4.8 \times 10^5$ for D612 and $1.1 \times 10^6$ for 17-1A. It can be seen from these studies that the D612 and 17-1A MAbs have equivalent effector functional activity while the D612 MAb has a better association constant.

Taken together, the tissue distribution, cytological location, and immunochemical characteristics of the antigen recognized by D612 indicate that the D612 MAb is different from a large variety of anti-colon carcinoma MAbs previously described in the literature. The D612 antigen has a fairly uniform expression within and among colorectal carcinomas, restricted normal tissue reactivity, and cell surface location--desirable properties for an antigen intended to serve as a target for immunotherapy. Furthermore, radiolabeled D612 was shown to localize colonic tumor xenografts. Its ability to mediate ADCC coupled with IL-2 augmentation of this property indicate that further studies of antibody-dependent cellular immunotherapy with this MAb would be worthwhile.

Retargeting of T-cell experiments were initiated using a MAb, designated B38.1 (IgG1), that was generated by immunization with membrane-enriched fractions of human metastatic mammary carcinoma cells. Cross-competition experiments between B38.1, 17-1A, and a second generation MAb, designated GA73.3, showed that these MAbs cross-blocked one another, with the B38.1 and GA73.3 pair showing identical activities. Furthermore, B38.1 immunoprecipitated three bands, from an intrinsically labeled colon carcinoma cell line, that migrated in the range of 37 to 40 kDa, similar to the profile revealed by GA73.3 immunoprecipitates. The tissue distribution properties of B38.1, and for comparison, the GA73.3 MAb, were

analyzed immunohistochemically with fresh, frozen specimens. Both MAbs reacted with a variety of normal tissues, such as thyroid, liver, kidney, pancreas, and colon, with a similar staining pattern. Collectively, these results indicate that the B38.1 MAb most likely recognizes the same antigen detected by 17-1A/GA73.3.

Heteroconjugates were prepared between B38.1 and OKT-3 (anti-CD3) by disulfide linkage using the heterobifunctional reagent SPDP. Heteroantibody preparations were made with intact MAb or $F(ab')^2$ fragments, and an essentially dimeric heteroantibody fraction was then isolated by molecular sieve chromatography. Heteroantibody-mediated cellular cytotoxicity (HMCC) against cultured colon carcinoma cell lines was measured in short-term chromium release assay after activation of normal human PBMNC with solid-phase OKT-3 followed by incubation with rIL-2 (100 U/ml). Monomeric B38.1 variably mediated low levels of ADCC with PBMNC activated with IL-2 alone, an expected finding based on the IgG1 isotype of B38.1, but was without activity in conjunction with effector cells exposed to anti-CD3 + IL-2. Heteroantibodies formed with intact Ig or $F(ab')_2$ fragments were equally effective in retargeting effector cells activated with anti-CD3 alone, or with anti-CD3 + IL-2. Depletion of FcR III-positive lymphoid cells before activation did not alter heteroantibody retargeting, demonstrating that natural killer cells were not the precursors for the effector population activated in this manner for HMCC. HMCC activity with effector cells produced by exposure to anti-CD3 and IL-2 was similar on a per cell basis to that of cells incubated with anti-CD3 or IL-2 alone. However, greater total HMCC activity was obtained with PBMNC cultures treated with anti-CD3 + IL-2, since this combination resulted in a 30- to 50-fold increase in cell number after 9 days of IL-2 as compared to a doubling of cell number in cultures exposed to anti-CD3 or IL-2 alone. These studies suggest that lymphoid cells, probably T cells for the most part, activated by anti-CD3 and IL-2 may be alternative candidates for immunotherapy when used in conjunction with retargeting heteroantibodies.

*Publications*

Muraro R, Nuti M, Natali PB, Bigotti A, Simpson JF, Primus FJ, Colcher D, Greiner JW, Schlom J. A monoclonal antibody (D612) with selective reactivity for malignant and normal gastrointestinal epithelium, Int J Cancer 1989;43:598-607.

Kuroki M, Greiner JW, Simpson JF, Primus FJ, Guadagni F, Schlom, J. Serologic mapping and biochemical characterization of the carcinoembryonic antigen epitopes using fourteen distinct monoclonal antibodies, Int J Cancer 1989;44:208-218.

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Hutzell P, Kashmiri S, Colcher D, Primus FJ, Horan-Hand P, Roselli M, Yarranton G, Bodmer M, Whittle N, King D, Loullis CC, McCoy DW, Callahan R, Schlom J. Generation and characterization of a recombinant/chimeric B72.3 (Human IgG1), Cancer Res 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 09026-03 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Characterization of Human Tumor-Associated Antigens

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

| | | |
|---|---|---|
| Philip D. Fernsten | Expert | LTIB, DCBDC, NCI |
| Jeffrey Schlom | Chief | LTIB, DCBDC, NCI |

COOPERATING UNITS *(if any)*

Dr. Andrew Raubitschek, Radiation Oncology Branch, Division of Cancer Treatment, NCI

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 1.1 | 1.1 | 0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Monoclonal antibody B72.3, which has been extensively characterized as to reactivity with a variety of carcinomas versus normal tissues, reacts with a mucin-like glycoprotein. The B72.3-reactive antigen, designated TAG (tumor-associated glycoprotein) -72, was partially purified and used as immunogen to produce 18 second generation anti-TAG-72 antibodies. Based on competitive binding studies, a number of the epitopes recognized by the anti-TAG-72 antibodies appear to be structurally or spatially related, but none appear to be identical. All of the anti-TAG-72 antibodies produced immune precipitate lines with purified antigen in double immunodiffusion assays, indicating that each epitope recognized by the antibodies is expressed in multiple copies on the TAG-72 molecule. All of the epitopes are sensitive to mild periodate oxidation but differ in their sensitivities to neuraminidase, and all of the epitopes have been shown to be expressed on the same molecule or population of molecules. One of the second generation antibodies was selected to develop procedures to purify preparative amounts of TAG-72 from xenografted human colon carcinoma cells and from malignant effusions. TAG-72, extracted from xenografted human colon carcinoma cells, was subjected to antibody affinity chromatography, size exclusion chromatography, and ion exchange chromatography. A double determinant radioimmunoassay revealed a greater than 1000-fold purification with a greater than 10% yield. SDS-PAGE analysis of radiolabeled purified TAG-72 revealed a polydisperse, but apparently homogenous, high molecular weight glycoprotein. The density of purified TAG-72, as determined by equilibrium centrifugation in a CsCl gradient, was 1.45 g/ml, indicating a high degree of glycosylation. Compositional analysis of the purified TAG-72 revealed amino acid and carbohydrate profiles and content highly similar to that reported for other purified mucins. TAG-72 purified from malignant effusions was shown to have properties highly similar to the TAG-72 purified from the colon carcinoma xenografts. The colon carcinoma-associated antigen recognized by monoclonal antibody D612 has been shown to be a 48 kilodalton glycoprotein, consisting of a 42 kilodalton polypeptide and three N-linked oligosaccharides. The D612 antigen has also been shown to be structurally and antigenically unrelated to the colon carcinoma-associated antigens defined by monoclonal antibodies CO17-1A, GA733, KS1/4, etc.





GPO 914-918

*Major Findings*

Xenografts of the LS-174T human colon carcinoma cell line have been shown in a double determinant radioimmunoassay to contain levels of the B72.3-reactive antigen, TAG-72, equivalent to the highest levels found in primary and metastatic human colon and breast carcinomas. Western blotting analyses of LS-174T extracts revealed that the antigen had a molecular size distribution highly similar to that of TAG-72 derived from human primary and metastatic breast and colon carcinomas. TAG-72 was partially purified from extracts of LS-174T xenografts by gel filtration on Sepharose CL-4B followed by B72.3 antibody affinity chromatography, and used as immunogen to produce 19 second generation anti-TAG-72 MAbs, designated CC antibodies. Based on competitive binding studies, a number of the epitopes recognized by the anti-TAG-72 MAbs appear to be structurally or spatially related, but none appear to be identical. Immunodiffusion analyses indicate that all of the epitopes are expressed on the same molecule or population of molecules. All of the epitopes are sensitive to mild periodate oxidation and 15 out of 19 are sensitive or partially sensitive to neuraminidase digestion. All of the anti-TAG-72 antibodies produced immune precipitate lines with purified TAG-72 in double immunodiffusion assays, indicating that each epitope recognized by the antibodies is expressed in multiple copies on the TAG-72 molecule.

Due to the high levels of TAG-72 found in LS-174T xenografts as well as its relative ease of availability, LS-174T xenografts were also chosen as the starting material for purification of preparative amounts of TAG-72. CC49, one of the second generation antibodies elicited by partially purified TAG-72, was selected for utilization in a larger scale purification scheme because of its higher affinity of binding to TAG-72 than MAb B72.3. LS-174T xenografts were homogenized in a non-ionic detergent and centrifuged at 10,000 × g and at 100,000 × g. Since TAG-72 is both heat and acid stable, the supernatants were then either extracted at 100 degrees or with perchloric acid. Following cooling or neutralization, the supernatants were centrifuged again, reduced with dithiothreitol, and alkylated with iodoacetamide. This material was then applied to a CC49 affinity column. After washing and elution with 6 molar guanidine HCl, the affinity purified TAG-72 was separated from the bulk of the remaining protein on a Superose 6 column equilibrated with 6 molar guanidine HCl. Final purification was achieved using a DEAE-5PW column equilibrated with 8 molar urea + 20 mM formic acid, pH 3.5. Under these conditions, the remaining contaminating protein failed to bind to the column matrix and the TAG-72 was recovered by elution with NaCl. A purification of greater than 1000-fold with a total yield of greater than 10%, as determined by amino acid analyses and double determinant radioimmunoassays, was achieved using this protocol. Autoradiographs of radioiodinated purified TAG-72 separated on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) showed a high molecular weight polydisperse, but apparently homogenous, band. Similar results were obtained by periodic acid-Schiff's reagent staining or Western blotting analyses using CC49 immunostaining of purified TAG-72 run on SDS-PAGE. The purified TAG-72 preparations did not contain material that stained with Coomassie blue. Compositional analysis of the purified TAG-72 revealed that the molecule consisted of approximately 80% carbohydrate by weight, primarily galactose, N-acetylglucosamine, N-acetylneuraminic acid, fucose, and N-acetyl-galactosamine. Amino acid analyses of the purified TAG-72 revealed a profile highly enriched in serine, threonine, glycine, proline, and glutamine + glutamate and containing only trace amounts of methionine, tyrosine, and phenylalanine. This composition is highly consistent with that reported for other purified mucins. TAG-72 has also been purified from malignant effusions of patients with different types of carcinomas and has been shown to have physical and serological properties highly similar to those of TAG-72 derived from the human colon carcinoma xenografts. Approximately 97% of the carbohydrate has been removed from purified TAG-72 using trifluoromethane sulfonic acid, and discrete peptides generated from this material will be subjected to amino acid sequence analysis.

The colon carcinoma-associated antigen recognized by monoclonal D612 has been analyzed in immunoprecipitates of radiolabeled cell extracts and has been shown to be a 48 kilodalton cell surface glycoprotein. The results of radiolabeling in the presence of tunicamycin and of endoglycosidase digestions of the immunoprecipitates reveal that the antigen consists of a 42 kilodalton polypeptide with three N-linked oligosaccharides. The epitope recognized by D612 consists of polypeptide only and appears to be conformation specific. The cysteine content of the antigen and its increased electrophoretic mobility under nonreducing conditions suggest that it possesses domain structure but not subunit structure. The D612 antigen binds to Concanavalin A, binds weakly to *Lens culinaris* agglutinin, and fails to bind to soybean agglutinin, *Ricinis communis* agglutinin I, or wheat germ agglutinin. The results of reciprocal immunodepletion studies reveal that the D612 antigen is unrelated antigenically, as well as structurally, to the colon carcinoma-associated antigens defined by monoclonal antibodies CO17-1A, GA733, and KS1/4.

*Publications*

Muraro R, Kuroki M, Wunderlich D, Poole DJ, Colcher D, Thor A, Greiner JW, Simpson JF, Molinolo A, Noguchi P, Schlom J. Generation and characterization of B72.3 second generation monoclonal antibodies reactive with the tumor-associated glycoprotein 72 antigen, Cancer Res 1988;48:4588-4596.

Sheer DG, Schlom J, Cooper HL. Purification and composition of the human tumor-associated glycoprotein (TAG-72) defined by monoclonal antibodies CC49 and B72.3, Cancer Res 1988;48:6811-6818.

Kuroki M, Fernsten PD, Wunderlich D, Colcher D, Simpson DF, Poole DJ, Ooley TG, Schlom J. Serologic mapping of the TAG-72 tumor-associated antigen employing 19 distinct monoclonal antibodies, Cancer Res 1990, in press.

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PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 09006-08 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Studies on the Nature and Function of the Phosphoprotein, Prosolin

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

| | | |
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| Rebecca Fuldner | Biotechnology Research Fellow | LTIB, DCBDC, NCI |
| Richard Braverman | Chemist | LTIB, DCBDC, NCI |

COOPERATING UNITS *(if any)*

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| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 3.0 | 2.5 | 0.5 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have extensively characterized prosolin, a major cytosolic protein of proliferating HL-60 promyelocytic leukemia cells that undergoes very rapid phosphorylation in response to treatment of the cells with tumor-promoting phorbol esters like TPA as an early step in a pathway leading to cessation of cell growth and onset of monocytoid differentiation. Prosolin has been identified in proliferating human peripheral blood lymphocytes (PBL) by tryptic peptide mapping of proteins isolated from two-dimensional electrophoretic gels. While virtually absent from resting PBL, prosolin expression is induced by mitogens as a late event, during the onset of S-phase. Its level of synthesis correlates with the prevalent level of DNA synthesis. Prosolin undergoes rapid phosphorylation in proliferating PBL in response to TPA or A23187 treatment, together with an inhibition of DNA synthesis. Phosphorylation of prosolin may be part of the mechanism by which naturally occurring growth inhibitory factors terminate normal lymphocyte proliferation. T-cell leukemia cell lines show a marked reduction in the phosphorylation of prosolin in response to A23187 and other agents. We are cloning and sequencing cDNAs to prosolin. Partial amino acid sequence data were obtained by analysis of tryptic peptides of prosolin isolated from two-dimensional electrophoretic gels. Degenerate oligonucleotide probes were synthesized and used to probe a lymphoblast cDNA library.

*Major Findings*

Prosolin is a major cytosolic protein of proliferating HL-60 promyelocytic leukemia cells that undergoes very rapid phosphorylation in response to treatment of the cells with tumor-promoting phorbol esters like TPA. We have characterized this protein extensively and have shown that its TPA-induced phosphorylation is an early step in a pathway leading to cessation of cell growth and onset of monocytoid differentiation in HL-60 cells.

Prosolin has now been identified in proliferating human peripheral blood lymphocytes (PBL) by tryptic peptide mapping of proteins isolated from two-dimensional electrophoretic gels. While virtually absent from resting PBL, prosolin expression is induced by mitogens as a relatively late event, during the onset of S-phase. Once induced in rapidly proliferating lymphocytes, prosolin becomes a major cytosolic protein and its level of synthesis correlates with the prevalent level of DNA synthesis. As in HL-60, prosolin undergoes rapid phosphorylation in proliferating PBL in response to TPA treatment, an effect which correlates with an inhibition of DNA synthesis. Thus, prosolin may play a necessary role in the S-phase of the cell cycle, and its phosphorylation may be part of the mechanism by which lymphocytes exit from S-phase and suspend proliferation. We are exploring the possibility that phosphorylation of prosolin may be part of the mechanism by which naturally occurring growth inhibitory factors terminate normal lymphocyte proliferation.

Rapid phosphorylation of prosolin has also been demonstrated upon treatment of lymphocytes with calcium ionophore (A23187), lymphocyte mitogens (PHA), and anti-CD3 monoclonal antibodies (which activate the T-cell receptor). Production of 4 distinct phosphorylated forms of the molecule was detected. Prosolin is evidently a target of the phosphorylation activities induced by activation of the T-cell receptor. Since this molecular target only occurs in proliferating cells, it provides the opportunity for the cell to respond differently to T-cell receptor activation depending on whether it is in the resting or proliferating state. This may have important implications for normal regulation of lymphocyte growth and may be the basis for the observation that T-cell receptor activation in resting cells provides a proliferative signal while similar activation in proliferating cells causes down-regulation of DNA synthesis.

Our studies suggest that different protein kinases are activated by the various phosphorylation-inducing agents, resulting in the production of the various forms of prosolin. Analysis of these protein kinases is in progress.

We have found that T-cell leukemia cell lines are markedly defective in their ability to phosphorylate prosolin in response to calcium ionophore, while they generally retain their ability to respond to TPA. They also have a reduced ability to down-regulate DNA synthesis in response to calcium ionophore. This may indicate a deficiency in the pathway by which calcium activates this kinase. The defect may relate to the loss of normal cell growth regulation in the leukemic cells.

We are currently cloning and sequencing cDNAs to prosolin. Partial amino acid sequence data were obtained by analysis of tryptic peptides of prosolin isolated from two-dimensional electrophoretic gels. Degenerate oligonucleotide probes were synthesized and used to probe a lymphoblast cDNA library. Positive clones are currently being analyzed. Deduced amino acid sequence information may allow us to compare the structure of prosolin with that of other proteins so as to improve our understanding of its function and the control of its phosphorylation. Full length cDNAs to prosolin will facilitate Northern and Southern blot analysis of prosolin expression and genomic organization and will permit production of recombinant prosolin. This will allow us to produce monoclonal antibodies, to conduct detailed phosphorylation studies, and to perform biochemical and molecular biological experiments on the function of prosolin.

Longer term projects in this area include more precise examination of the cell-cycle specificity of the synthesis and phosphorylation of prosolin, and attempts to clarify its function by studying its interactions, *in vivo* and *in vitro*, with other molecules. Finally, the possible diagnostic and prognostic significance of the reduced phosphorylation response of prosolin to TPA and other agents in lymphoid leukemia cells will be explored.

*Publications*

Cooper HL, Fuldner R, McDuffie E, Braverman R. A specific defect of prosolin phosphorylation in T-cell leukemic lymphoblasts is associated with impaired down-regulation of DNA synthesis, J Immunol, in press.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 09022-04 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*


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COOPERATING UNITS *(if any)*

David S. Salomon, LTIB

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|---|---|---|
| 2.0 | 1.5 | 0.5 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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


We are examining the relationship between neoplastic transformation and biochemical derangements of expression or utilization of tropomyosins (TMs). In NIH/3T3 fibroblasts transformed by a number of related and unrelated retroviral oncogenes, expression of the transformed phenotype was reproducibly correlated with suppression of synthesis of two specific TM isoforms ("muscle type"). Of all the proteins detectable on two-dimensional gel electrophoresis of whole-cell proteins, only the TMs behaved in this way. Treatment of rat fibroblasts with tumor growth factor-α (TGF-α), in addition to inducing the transformed phenotype, specifically suppressed synthesis of muscle-type TMs and inhibited their utilization in the cytoskeleton, strengthening the connection between suppression of TM and expression of the transformed phenotype and adding support at the biochemical level for an autocrine pathway in oncogene action. The findings point out the close biochemical relationship between epidermal growth factor (EGF) receptor activation and microfilament organization. Tropomyosin expression in relation to oncogene expression and neoplasia is being studied in murine and human epithelial cell systems. In mouse mammary epithelial cells transformed by the Ha-*ras* oncogene, the ratio of TM to actin accumulating in the cytoskeleton was reduced, indicating the production of microfilaments deficient in TM. The TM isoforms expressed by normal diploid human mammary epithelial cells have been compared with the pattern expressed by breast carcinoma cell lines. Defects in expression of particular TM isoforms occurred in all of the breast carcinoma cell lines studied, suggesting a possible role of deranged TM expression in human neoplasia.

*Major Findings*

We are examining the relationship between neoplastic transformation and biochemical derangements of expression or utilization of tropomyosins (TM), a family of microfilament-associated proteins now known to occur in all cell types. TMs are an essential component of microfilaments, present in a strict stoichiometric relationship to the major microfilament protein, actin. In a study of NIH/3T3 fibroblasts transformed by a number of related and unrelated retroviral oncogenes, we found that expression of the transformed phenotype was reproducibly correlated with suppression of synthesis of two specific TM isoforms ("muscle type"). Of all the proteins detectable on two-dimensional gel electrophoresis of whole-cell proteins, only the TMs behaved in this way. Similar observations were made with NIH/3T3 fibroblasts transformed by chemical mutagens.

Transformed fibroblasts are known to elaborate transforming growth factors, particularly TGF-α, which has been proposed as a mechanism of transformation utilizing an autocrine loop. We found that treatment of rat fibroblasts with TFG-α, in addition to inducing the transformed phenotype, specifically suppressed synthesis of muscle-type TMs and inhibited their utilization in the cytoskeleton. This further strengthened the connection between suppression of TM and expression of the transformed phenotype and added support at the biochemical level for an autocrine pathway in oncogene action. The findings point out the close biochemical relationship between EGF receptor activation and microfilament organization.

Since most human malignancies occur in cells of epithelial rather than fibroblast lineage, we examined a murine epithelial cell model system. Immortalized "normal" mouse mammary epithelial cells transformed by the Ha-*ras* oncogene showed no reduction in TM synthesis. However, the synthesis of actin was increased, and the ratio of TM to actin accumulating in the cytoskeleton was reduced, indicating the production of microfilaments deficient in TM. Thus, TM deficiency in microfilaments may be the disturbance of TM metabolism that is common to transformation in both fibroblasts and epithelial cells.

The relevance of disturbed TM expression to human carcinoma is being studied in cultured lines derived from human breast carcinomas. The array of TM isoforms expressed by normal diploid human mammary epithelial cells has been characterized and compared with the pattern expressed by cell lines derived from breast carcinoma. Defects in expression of particular TM isoforms, homologous to the muscle-type TMs suppressed in transformed fibroblasts, occurred in all of the breast carcinoma cell lines studied. We plan to extend these studies to fresh specimens of normal and neoplastic breast tissue to determine whether such defects occur in tumors as well as in established tumor cell cultures. Other studies will examine the effect of transforming oncogenes on TM expression in mammary epithelial cells to determine if defects observed in breast cancer tissues and cell lines resemble those induced by transforming oncogenes. This will help clarify the question of the prevalence of oncogene-related mechanisms in carcinogenesis.

In related work, we are developing cDNA clones specific for the TMs expressed by epithelial cells. These will serve as probes in studies of expression of the various TM isoforms and in elucidating the mechanisms controlling them. They will also be used in Southern blot analyses of DNAs from breast cancer cells to determine whether deletion or rearrangement of TM genes is responsible for altered expression of particular isoforms. Together with the preceding study, the information to be obtained will furnish perspective on the question of the relative importance of mutagenic *versus* oncogene-related processes in carcinogenesis.

To determine whether disturbed TM expression plays a causal role in transformation, we have begun a study aimed at specifically suppressing TM

synthesis by the use of genetic insertions that produce antisense RNA sequences to specific TM transcripts. A full length cDNA encoding TM1 has been introduced, in both sense and antisense orientations, into the retroviral vector, pBNC. These constructs are being used for both transfection and infection of NIH3T3 cells to determine the effect of antisense expression on TM production and on cell growth characteristics. Conversely, these same constructs are being used to enhance expression of TM1 in the retrovirally transformed DT cell line in order to determine whether such enhanced expression of TM1 will alter cell growth characteristics toward normality.

*Publications*

Bhattacharya B, Prasad GL, Valvarius EM, Salomon DS, Cooper HL. Tropomyosins of human mammary epithelial cells: consistent defects of expression in mammary carcinoma cell lines, Cancer Res 1990;50:2105-2112.

Yanagihara K, Ciardiello F, Talbot N, McGready M, Cooper HL, Benade L, Salomon DS, Bassin RH. Isolation of a new class of "flat" revertants from *ras*-transformed NIH/3T3 cells using *cis*-4-hydroxy-L-proline, Oncogene, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 04848-18 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

| | | |
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| R. Bassin | Chief, Biochemistry of Oncogenes Sect. | LTIB, DCBDC, NCI |
| B. Blondel | Visiting Fellow | LTIB, DCBDC, NCI |
| D. Salomon | Research Biologist | LTIB, DCBDC, NCI |
| R. Callahan | Chief, Oncogenetics Section | LTIB, DCBDC, NCI |
| N. Talbot | Biochemistry of Oncogenes Section | LTIB, DCBDC, NCI |

COOPERATING UNITS *(if any)* Dr. Len Benade, Head, Virus Group, American Red Cross, Rockville, MD; Dr. Arie Moran, Ben Gurion University of the Negev, Israel; Dr. Makoto Noda, Riken Institute of Science, Japan; Dr. Yanagihara, Hiroshima University, Japan

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Biochemistry of Oncogenes

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| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 5.2 | 3.2 | 2.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have developed a new technique for isolating cells that resist transformation by specific oncogenes using 4-*cis*-hydroxy-L-proline as a selective agent. We have begun to characterize additional revertants from *ras*-transformed cells. The new revertants are resistant to transformation by members of the *ras* gene family and to certain tyrosine kinase oncogenes, but they are sensitive to retransformation by *mos*, *raf*, and *fos*. Combining the retransformation data observed here with those from our ouabain-derived revertants isolated previously (C-11 and F-2), we find that all oncogenes can be placed into one of four groups, and we suggest that this represents a primitive functional classification. If true, the *ras* gene would require the role of at least two cellular 2 functions (such as attachment to the cell membrane and kinase activity) to successfully transform mouse cells.

Transfection of a cDNA library prepared from one of the revertants, CHP 9CJ, has resulted in the isolation of several DNA sequences that may be associated with the revertant phenotype. One of these sequences may be identical to the K-rev-1 gene previously isolated from *ras*-resistant human cells.

*Major Findings*

The goal of this project is a fundamental understanding of the mechanisms by which specific oncogenes transform cells, through the isolation and analysis of cells genetically resistant to transformation. A major effort during the past several years has involved the isolation and analysis of "flat" revertant cells with defects in cellular genes or proteins that are necessary for transformation by selected oncogenes. We have previously isolated two such revertant cell lines with ouabain as a selective agent. These revertants have been used to separate oncogenes that are able to transform a given revertant from onocogenes that cannot. From our previous work, it seems likely that revertants resist transformation because they have defects in an as yet unknown cell-encoded pathway necessary for transformation by the *ras* gene and by other oncogenes that use the same pathway--this interpretation implies that the oncogenes behave differently because they are functionally different. If this is true, then isolation of additional revertants with defects in different pathways could be used to define major functional groups of oncogenes. Since these two initial revertants (C-11 and F-2) exhibited identical patterns of susceptibility and resistance when tested in retransformation assays with a panel of oncogenes, we attempted to isolate additional oncogenes with different behavior patterns. Since the properties of each revertant cell line might reflect the type of selective procedure used in its isolation, we developed a new selective procedure using *cis*-hydroxyproline (CHP). With CHP as the selective agent, two similar revertants were isolated. The new CHP-derived revertants exhibit a very different resistance pattern than the original ouabain-derived revertants.

If we combine the data from the ouabain-resistant revertants with that from the new CHP-derived revertants, the following pattern emerges:

RESISTANCE TO CHP-DERIVED REVERTANTS

| RESISTANCE TO OUABAIN-DERIVED REVERTANTS | | yes | no |
|---|---|---|---|
| | yes | Ha-*ras* Ki-*ras* N-*ras* *bas* 1 | *src* *abl* *fgr* *fes* 2 |
| | no | *raf* *fos* *mos* 3 | *fms* *sis* 4 |

Thus, these two sets of revertants can be used to define four groups of oncogenes, hopefully according to the cellular pathways with which they interact. Therefore, despite an almost unlimited number of oncogenes and growth factors, there appear to be a relatively limited number of pathways involved in cell transformation. Note that, except for *fms* and *sis*, each of which may act through its own unique receptor pathway (the *sis* protein p28 is identical to the B chain of PDGF, and *fms* is related to the CSF-1 receptor), all of the tyrosine kinase-encoding oncogenes fall into the same group. This indicates that they all use the same pathway to transform cells and that tyrosine kinase activity is a critical function in this process. The group labeled "1" contains only the *ras*-related genes. To account for the ability of *ras*-derived resistant cells to suppress transformation by other oncogenes and to explain some additional data of Smith *et al.*, using micro-injection of antibodies against $p21^{ras}$, it can be assumed that $p21^{ras}$ interacts with the cellular pathway involved in transformation at a point distal to the action of many other oncogene products.

In an extension of our studies with the ouabain revertants resistant to transformation by the *ras* oncogene, Dr. Makoto Noda, a former Visiting Fellow in this section, has recently isolated sequences from a cDNA library described made by Chen and Okoyama from normal human fibroblasts that have the capability of suppressing transformation by *ras* in mouse cells. When sequenced, this activity turns out to be associated with a gene, K-*rev* 1, that is distantly related to the *ras* gene itself and makes its own version of $p21^{ras}$. Thus, the human cells may be resistant to transformation by *ras* because they have a p21-related product that successfully competes with authentic p21 for the immediate cell-encoded "target" used by the *ras* gene product to transform cells. Other explanations for the activity of K-*rev* 1 are possible.

Although K-*rev* 1 does not seem to involve a change in the cellular transformation pathway as we had hoped, this result clearly indicates that we can identify and isolate cDNA sequences capable of reversing the transformed phenotype using the methods we have developed. Because of Dr. Noda's work, which has been confirmed by Drs. Chardin and Tavitian in Paris, we will change our original strategy of transfecting genomic DNA from revertants and will now concentrate on efforts to generate cDNA libraries with revertant activity. We also have isolated several new resistant cell lines that should be useful in generating subtraction cDNA libraries enriched for genes that can induce the revertant phenotype. These include (1) revertant cDNA-NIH/3T3 RNA or transformed NIH/3T3 DNA; (2) revertant cDNA-RNA extracted from re-revertants; and (3) revertant cDNA-RNA from azacytidine-treated revertant cells (revertants treated with azacytidine become stably transformed by an as yet unknown mechanism, perhaps involving a loss of expression of the target gene cell-encoded "target" gene).

Transfection into *ras*-transformed cells of a cDNA library prepared from CHP 9CJ extracts results in rare colonies of nontransformed cells that are resistant to transformation by *ras*. We have isolated 3 different sequences from such colonies. One of them shows homology with the K-*rev* 1 gene previously isolated from resistant human cells. The other 2 sequences, which appear as single copy genes in CHP 9CJ cells, are currently under study.

*Publications*

Ciardiello F, Kim N, Valverius EM, Colucci-d'Amato G, Bassin RH, Salomon DS. Differential growth factor expression in transformed NIH-3T3 cells, J Cell Biochem, 1990;42-1-13.

Bassin RH, Benade LE. Defining the mechanisms of transformation through analysis of revertant cells. In: Klein, G, ed. Genes that counteract neoplastic transformation, in press.

Blondel B, Talbot N, Merlo GR, Wychowski C, Yokozaki H, Valverius EM, Salomon DS, Bassin RH. Efficient induction of focus formation in a subclone of NIH 3T3 cells by c-*myc* and its inhibition by serum and by growth factors, Oncogene, in press.

Yanagihara K, Ciardiello F, Talbot N, McGeady ML, Cooper H, Benade L, Salomon DS, Bassin, RH. Isolation of a new class of "flat" revertants from *ras*-transformed NIH/3T3 cells using *cis*-4-hydroxy-L-proline, Oncogene, in press.

PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 09003-08 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

The Role of TGFα in the Etiology and Progression of Breast Cancer

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

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| Fortunato Ciardiello | Visiting Fellow | LTIB, DCBDC, NCI |
| Toshiaki Sacki | Visiting Fellow | LTIB, DCBDC, NCI |

COOPERATING UNITS *(if any)* Dr. Robert H. Bassin, Chief, Biochem. Onc. Sec., LTIB, DCBD, NCI; Dr. Robert Callahan, Chief, Oncogen. Sec., LTIB, DCBD, NCI; Dr. Marc Lippman, Dir., Lombardi Cancer Ctr., Georgetown University, Washington, DC; Dr. Rik Derynck, Dept. Molecular Biology, Genentech, Inc., San Francisco, CA.

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| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 3.7 | 2.7 | 1.0 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Transforming growth factor alpha (TGFα) has been circumstantially implicated in the autocrine growth of a number of different rodent and human tumor cells *in vitro*. However, its distribution and role in the etiology and/or progression of rodent and human breast cancer are relatively unknown. The present studies have demonstrated that biologically active and immunoreactive TGFα can be detected in hormone (estrogen)-dependent, DMBA- or NMU-induced rat mammary adenocarcinomas and that these tumors possess a specific 4.8-kb TGFα mRNA. These tumors also express elevated levels of c-Ha-*ras* protein (DMBA tumors) or possess a point-mutated c-Ha-*ras* gene (NMU tumors). Ovariectomy results in tumor regression and is preceded by a specific decrease in TGFα mRNA and protein. Transformation of nontransformed mouse mammary epithelial cell lines with a point-mutated c-Ha-*ras* protooncogene results in the loss in mitogenic responsiveness of these cells to exogenous epidermal growth factor (EGF) and a reduction in the number of unoccupied EGF receptors on these cells due in part to an enhanced production and secretion of TGFα. Several human breast cancer cell lines also secrete TGFα and possess TGFα mRNA. In the estrogen-responsive breast cancer cell lines, estrogen can induce a 3- to 5-fold increase in TGFα mRNA and protein. Treatment of these cells with a polyclonal anti-TGFα antibody or with a monoclonal anti-EGF receptor antibody can inhibit the growth of these cells *in vitro*. Elevated levels of TGFα protein or expression of TGFα mRNA can be detected in 50-60% of primary human breast tumors. No evidence was found for any gross amplifications and/or rearrangements of the TGFα gene in these tumors. Additionally, overexpression of a human TGFα gene in a cloned immortalized population of mouse and human mammary epithelial cells can lead to the malignant transformation of these cells *in vitro* and *in vivo*. Collectively, these results suggest that TGFα can function as an autocrine growth factor for a subset of rodent and human breast tumors, that estrogens or activated protooncogenes such as *ras* can enhance the expression of TGFα, and that enhanced production of TGFα can lead to the transformation of mammary epithelial cells that have a sufficient complement of functional EGF receptors.

*Major Findings*

We have demonstrated that primary dimethylbenz(α)anthracene (DMBA)- or nitrosomethylurea (NMU)-induced rat mammary adenocarcinomas produce biologically active TGFα that can compete in an EGF radioreceptor assay for binding to the EGF receptor and elaborate an equivalent amount of immunoreactive TGFα. In addition, a specific 4.8-kb TGFα mRNA species could be detected in these tumors after Northern blot hybridization with a labeled nick-translated human TGFα cDNA insert. The primary, carcinogen-induced rat mammary tumors are estrogen dependent for their growth *in vivo* and are estrogen responsive *in vitro*. Ovariectomy results in a rapid and specific reduction in the levels of TGFα mRNA and bioactive TGFα protein in the tumors within 6-12 hr post-ovariectomy. Furthermore, estrogen treatment of primary cultures of DMBA or NMU rat mammary epithelial cells results in a 3- to 4-fold increase in the levels of TGFα mRNA. These results demonstrate that the levels of TGFα in a subset of rat mammary tumors may be regulated by estrogens *in vivo* and *in vitro*. The DMBA-induced rat mammary tumors express elevated levels of c-Ha-*ras* mRNA and $p21^{ras}$ protein, whereas the NMU-induced tumors possess a point-mutated c-Ha-*ras* gene. In immortalized rodent fibroblasts transformed by *ras*, there is generally an enhanced level of production of TGFα that can be detected in these cells. To determine whether a similar phenomenon may occur in mammary epithelial cells, we have examined the levels of TGFα production in two immortalized mouse mammary epithelial cell lines transformed with a point-mutated, human c-Ha-*ras* protooncogene. We feel that this may be particularly relevant to human breast cancer, because approximately 60-70% of primary human breast tumors express elevated levels of c-Ha-*ras* mRNA or $p21^{ras}$ protein. Mouse mammary NMuMg cells or cloned NOG-8 cells were transfected with the point-mutated c-Ha-*ras* gene in plasmids containing either an SV40 promoter (NMuMg) or the glucocorticoid-inducible mouse mammary tumor virus (MMTV) promoter (NOG-8). After transformation of the NMuMg cells with the SV40 plasmid in which overexpression of the point-mutated c-Ha-*ras* gene is constitutive, there was a 3- to 4-fold increase in the levels of TGFα that could be detected in the conditioned medium (CM). Likewise, after dexamethasone treatment of NOG-8 cells that are carrying the MMTV-inducible construction, there was a rapid increase in the levels of a 1.4-kb specific c-Ha-*ras* mRNA and $p21^{ras}$ protein within 3-12 hr. During the same interval, there was a concomitant increase in the levels of TGFα mRNA that was followed by a subsequent elevation in the amounts of TGFα in the CM. Normally, NMuMg or NOG-8 cells are mitogenically responsive to exogenous EGF in monolayer cultures and will grow in soft agar to a limited degree in response to exogenous EGF or TGFα. However, after *ras* transformation, the cells become refractory to these exogenously added growth factors. This may be due in part to the enhanced levels of endogenous TGFα that are secreted and occupy cell surface EGF receptors on these cells. This may occur because the number of unoccupied EGF receptors on these cells is reduced by 80-90%, suggesting that there has been a chronic receptor down-regulation induced by overexpression of *ras*. There is also an increase in TGFβ and insulin-like growth factor type I expression following *ras* transformation in the NOG-8 cells, suggesting that a spectrum of complementing growth factors are induced in mammary epithelial cells by this activated protooncogene and that part of the enhanced anchorage-dependent and -independent growth may be mediated through these growth factors.

TGFα is not entirely restricted to malignant rodent mammary epithelial cells. Biologically active and immunoreactive TGFα (range 0.5 to 50 ng/$10^8$ cells/48 hr) has been detected in the CM obtained from the human breast cancer cell lines MCF-7, ZR-75-1, T47-D, and MDA-MB-231. In the estrogen-responsive and estrogen receptor (ER)-positive and progesterone receptor (PgR)-positive breast cancer cell lines MCF-7, ZR-75-1, and T47-D, 17-β estradiol ($10^{-8}$ M) produced a 3- to 5-fold increase in the CM levels of TGFα within 24 hr after treatment of the cells. Preceding this increase in secreted TGFα protein, there was a corresponding elevation in the level of expression of a major 4.5- to 4.8-kb TGFα mRNA species and a minor 1.6-kb transcript that could be detected within 6 hr. Optimal induction occurs between $10^{-9}$ and $10^{-8}$ M estrogen. Biosynthetic labeling of MCF-7

or MDA-MB-231 cells with [$^{35}$S]cysteine followed by immunoprecipitation of the culture medium with a specific polyclonal rabbit anti-TGFα antibody demonstrated the presence of several high molecular weight TGFα species between 17,000 and 30,000. Treatment of several of these breast cancer cell lines with a neutralizing polyclonal anti-TGFα antibody or with a blocking mouse monoclonal anti-EGF receptor antibody produced a 60-70% inhibition in the anchorage-dependent and -independent growth of these cells, suggesting that this growth is functioning through the EGF receptor in an autocrine fashion to regulate the proliferation of these cells. To ascertain if overexpression of the TGFα gene in a population of nonmalignant, immortalized mammary epithelial cells is sufficient to induce their neoplastic transformation, we co-transfected an SV40-human TGFα cDNA expression vector plasmid and the pSV2*neo* plasmid into the NOG-8 mouse mammary epithelial cell line. After selection with G418 for several weeks, we isolated a number of G418-resistant clones. Analysis of the CM from a number of these isolates demonstrated that several clones were secreting high levels of TGFα in the range of 200 to 600 ng/$10^8$ cells/48 hr as compared with 5-10 ng/$10^8$ cells/48 hr for the parental nontransfected or only the pSV2*neo*-transfected cells. Those clones that were secreting elevated levels of the growth factor grew aggressively in soft agar and formed undifferentiated, invasive carcinomas when injected into nude mice at a frequency of 80-90% within a period of 2-3 weeks. Additionally, there was an 80-90% reduction in the number of free, unoccupied EGF receptors on these cells compared with the parental NOG-8 cells. Neutralizing mouse monoclonal anti-TGFα antibodies were able to produce a 60-70% inhibition in the soft agar growth of these cells. Collectively, these results demonstrate that overexpression of this growth factor in an immortalized population of mammary epithelial cells that also possesses a sufficient complement of functional EGF receptors is capable of transforming these cells.

We have also initiated a series of studies to ascertain whether TGFα is produced by primary human breast carcinomas and to determine whether there are any genomic alterations in this gene within these tumors. In detergent extracts prepared from normal or benign mammary tissues and from 22 primary infiltrating ductal carcinomas, the immunoreactive TGFα concentrations were found to range from 1.5 to 6 ng/mg cell protein, with approximately 50% of the breast tumors possessing TGFα levels that were >2.5 ng/mg cell protein, the level that was at the upper end for the normal and benign tissues. We also analyzed 54 infiltrating ductal carcinomas for TGFα mRNA expression. Approximately 50-70% of these tumors obtained from two separate clinical sources expressed a detectable 4.8-kb TGFα mRNA species. However, no significant association could be discerned between TGFα mRNA expression and ER and/or PgR status, axillary lymph node involvement, c-Ha-*ras* expression, and patient relapse due to the recurrence of the primary tumor or distal metastasis. Furthermore, in a total of 79 breast tumors that were analyzed by Southern blot hybridization after digestion of high molecular weight DNA with *Bam*HI, no gross rearrangements or amplifications of the TGFα gene could be detected when compared with the DNA restriction pattern that was obtained from samples of matched peripheral blood lymphocytes from the same breast cancer patients. We have been able to demonstrate the presence of significantly higher levels (approximately 2- to 3-fold) of biologically active and immunoreactive TGFα in the serous effusions (pleural and ascites) from breast cancer patients compared with the levels found in the effusions from noncancer patients. In addition, there appears to be a significant positive correlation between elevated levels of TGFα in these effusions and the presence of two other tumor-associated antigens, CEA and TAG-72. These data suggest that TGFα levels in effusions may serve as a prognostic marker for breast cancer recurrence.

*Publications*

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PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 04829-15 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

| | | |
|---|---|---|
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| Giorgio Merlo | Visiting Associate | LTIB, DCBDC, NCI |
| Danielle Liscia | Visiting Associate | LTIB, DCBDC, NCI |
| Craig Cropp | Biotechnology Fellow | LTIB, DCBDC, NCI |
| Kazanubu Fujita | Guest Worker | LTIB, DCBDC, NCI |

COOPERATING UNITS *(if any)* Dr. Carleton Garrett, George Washington Univ. Med. School, Washington, DC; Dr. Rosette Lidereau, Centre Rene Huguenin, St. Cloud, France; Dr. Mariani-Costantini, Univ. of Rome, Italy; Dr. Gregory Campbell, Lab. of Statistics and Mathematical Methodology, NIH; (see attached sheet)

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Oncogenetics Section

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.4 | 4.4 | 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 etiology of human breast cancer is thought to involve a complex interplay of genetic, hormonal, and dietary factors that are superimposed on the physiological status of the host. Attempts to derive a cohesive picture of how these factors participate in the etiology of breast cancer have been confounded by a lack of information on specific mutations associated with the initiation and progression of the disease. We have undertaken a strategy that is aimed at determining, on a molecular level, those genetic alterations in primary breast tumor DNA that have a statistically significant association of patient history, tumor characteristics, and patient postsurgical prognosis. The *int*-2 gene is frequently activated in mouse mammary tumors by the mouse mammary tumor virus (MMTV) *via* insertional mutagenesis. We previously found that *int*-2 is amplified 2- to 15-fold in 14% of primary human breast tumor DNAs (n = 118) and has a significant association ($p < 2 \times 10^{-6}$) with a poor prognosis for the patient. We have now defined the amplification unit on chromosome 11q13 and found that 17 of 18 tumors amplified for *int*-2 were also amplified for *hst* and *bcl*-1. The *hst* and *int*-2 genes are both related to basic fibroblast growth factor. The *bcl*-1 locus defines the translocation t(11;14) breakpoint in B-cell chronic lymphocytic leukemias. In a lymph node metastasis of a breast cancer patient, *int*-2 but not *hst* was expressed. In 10% of the primary breast tumor DNAs (n = 122) the c-*erb*B-2 gene was amplified. The c-*erb*B-2 gene is related to the gene encoding the epidermal growth factor receptor. However, in contrast to one published report, we found no significant association with patient postsurgical prognosis or other aspects of patient history and tumor characteristics. In other studies, we detected the expression of MMTV-related human RNA sequences in breast tumor-derived cell lines and have obtained recombinant cDNA clones of these sequences for further analysis.

Cooperating Units (continued)

Dr. Henry Lynch, Creighton Univ., Omaha, NE; Dr. Fredrick Li, Epidemiology Branch, DCBD, NCI; Dr. Alberto P.M. Cappa, Pathology Service, Ospedale Maggiore S. Giovanni, Turin, Italy; Dr. Bruce Roe, Univ. of Oklahoma, Norman, OK; Dr. Gordon Peters, Imperial Cancer Research Fund, London, England.

*Major Findings*

In previous work on frequently occurring mutations in primary human breast tumor DNA, we found the c-*myc* proto-oncogene amplified in 32%, and the *int*-2 proto-oncogene amplified in 16% of the tumors, respectively. We have further characterized the *int*-2 amplification unit on chromosome 11q13 by testing other closely linked markers. These include the proto-oncogenes c-*sea* and *hst*, the t(11;14) translocation breakpoint *bcl*-1, and the apolipoprotein gene complex APOL-1, as well as the progesterone receptor gene (chromosome 11q21) and the *ets*-1 proto-oncogene (chromosome 11q23). In 17 of 18 tumors containing an *int*-2 gene amplification only *bcl*-1 and *hst* were coamplified. More recently, we have obtained preliminary evidence, by RNA:RNA *in situ* hybridization, suggesting that *int*-2 but not *hst* RNA is expressed in a lymph node metastasis from a breast cancer patient.

We have found that the c-*erb*B-2 proto-oncogene is amplified in approximately 10% of our primary breast tumor panel. Univariant and multivariant analysis of various clinical parameters, including patient prognosis and overall survival, did not reveal a significant association with c-*erb*B-2 amplification. This is in contrast to a recent report by Slamon *et al.* (*Science* 235:177, 1987) claiming that c-*erb*B-2 gene amplification has a highly significant association with relapse and overall survival. This apparent conflict may reflect the absence of certain controls in the case of Slamon *et al.* as well as aspects of their statistical methodology.

In previous work, we identified and characterized a family of human endogenous retroviral genomes (designated HERV-II) that share significant homology with MMTV structural genes. Recently, we have identified two breast tumor cell lines (MDA-MB-468 and MDA-MB-231) that express RNA species (5.0 and 2.0 kb) related to the HERV-II genomes. In addition, the MDA-MB-468 cell line also expresses another novel 1.7-kb RNA species that hybridizes only with HERV-II long terminal repeats. Recombinant cDNA libraries from these cell lines have been constructed and are being screened for HERV-II-related clones.

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PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 05148-11 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Mammary Tumorigenesis in Inbred and Feral Mice

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

| | | |
|---|---|---|
| Robert Callahan | Chief, Oncogenetics Section | LTIB, DCBDC, NCI |
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LAB/BRANCH

Laboratory of Tumor Immunology and Biology

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Oncogenetics Section

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National Cancer Institute, NIH, Bethesda, MD 20892

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|---|---|---|
| 2.5 | 1.5 | 1.0 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The study of experimentally induced mammary tumors has focused primarily on several mouse strains that are infected with mouse mammary tumor virus (MMTV) and have been inbred for a high incidence of mammary tumors. We have expanded this study to include other species of MMTV-infected feral mouse strains that have not been bred for high mammary tumor incidence. MMTV appears to induce mammary tumors by acting as an insertional mutagen that leads to the activation of previously silent cellular genes (*int* genes). We have previously shown that the inbreeding program used to develop the high-incidence mouse strains may also have fixed other cellular mutations that affect the frequency with which *int*-1 and *int*-2 are activated. The *int*-1 and *int*-2 genes are much less frequently activated in feral mouse mammary tumors, raising the possibility that other *int* loci are active in these tumors. We described one such locus, designated *int*-3, in CZECHII $V^+$ feral mice. The organization of *int*-3 differs from that of *int*-1 or *int*-2. MMTV insertions occur within a 500-bp region of the cellular genome on chromosome 17. Viral insertion at this locus leads to the activation of the expression of two cellular RNA species that correspond to host flanking sequences located 5' and 3' of the viral insertion site, respectively. We are currently determining the nucleotide sequence of recombinant cDNA clones corresponding to each of these RNA species. Mice chronically infected with MMTV frequently contain preneoplastic hyperplastic alveolar nodules (HAN), which can be transplanted as hyperplastic outgrowth (HOG) lines. We have found that in three of four CZECHII HOG lines, *int*-1 is occupied by an MMTV genome. This suggests that activation of *int*-1 is an early event in MMTV-induced mammary tumorigenesis. Human breast tumors frequently express tumor-associated antigens. One of these, the carcinoembryonic antigen (CEA) is a member of the family of related glycoproteins that are expressed normally in fetal tissue, the placenta, and other adult tissues. We have found that MMTV-induced CZECHII $V^+$ mammary tumors express a 4.0-kb RNA species that is related to human CEA.

*Major Findings*

We have previously found that 18% of CZECHII $V^+$ mammary tumors ($n$ = 44) contain a MMTV proviral genome integrated at the *int*-3 locus. MMTV integration at *int*-3 activates the expression of a 6.5- and 2.6-kb RNA species corresponding to cellular sequences located 5' and 3', respectively, of the viral insertion site. In two of the tumors, however, only the 2.6-kb RNA species was detected. In these tumors the MMTV proviral genome at the *int*-3 locus was truncated, containing only long terminal repeat related sequences. At the present time, we are determining whether or not the cellular sequences located 5' of the viral insertion site are expressed in these tumors. We have obtained recombinant cDNA clones of portions of the 6.5- and 2.6-kb *int*-3 RNA species and are now determining their nucleotide sequences.

In *M. spretus* (MS) and CZECHII $V^+$ mammary tumors, the *int*-1 and *int*-2 genes are activated at a significantly lower frequency (*int*-1 0% and 24% respectively, *int*-2 2% and 6% respectively) than in mammary tumors of high-incidence mouse strains. This may reflect differences in the tropism of the feral mouse MMTV strains for subpopulation of mammary epithelium that are sensitive to the activation of other *int* loci. To test this possibility, we have developed new mouse strains by foster nursing BALB/c and CZECHII $V^-$ newborns on MS mice and BALB/c newborns on CZECHII $V^+$ mice. We have immunological evidence of virus transmission in each of these foster-nursed mouse strains and have begun to collect mammary tumors to determine the frequency with which the known *int* genes are activated. If the frequency of *int* gene activation is the same as in the virus donor strain, it would strongly suggest that the frequency of *int* gene activation is, in part, a function of the particular virus strain.

Mammary epithelium of mice chronically infected with MMTV frequently contain preneoplastic lesions termed hyperplastic alveolar nodules (HANs). HANs are composed of immortalized cells capable of repopulating mammary fat pads that have been cleared of epithelium. HANs transplanted in this manner are referred to as hyperplastic outgrowth (HOG) lines. We have found that the *int*-1 locus in three of four CZECHII HOG lines is occupied by an acquired MMTV proviral genome. This suggests that activation of *int*-1 represents an early event in mammary tumorigenesis which contributes to the immortalization of mammary epithelium.

CEA is frequently expressed in primary human breast tumors as well as other neoplasias. It is a member of a family of related high molecular weight glycoproteins, some of which are expressed in fetal tissue, the placenta, and certain normal adult tissues. Using a recombinant cDNA probe representing the 3' portion of the human CEA gene, we have detected a related 4.0-kb species of RNA in MMTV-induced CZECHII $V^+$ mammary tumors. We have obtained a recombinant cDNA clone representing a portion of the mouse CEA-related RNA and are presently determining its nucleotide sequence.

*Publications*

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PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 09023-04 LTIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Cloning of Anti-Tumor Antigen Immunoglobulin Genes

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

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| Benjamin Calvo | Staff Fellow | LTIB, DCBDC, NCI |
| Anna Maria Masci | Visiting Fellow | LTIB, DCBDC, NCI |
| Limming Shu | Staff Fellow | LTIB, DCBDC, NCI |
| Robert Callahan | Chief, Oncogenetics Section | LTIB, DCBDC, NCI |
| Jeffrey Schlom | Chief | LTIB, DCBDC, NCI |

COOPERATING UNITS (if any)

Dr. Mark Bodmer, Celltech, Inc., England

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Laboratory of Tumor Immunology and Biology

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Oncogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 5.2 | 4.2 | 1.0 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The main objective of this research project is to genetically engineer immunoglobulin genes to generate useful reagents for *in vivo* localization and therapy of human tumors. Several hybridoma cell lines have been developed in this laboratory that produce monoclonal antibodies (MAbs) that selectively react with tumor-associated antigens. These include the carcinoembryonic antigen and a tumor-associated glycoprotein, TAG-72, which is present in a variety of carcinomas. The MAbs are currently being used in a number of diagnostic and therapeutic trials on breast, colon, and ovarian cancers. In clinical tests, one such MAb, B72.3, which was raised against a membrane-enriched extract of a human metastatic breast carcinoma and recognizes TAG-72, has shown promise for being developed into a diagnostic and therapeutic agent. The usefulness of mouse MAbs for *in vivo* therapy and diagnosis of human tumors, however, is limited because of their immunogenicity in patients. This problem can be reduced by replacing the constant region of the mouse antibody with the constant region of the human antibody, using recombinant DNA techniques. To that end, we have cloned the rearranged heavy and 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. They were inserted separately into appropriate expression vectors. Stable chimeric antibody-producing cell lines were developed by introducing these constructs into both fibroblast and myeloma cell lines. Chimeric antibody produced either from cDNA clones or clones of the rearranged genes retained specificity and binding properties of the parental antibody.

*Major Findings*

We have prepared mouse-human chimeric B72.3 antibodies from cells transfected with expression constructs of chimeric cDNA or genomic B72.3 clones.

A cDNA library was constructed, in λt10 vector, using cDNA synthesized from mRNA isolated from the hybridoma cell line, B72.3. The library was screened with radiolabeled probes specific to the CH1 domain of mouse γ1 and Ck sequences. Two *Eco*RI fragments, one of 980 bp and the other of approximately 1700 bp, containing intact coding sequences for leader, variable, and constant regions of light (κ) and heavy chains, respectively, were detected and cloned. The *Eco*RI cDNA inserts were subcloned in M13 vectors, and the variable regions of heavy and κ chain clones were sequenced by the chain termination procedure of Sanger. Chimeric mouse-human constructs were generated by replacing the constant region of B72.3 heavy and κ chain cDNA clones with the rearranged human γ4 and human Ck region, respectively. In order to make a chimeric mouse-human heavy chain construct, a *Hind*III site was created by site-directed mutagenesis at the start of the CH1 exon of a genomic clone of human Cγ4. The B72.3 variable region of heavy chain cDNA clone, which has a *Bgl*II site near its 3' end, was fused to the human Cγ4 region using a *Bgl*II-*Hind*III synthetic linker. Construction of mouse-human light chain clone was straight forward because both mouse and human Ck regions have a site for *Mbo*II 18 bp downstream from the junction of variable and constant domains. The chimeric genes were inserted separately in an expression vector that contains a strong promoter-enhancer transcriptional control element from human cytomegalovirus. The expression constructs were introduced either singly or in heavy/light chain gene pairs in COS-1 cells that allowed high transient expression of the chimeric antibody molecules. An antigen-based ELISA assay showed that the chimeric antibody molecules retained the specific binding characteristics of the parent B72.3 antibody. SDS-PAGE analysis of the supernatant from transfected COS-1 cells labeled with [$^{35}$S]-methionine showed that transiently expressed antibodies were correctly synthesized and assembled in tetrameric form. To develop a stable cell line producing chimeric antibody, an expression construct of the mouse-human chimeric B72.3 light chain gene was introduced into a vector carrying the *neo* resistant gene and was electroporated into CHO-K1 cells. Transfectants were selected in medium supplemented with G418 and screened for light chain producers by an ELISA assay. Expression constructs of chimeric B72.3 heavy chain gene carrying *gpt* as a selectable marker were then electroporated into CHO-K1 cells producing light chain. Mycophenolic acid-resistant colonies were assayed for antibody production by antigen-binding assay. Stable transfectants producing 0.1-40 μg/ml of chimeric antibody were identified. Chimeric antibody produced by the transfectant maintained the tissue binding and idiotypic specificity of the native B72.3 antibody.

As a first step toward cloning the rearranged gene, the unique restriction fragments carrying functionally rearranged genes were to be identified. To that end, Southern blot analysis of genomic DNA of NS1, Balb/c, and hybridoma cell line B72.3 was undertaken. To identify the light chain, a genomic $J_k$ fragment was used as a probe. The rearranged B72.3 light chain gene was identified on a unique 18-kb *Bam*HI fragment of B72.3 genomic DNA. A partially enriched genomic library was prepared from the 18-kb fraction of *Bam*HI-digested B72.3 DNA in EMBL3 vector. The library was screened with both the J region probe and a variable region probe derived from B72.3 light chain cDNA clone. Southern blot analysis of *Eco*RI-digested B72.3 genomic DNA with $J_H$ probe showed 3-kb and 6.5-kb fragments in B72.3 DNA that were not present in NSI and Balb/c genomic DNA fragments. A partially enriched genomic library was constructed from the 3-kb fraction of *Eco*RI-digested B72.3 genomic DNA in λ Zap vector. The library was screened with $J_H$ and variable region probe derived from the cDNA clone. Clones of rearranged light and heavy chain genes were isolated, their inserts were characterized by restriction endonuclease mapping, and they were subcloned in M13 vector for sequencing. Sequence analysis of light and heavy chain gene clones showed their sequences to be identical to the B72.3 light and heavy chain cDNA clones. Construction of mouse-human chimeric light chain gene was performed by inserting a 5.6-kb *Hind*III fragment containing κ VJ region of B72.3 into the

*Hind*III site of pACYC 184/HNEK vector, which contains sequences encoding the human Ck region and the *neo* resistant gene as a selectable marker. Similarly, the mouse-human chimeric heavy chain gene was constructed by inserting an approximately 3-kb *Eco*RI fragment containing rearranged VDJ sequences of B72.3 into *Eco*RI sites of PSV2 *gpt*/Hu $IgG_1$ vector that includes constant region of the genomic human $IgG_1$. SP2/0 cells were electroporated with expression construct carrying chimeric light chain genes. Transfectants were selected for G418 resistance, and expression of light chain gene was analyzed by Western blot analysis. The chimeric $H_2L_2$-producing cell line was developed by electroporating the chimeric heavy chain expression construct into SP2/0 cells producing light chain. Mycophenolic acid-resistant transfectants were assayed for $H_2L_2$ production by TAG-72 antigen-based radioimmunoassay. Several $H_2L_2$-producing colonies were picked that produced 5.0-10.0 µg/ml of chimeric B72.3 MAb.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 09027-02 LTIB

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October 1, 1989 to September 30, 1990

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

Biological Effect(s) of Oncogenetic Elements on Mammary Cell Growth and Senescence

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

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COOPERATING UNITS *(if any)* Robert Bassin, LTIB, DCBD, NCI; David Salomon, LTIB, DCBD, NCI; Dennis Roop, Baylor College of Medicine, Houston, TX; Daniel Medina, Baylor College of Medicine, Houston, TX; Jaime Zweibel, Georgetown University School of Medicine, Washington, DC

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| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.0 | 1.0 | none |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

A number of genes have been implicated in the process of malignant transformation in the mammary glands of experimental animals and humans. The mouse mammary gland provides an excellent experimental model within which to elucidate the function of these putative mammary oncogenes during the growth, development, and differentiation of mammary tissue *in vivo*. Any portion of the adult mouse mammary gland at any age and throughout all stages of differentiation can repopulate the gland-free mammary fat pad of syngeneic mice upon transplantation. Similar results are obtained when dissociated glandular epithelium is injected into the fat pad or alternatively grown in culture under prescribed conditions prior to reintroduction into the mammary fat pad. We have initiated experiments designed to evaluate the feasibility of introducing functional genetic elements into mouse mammary cells with recombinant retroviral expression vectors antecedent to the reintroduction of these cells into a gland-free fat pad. Paramount to the success of such a project is the maintenance of the "repopulation potential" of the mammary epithelial cells. Our studies and those of others indicate that the number of cells capable of repopulation of the fat pad is small initially and the ability to repopulate is gradually lost through mitotic events. We have morphologically identified a potential candidate for these "mammagenic" cells *in situ* and in explant cultures. These cells seem to differentially express certain cytokeratins during growth and expansion of the mammary gland *in vivo*, suggesting that specific keratin expression could serve as a marker for these "stem" cells. In addition, primary mammary cell cultures are being evaluated for the efficiency of retroviral gene transfer, the level of recombinant *trans*-gene expression in the infected cultures, and the stability of recombinant retroviral gene expression in "mammagenic cell populations" subsequent to repopulation of the gland-free mammary fat pads. We have developed a number of partially transformed preneoplastic mammary outgrowth lines which are carried serially *in vivo* by transplantation into gland-free mammary fat pads. Cells from these lines have been grown *in vitro* and are capable of repopulating mammary fat pads subsequently. These cells are being tested for retroviral gene transfer studies.

*Major Findings*

Sixteen hyperplastic mammary outgrowth lines have been established from Czech II $V^+$ as well as Czech II $V^-$ mice that had been foster-nursed on GR and C3H high-incidence inbred mouse strains. Five of these serially transplantable preneoplastic mammary populations have begun to produce mammary tumors in a stochastic manner. The cellular DNA from 4 of these 5 lines has been analyzed for MMTV DNA insertions which result in the rearrangement of the known *int* genes, *int*-1, *int*-2, and *int*-3. Three hyperplastic lines were found to possess a rearrangement at *int*-1; the tumors arising within these lines possess the same *int*-1 rearrangement. These observations suggest that these lines are clonal. Analysis of DNA from the original outgrowth confirmed this conclusion. The fourth hyperplastic line that was analyzed did not have MMTV rearrangements near *int*-1, *int*-2, or *int*-3; but did contain 2 newly acquired MMTV genomes. The tumors arising from this line also contain these same integrations, again suggesting that this hyperplasia developed clonally. Analysis of the regions flanking these MMTV insertions may reveal additional *int* genes important in the early stages of mammary tumorigenesis.

Transplantation experiments were conducted to test the "repopulation potential" of the normal mammary gland in various stages in its development and differentiation. All portions of the gland were capable of repopulating gland-free mammary fat pads within 12 weeks of implantation. The estimated minimum number of cells required for this activity was approximately $2.3 \times 10^3$, but these fragments contained the growing points (end buds) of actively developing mammary epithelium in pubescent female mice. From mature virgins the smallest number of cells that was capable of producing a repopulated gland was a fragment of the tertiary duct or about $7.2 \times 10^3$ epithelial cells. In contrast approximately $3.3 \times 10^4$ epithelial cells were contained within the fragments of lactating mammary gland implanted within the gland-free pad. Interestingly, these latter fragments were less efficient at repopulation although possessing larger numbers of epithelial cells. In agreement with these transplantation studies we were able to detect morphologically distinct cells present among the epithelium which persisted throughout all stages of development and differentiation. These cells were more prevalent in the fragments of growing end buds and tertiary ducts of 6 week old mice. These fragments were able to repopulate the cleared fat pad most efficiently. In explant culture, these "stem" cells underwent mitosis within 4 hrs even in the absence of DNA synthesis, giving rise to groups of morphologically distinct, pale-staining cells. In the presence of lactogenic hormones, these daughter cells differentiated to a secretory phenotype. We believe these cells represent dormant mammary epithelial-specific precursor cells. We are currently evaluating keratin gene expression in the developing and differentiated mammary epithelium in an effort to determine if these gene products could serve as cellular markers for these putative mammary epithelial stem cells.

Frozen sections of normal mouse mammary gland were stained with antibodies raised against the synthetic peptides unique to the following epidermal keratins (K1, K10, K5, K14, K6, K16, K18, K13). These studies were carried out in collaboration with Dr. Dennis Roop (Baylor). Two pairs of keratin genes were expressed differentially in mouse mammary epithelium, K6 and K16 and K5 and K14. The polypeptides encoded by these genes combine within the cellular cytoplasm to form keratin-specific intermediate filaments. K5 and K14 were expressed in basally located epithelia which apparently corresponded to the myoepithelial cells. K6 and K16 were found associated with a subset of luminal epithelial cells which possessed the same distribution in developing, differentiating, and functioning mammary epithelium as the putative mammary stem cells we described earlier. The numbers of these K6-positive cells increased during periods of epithelial growth and expansion in a manner similar to the pale-staining morphologically distinct cells. Expression of K6 by these cells was confirmed directly by immunoperoxidase staining of semi-thin sections of plastic-embedded

mammary explants. We are currently examining preneoplastic and neoplastic mammary tissues for the expression of these keratins.

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PROJECT NUMBER

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 CB 05216-19 LTIB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Site-Selective cAMP Analogs as Antineoplastics and Chemopreventives

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

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| T. Clair | Chemist | LTIB, DCBDC, NCI |
| H. Yokozaki | Visiting Fellow | LTIB, DCBDC, NCI |
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|---|---|---|
| 2.5 | 2.0 | 0.5 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Cyclic AMP (cAMP) in mammalian cells functions by binding to cAMP receptor protein, the regulatory subunit of cAMP-dependent protein kinase. The cAMP receptor protein has two different cAMP binding sites, and cAMP analogs that selectively bind to either one of the two binding sites are known as Site 1-selective (C-2 and C-8 analogs) and Site 2-selective (C-6 analogs), respectively. Such site-selective cAMP analogs at micromolar concentrations exerted potent growth inhibition, with no sign of toxic effects, in a spectrum of human cancer cell lines, including those that are resistant to previously tested cAMP analogs, leukemia lines, v-*ras*-H oncogene-transformed NIH/3T3 cells, and v-*ras*$^{Ki}$ oncogene-or TGFα-transformed normal rat kidney (NRK) cells; the combination of a C-8 with a C-6 analog showed synergistic effects. The growth inhibition was not due to a block in a specific phase of cell cycle but paralleled selective modulation of type I versus type II protein kinase isozymes, suppression of cellular proto-oncogene expression, inhibition of TGFα production, phenotypic change, and differentiation (leukemic cells). It is suggested that the site-selective cAMP analogs, substituting for endogenous cAMP and binding to protein kinase, suppress the cancer cell growth not via directly inhibiting cell division but rather by promoting cell differentiation. Site-selective cAMP analogs thus provide new biological tools for investigating cell proliferation and differentiation and also for the improved management of human cancer.

8-Cl-cAMP is now in preclinical phase I studies at the National Cancer Institute. A U.S. patent for the cAMP analogs, entitled "Derivatives of cyclic AMP as treatment for cancer," was applied for on May 25, 1988.

*Major Findings*

New site-selective cAMP analogs demonstrated the growth inhibition with many-fold greater potency than previously studied analogs in a spectrum of 20 human cancer cell lines. Of 24 site-selective analogs tested, the majority produced growth inhibition (30-80%) with no sign of toxicity in all 20 cancer cell lines at micromolar concentrations. The growth inhibition was not due to a block in cell cycle progression but paralleled a change in cell morphology, an augmentation of the RII cAMP receptor protein, and a decrease of p21*ras* protein. Since the adenosine counterpart of the analog produced cell death and $G_1$ synchronization without affecting the RII and p21*ras* protein levels, it is likely that an adenosine metabolite is not involved in the analog effect. The effect of site-selective cAMP analogs on the growth of rat mammary tumors and human tumor xenografts, MX-1 and MDA-MB-231 mammary carcinomas, LOVO colon carcinoma, and LX-1 lung carcinoma, in athymic mice was also tested. These *in vivo* models were used to determine that the site-selective analogs that are most potent in the growth inhibition of the cancer cell lines in culture are also most effective in producing tumor regression *in vivo*.

The effect of site-selective cAMP analogs on the expression of differentiation markers was determined in leukemic cells. Treatment of HL 60 cells for 3 days with 8-Cl-cAMP at concentrations where 90% cell visibility is present, induced a marked increase in the expression of monocyte-specific surface antigens ($MO_2$ and $OKM_5$) and a decrease in markers related to the immature progenitor cells My7 and My9). Disappearance of cellular terminal deoxynucleotidyl transferase (TdT) has been considered as a differentiation marker for human T-lymphocytic leukemia. Treatment of MOLT-4 (acute T lymphocytic) leukemia cells with 8-Cl-cAMP (10 μM) caused a time-dependent decrease in TdT activity; by Day 4, the activity decreased to 10% of the untreated control levels. Moreover, treatment for 4 days with 8-Cl-cAMP in combination with $N^6$-benzyl-cAMP (20 μM) brought about almost complete loss (over 95%) of TdT activity. In the K-562 chronic myelogenous leukemia cell line, site-selective cAMP analogs induced megakaryocytic differentiation. Treatment for 3 days with 8-Cl-cAMP (30 μM) induced a marked increase in the fraction of cells positive for the glycoprotein $II_b$-$III_a$ antigen, a specific megakaryocytic marker expressed in the differentiation of this lineage. $N^6$-benzyl-cAMP treatment alone did not induce an appreciable increase of this antigen expression, but when $N^6$-benzyl-cAMP was added with 8-Cl-cAMP, the majority of cells expressed this antigen.

Transforming growth factor alpha (TGFα) is a potent mitogen for fibroblasts and epithelial cells. 8-Cl-cAMP (50 μM) exerted potent inhibition of both the monolayer and soft agar growth of NRK fibroblasts that had been transformed with the v-$ras^{Ki}$ oncogene or treated with TGFα. The growth inhibition was dose dependent and reversible and was accompanied by reversion of the transformed phenotype, suppression of TGFα production, and a decrease in p21*ras* protein levels. These effects were linked to the cAMP analog's selective modulation of the type I *versus* type II protein kinase regulatory subunits present in these cells. These data support the direct role of 8-Cl-cAMP in the control of malignancy induced by certain growth factors and/or cellular oncogenes.

These data show that site-selective cAMP analogs provide a biological tool for control of malignant growth.

*Publications*

Cho-Chung YS. Site-selective cAMP analogs in the arrest of cancer cell growth. In: Developments of cancer chemotherapy Vol II. Glazer R, ed. Boca Raton, FL: CRC Press, 1989;78-93.

Tortora G, Ciardiello F, Ally S, Clair T, Salomon DS, Cho-Chung YS. Site-selective 8-chloroadenosine 3',5'-cyclic monophosphate inhibits transformation and transforming growth factor α production in Ki-*ras*-transformed rat fibroblasts, FEBS Lett 1989;2:363-367.

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Cho-Chung YS, Clair T, Tagliaferri P, Ally S, Katsaros D, Tortora G, Neckers L, Avery T, Crabtree G, Robins RK. Site-selective cAMP analogs as a new biological tool in growth control, differentiation, and proto-oncogene regulation—basic science review, Cancer Invest 1989;7:161-177.

Ally S, Clair T, Katsaros D, Tortora G, Finch RA, Avery TL, Cho-Chung YS. Inhibition of growth and modulation of gene expression in human lung carcinoma in athymic mice by site-selective 8-Cl-cAMP, Cancer Res 1989;49:5650-5655.

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PROJECT NUMBER

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**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 CB 08281-08 LTIB

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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)*

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| 2.5 | 2.0 | 0.5 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

In prokaryotes, cAMP is known to regulate the transcription of specific genes by a well-defined mechanism. cAMP binds to the prokaryotic cAMP binding protein CRP (cAMP receptor protein)/CAP (catabolite gene activator protein), and this complex interacts with specific DNA sequences at the 5 ends of targeted genes, modulating the ability of RNA polymerase to bind to the promoters. In mammalian cells, gene expression is altered in response to stimulation by hormones. Stimuli whose action is mediated through adenylate cyclase and cAMP-dependent protein kinase influence the transcriptional efficiency of several genes through the binding of specific factors to distinct *cis*-acting DNA sequences, CRE (cAMP responsive element), located in the 5 noncoding (promoter) region. Recently, a region containing the CRE has been shown to bind specific nuclear proteins from various tissues. Recent evidence suggests that transcriptional regulation of eukaryotic genes by cAMP requires a cAMP-dependent protein kinase. In this study, we examined the role of cAMP-dependent protein kinase in gene transcription. Since cAMP-dependent protein kinase is exclusively present in the cytoplasm, any nuclear function of the protein kinase must be accompanied by its translocation to the nucleus. It was found that the *ras* gene suppression, growth inhibition, differentiation (leukemic cells), and phenotypic reversion of Ha-MuSV-transformed NIH/3T3 cells by site-selective cAMP analogs correlated with the nuclear translocation of the RII cAMP receptor protein, the regulatory subunit of protein kinase type II, which was detected within 10 min after the analog treatment. Thus, the nuclear translocation of the RII cAMP receptor protein is an early event in the cAMP regulation of growth and differentiation. The goal of this study is to provide direct evidence of interaction between the cAMP consensus sequences and cAMP receptor protein (RII) and/or a phosphoprotein substrate of protein kinase.





GPO 914-918

*Major Findings*

Type I and type II cAMP-dependent protein kinases are distinguished by their regulatory subunits (RI and RII, respectively). Four different regulatory subunits ($RI_\alpha$ [previously designated RI], $RI_\beta$, $RII_\alpha$ [$RII_{54}$] and $RII_\beta$ [$RII_{51}$]) have been identified at the gene/mRNA level. Two different catalytic subunits ($C_\alpha$ and $C_\beta$) have also been identified; however, preferential coexpression of either one of these catalytic subunits with either the type I or type II protein kinase regulatory subunit has not been found. The changing levels of protein kinase isozymes, therefore, can be measured using the photoaffinity ligand 8-$N_3$-[$^{32}$P]cAMP, which binds covalently to their regulatory subunits, the cAMP receptor proteins. The untreated breast (MDA-MB-231 and MCF-7) and colon (LS-174T) cancer cells and HL-60 leukemia cells contained a major cAMP receptor protein with a $M_r$ of 48,000, the $RI_\alpha$ cAMP receptor protein. When the cells were treated for 3 days with 8-Cl-cAMP, the cAMP receptor protein of 52,000 $M_r$, $RII_\beta$, increased appreciably with a concomitant decrease of the $RI_\alpha$ protein. The concentration, subcellular localization, and transcriptional control of the expression of these cAMP receptor proteins have been examined during treatment of LS-174T human colon cancer cells with 8-Cl-cAMP. Two species of RII with an apparent $M_r$ of 52,000 ($RII_\beta$) and 56,000 ($RII_\alpha$) and a single species of RI with a $M_r$ of 48,000 ($RI_\alpha$) were identified in the cancer cells. $RI_\alpha$ and both forms of RII were covalently labeled with 8-$N_3$-[$^{32}$P]cAMP, and two different anti-RII antibodies that exclusively recognize either $RII_\beta$ or $RII_\alpha$ resolved two forms of the RII receptors. 8-Cl-cAMP treatment induced a decrease of $RI_\alpha$, an increase of both $RII_\beta$ and $RII_\alpha$ in the cytosols of cancer cells, and rapid translocation (within 10 min) of $RII_\beta$ from the cytosol to nucleus. Nuclear run-off assay demonstrated that 8-Cl-cAMP caused transcriptional activation of the $RII_\beta$ receptor gene and inactivation of the $RI_\alpha$ receptor gene. The transcriptional control of these cAMP receptor genes, however, did not appear to be the initial event in 8-Cl-cAMP-induced growth inhibition. Within 10 min after 8-Cl-cAMP treatment, both $RI_\alpha$ and $RII_\beta$ receptor levels increased 2-fold even though the transcriptional changes of these genes could not be detected yet. Thus, 8-Cl-cAMP initially enhanced, at least transiently, the stability of both $RI_\alpha$ and $RII_\beta$ receptors and simultaneously induced a rapid nuclear translocation of $RII_\beta$ but not $RI_\alpha$ receptor protein. In K562 chronic myelogenous leukemic cells, Northern blot analyses demonstrated that 8-Cl-cAMP treatment induced an increase in the $RII_\beta$ mRNA level along with a decrease in the $RI_\alpha$ mRNA level but did not affect the mRNA level of the catalytic subunit. 8-Cl-cAMP treatment also brought about a rapid decrease in c-*myc* mRNA level. These changes occurred within a few hours of analog treatment, indicating that the changes are early events rather than the consequences of growth inhibition and differentiation. The growth inhibition induced by 8-Cl-cAMP of a human lung carcinoma, LX-1, in athymic mice also correlated with a selective modulation of different forms of cAMP receptor proteins. $RI_\alpha$ and $RII_\beta$ levels changed in an inverse manner during 8-Cl-cAMP treatment. Northern blotting analyses demonstrated that the changing levels of $RI_\alpha$ and $RII_\beta$ receptors paralleled the changing levels of their mRNAs. Also, only $RII_\beta$, not $RI_\alpha$, accumulated in the nucleus in regressing tumors after 8-Cl-cAMP treatment. 8-Cl-cAMP treatment also caused a sharp decrease in both N-*ras* and c-*myc* mRNA levels in the tumors.

These results suggest that the fundamental basis for the antineoplastic activity of 8-Cl-cAMP resides in the restoration of normal gene regulation in neoplasms in which cAMP receptor proteins may play a role. To further explore the role of cAMP receptor proteins in the gene regulation of cancer cells, we are currently conducting experiments that employ antisense strategy and gene transfer technology.

*Publications*

Cho-Chung YS, Clair T, Tagliaferri P, Ally S, Katsaros D, Tortora G, Neckers L, Avery T, Crabtree G, Robins RK. Site-selective cAMP analogs as a new biological tool in growth control, differentiation, and proto-oncogene regulation--basic science review, Cancer Invest 1989;7:161-177.

Tortora G, Ciardiello F, Ally S, Clair T, Salomon DS, Cho-Chung YS. Site-selective 8-chloroadenosine 3',5'-cyclic monophosphate inhibits transformation and transforming growth factor $\alpha$ production in Ki-*ras*-transformed rat fibroblasts, FEBS Lett 1989;2:363-367.

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Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1989 to September 30, 1990

The genetic basis of human renal cell carcinoma has been the major focus of the research effort of the Cellular Immunity Section. Previously, we presented evidence for the involvement of a specific gene in the pathogenesis of renal neoplasia. To develop a better understanding of this "renal cell carcinoma gene" 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 trait characterized by a predisposition to develop retinal angiomas, cerebellar and spinal hemangioblastomas, renal cell carcinomas, pheochromocytomas and benign tumors of the epididymis and pancreas. We have presented evidence that supports the concept that the VHL gene is a recessive oncogene. Renal cell carcinomas obtained from patients with von Hippel-Lindau disease showed 3p allele loss from the chromosome 3 bearing the wild-type allele of the VHL gene. We interpreted these findings to mean that the unaltered (normal) form of the VHL gene is essential for normal growth of several cell types. Loss of function of both copies of this gene is a critical early step in the development of certain benign and malignant tumors.

We have determined more precisely the location of VHL on chromosome 3. Linkage analysis were performed in 25 VHL families. We found that VHL was linked to RAF1, confirming previous observations, and to two polymorphic DNA markers, D3S18 and D3S191. A genetic map of the distal portion of 3p was constructed with the families supplied by CEPH to serve as a framework for locating VHL. The results of multipoint linkage analysis suggested that VHL is located between RAF1 and D3S18. This conclusion is supported by characterization of the recombinant chromosome 3's in individuals with VHL.

The D3S18 marker was inherited with the disease gene in about 95% of affected individuals. Early observations indicated that D3S18 is accurate and reliable in identifying asymptomatic gene carriers in VHL families. Asymptomatic individuals, predicted to be gene carriers by genetic analysis, were shown to have one or more of the manifestations of the illness when screened at the National Institutes of Health. These manifestations included renal cell carcinoma, retinal angiomas, cerebellar hemangioblastomas and pheochromocytomas. Identification of symptomatic individuals with disease manifestations has led to early treatment of ocular, central nervous system and renal neoplasms.

The results suggesting the VHL is located between RAF1 and D3S18 have been guiding efforts to isolate the VHL gene. A small region of human chromosome 3 (from RAF1 to D3S18) has been found to be conserved and located on mouse chromosome 6. This conserved region of human-mouse homology will help identify human DNA fragments that are close to the VHL gene. A large number of new polymorphic probes are being tested in VHL families to find probes that do not recombine with the disease gene.

Cohen et al (New England Journal of Medicine, 1979) identified a family with a 3;8 translocation associated with the development of renal cell carcinoma. Ninety percent of family members with the translocation developed renal cell carcinoma by age 60. We used cytogenetic and molecular techniques to test for the presence and source of 3p loss in renal cell carcinomas associated with the 3;8 translocation. We found that the derivative chromosome 8 (bearing the distal portion of 3p) was lost in the renal cell carcinomas of two patients with this form of hereditary renal cell carcinoma. This result suggests that there are at least three events in the origin of renal cell carcinoma associated with the 3;8 translocation: (1) inheritance of the translocation chromosome; (2) a somatic mutation of the renal cell carcinoma gene located on the "normal" chromosome 3; and (3) loss of the derivative 8 chromosome.

Tumor tissue from 60 patients with sporadic renal cell carcinoma were tested for loss of heterozygosity on chromosome 3p. Loss was found in 88% of informative patients. The region between D3S2 and D3S18 was deleted in all renal cell carcinomas that showed loss. Loss of heterozygosity at loci on 3p was found in all clinical stages of the disease and with all histological types.

A collection of 2,000 bacteriophage containing human single copy inserts were isolated from two chromosome 3 flow-sorted libraries. The single copy DNA fragments were first sorted into 3p and 3q locations and then about 800 3p fragments were regionally mapped with a somatic cell hybrid panel. About 100 fragments from the distal half of 3p were found to detect restriction fragment length polymorphisms. Several probes have been found to be adjacent to RAF1 or D3S18. As yet, no probe has been identified between D3S18 and RAF1 .

The collection of single copy probes served as the basis of a proposal to construct a high resolution genetic map of human chromosome 3. A grant for this work was awarded to the Laboratory of Immunobiology by the National Center for Human Genome Research.

Immunopathology Section. Last year we purified, sequenced and cloned a novel chemotactic cytokine, monocyte chemoattractant protein-1 (MCP-1), from the culture supernatants of either human malignant glioma cell line cells or mitogen-stimulated human peripheral blood mononuclear cells. Human MCP-1 has protein and nucleotide sequence similarity to mouse JE , which was originally defined as a gene expressed by mouse fibroblasts after stimulation by platelet-derived growth factor (PDGF). This suggested that human MCP-1 was a human homologue of mouse JE, which was confirmed when Rollins et al. cloned human JE from human fibroblasts with mouse JE cDNA as a probe and found that human JE was identical to human MCP-1. We therefore investigated MCP-1 production by three lines of human fibroblasts. Monocyte chemotactic activity (MCA) was detected in the culture supernatants of all three cell lines. MCA was eluted between cytochrome c and chymotrypsinigen by gel filtration HPLC; it was completely absorbed by an anti-MCP-1 polyclonal antibody column, suggesting that MCA was due exclusively to MCP-1. PDGF stimulation caused an increase in MCA production. Fibroblast production of MCP-1 was confirmed by immunoprecipitation of metabolically labeled MCP-1. Thus, human fibroblasts secrete MCP-1, and production is regulated by PDGF. In collaboration with Dr. Rollins, we also found that human endothelial cells produce MCP-1. We are now attempting to establish an animal model, to determine the significance of MCP-1 in vivo.

We have identified MCP-1 receptors on human monocytes. MCP-1 was radioiodinated without significant loss of biologic activity. Binding to monocytes occurred within 10 minutes at 0°C. Binding was inhibited by unlabeled MCP-1 but not by other chemoattractants, such as N-formyl-methionyl-leucyl-phenylalanine or neutrophil attractant/activation protein-1 (NAP-1). No significant binding of iodinated MCP-1 to neutrophils or lymphocytes was found. By Scatchard plot analysis, the Kd was $2 \times 10^{-9}$ M and the receptor number was about 2,000 per monocyte.

Our approaches to the role of NAP-1 in vivo include injection of NAP-1 into human skin and measurement of NAP-1 in human inflammatory exudates. We reported that intradermal NAP-1 caused infiltration of neutrophils, but not lymphocytes. We confirmed the absence of lymphocyte infiltration with immunohistochemical lymphocyte markers. Thus, the primary effect of intradermal NAP-1 is to cause infiltration of neutrophils. In collaboration with Dr. John Rankin at Yale, a time course study showed that culture fluids of LPS-stimulated human bronchoalveolar lavage macrophages have neutrophil chemotactic activity as early as 3 hrs after stimulation, which persists for the 24 hr observation period. The chemotactic activity is due primarily to two different chemoattractants: secreted leukotriene $B_4$ ($LTB_4$) accounts for chemotactic activity at 3 hrs. $LTB_4$ is completely inactivated by 24 hrs, and chemotactic activity at this time is due to NAP-1.

NAP-1 is structurally related to several host defense cytokines, including two platelet-derived proteins, platelet factor 4 (PF-4) and connective tissue activating protein-III (CTAP-III). It was reported that cleavage by elastase of the first fifteen N-terminal residues of CTAP-III yields a chemotactically active protein, called NAP-2. We have compared chemotactic efficacy, potency, and NAP-1 receptor binding of these cytokines. NAP-2 interacts with the neutrophil NAP-1 receptor. Although chemotactic potency of NAP-2 is two orders of magnitude less than that of NAP-1, its activity may be biologically significant because of the high concentration of the CTAP-III precursor at sites of platelet granule release. Thus, NAP-2, like $LTB_4$, is a neutrophil attractant that can be generated rapidly in response to tissue injury.

We have shown that macrophage stimulating protein (MSP) comprises two chains (A and B), linked by a disulfide bond. Partial sequencing of MSP showed a 12 residue sequence that was identical to a sequence in the A chain of bovine prothrombin, and another residue that was similar to a residue in the B chain of plasminogen. Plasminogen is activated by proteolysis at a single site, to yield disulfide-linked A and B chains with molecular masses similar to those of MSP A and B chains. The findings suggest that MSP may also exist in the circulation as a biologically inactive single chain precursor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08552-24 LIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 21701

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.0 | 1.0 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

SUMMARY OF WORK *(Use standard unreduced 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.



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

Major findings: Studies on the inactivation of HIV-1 by the classical pathway of complement in the absence of antiviral antibodies continued. Progress was hampered by lack of personnel to assist PI in this endeavor. Important findings were added to the previous studies to strengthen the idea that the classical components of complement directly inactivated HIV-1. One of these indicated that lack of inactivation by human complement was not due to lack of activation of C1 for when gp120 was bound to sheep red cells, both human and rat complement induced passive lysis of the labelled cell. This finding also indicated that gp120 may be one of the viral proteins that activcate complement and is involved in inactivating the virus. Another was that mouse serum lacking C5 (the fifth component of complement) inactivated HIV-1 as well as serum containing C5. This indicated that components beyond C3 were not necessary for viral inactivation. This proposition was further strengthened by the observation that virus neutralization by rat serum was greatly inhibited by rabbit anti-rat-C3 antibody.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08575-17 LIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

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INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, MD 21701

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 4.0 | 3.0 | 1.0 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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. Human monocyte chemoattractant protein-1 (MCP-1) was sequenced and cloned by us last year. MCP-1 has protein and nucleoside sequence similarity to mouse JE, which was originally defined as a gene expressed by mouse fibroblasts after stimulation by platelet-derived growth factor (PDGF). Rollins et al. cloned human JE from human fibroblasts with mouse JE cDNA as a probe and found that human JE was identicalto human MCP-1. We therefore investigated MCP-1 production by three lines of human fibroblasts. Monocyte chemotactic activity (MCA) was detected in the culture supernatants of all three cell lines. MCA was eluted between cytochrome c and chymotrypsinigen by gel filtration HPLC; it was completely absorbed by an anti-MCP-1 polyclonal antibody column, suggesting that MCA was due exclusively to MCP-1. PDGF stimulation caused an increase in MCA production. Fibroblast production of MCP-1 was confirmed by immunoprecipitation of metabolically labeled MCP-1. Thus, human fibroblasts secrete MCP-1, and production is regulated by PDGF. In collaboration with Dr. Rollins, we also found that human endothelial cells produce MCP-1. We are now attempting to establish an animal model, to determine the significance of MCP-1 in vivo.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08577-05 LIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

| | | | |
|---|---|---|---|
| PI: | B. Zbar | | |
| Other: | M.I. Lerman | Expert | LIB NCI |
| | H. Brauch | Guest Worker | LIB NCI |
| | L. Daniel | Bio Tech Fellow | LIB NCI |
| | G. Glenn | Medical Staff Fellow | LIB NCI |
| | S. Hosoe | Visiting Fellow | LIB NCI |
| | F. Latif | Visiting Fellow | LIB NCI |

COOPERATING UNITS *(if any)*

M. Linehan, Surgery Branch, DCT, NCI; F. Li, Epidemiology, NCI, Boston; E. Oldfield, Surgical Neurology Branch, NINCDS; E. Maher, Department of Pathology, Cambridge University, Cambridge, England.

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Cellular Immunity Section

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| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 9.0 | 7.0 | 2.0 |

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

☒ (a1) Minors

☐ (a2) Interviews

B

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


The major goal of the research effort was to precisely locate the von Hippel-Lindau disease (VHL) gene. Multipoint linkage analysis was performed with a panel of 25 families with von Hippel-Lindau disease. The results of this analysis indicate that the most likely location for the von Hippel-Lindau disease gene is on chromosome 3 in the interval between RAF1 and D3S18.

A probe was identified that is inherited with the von Hippel-Lindau disease gene in 96% of genetic events (meiosis). Because probe CRI-L162 (D3S18) is consistently inherited with the disease gene it should prove to be useful in presymptomatic diagnosis of von Hippel-Lindau disease. A prospective trial to test this hypothesis is in progress.

Asymptomatic, at-risk individuals were examined for evidence of von Hippel-Lindau disease. Twenty-two asymptomatic individuals were found to have the disease. These individuals were treated at the National Cancer Institute or were referred for treatment by physicians in their communities.

Major findings:

von Hippel-Lindau disease

Multipoint linkage analysis was used to determine the location of the von Hippel-Lindau disease gene. The gene was localized to a small region on chromosome 3 between the markers RAF1 and D3S18.

A prospective trial was begun to determine whether it is feasible to do presymptomatic diagnosis of von Hippel-Lindau disease by DNA polymorphism analysis. To date, 12 individuals at risk of developing von Hippel-Lindau disease have been tested. Each of 3 individuals predicted to be gene carriers by DNA polymorphism analysis have been shown on clinical examination to have evidence of the disease. Each of 9 individuals predicted not to be gene carriers by DNA polymorphism analysis have not shown any evidence of the disease on clinical examination.

Several new DNA polymorphisms have been identified at the D3S18 locus on chromosome 3. These new polymorphisms will increase the usefulness of this locus for genetic studies of families with von Hippel-Lindause disease.

An international collaborative study has been started to test the usefulness of new markers developed in the Laboratory of Immunobiology for the presymptomatic diagnosis of von Hippel-Lindau disease. Collaborating groups include Dr. Eammon Maher, Department of Pathology, Cambridge University and Dr. Jeffrey Vance, Department of Neurology, Duke University School of Medicine.

Two clinical forms of von Hippel-Lindau disease were recognized. The common form of von Hippel-Lindau disease is characterized by the predisposition to develop retinal angiomas, spinal and cerebellar hemangioblastomas, renal cell carcinomas and cysts in the pancreas; pheochromocytomas are rare. We identified a large (40 affected members) family with von Hippel-Lindau disease. The illness in this family was characterized by retinal angiomas, spinal hemangioblastomas and pheochromocytomas; no individual in this family developed renal cell carcinoma. Differences in incidence of pheochromocytoma and renal cell carcinoma in the two types of von Hippel-Lindau disease were significant. Pheochromocytomas were linked to RAF1 and D3S18, the loci previously shown to be linked to the common form of von Hippel-Lindau disease.

Hereditary renal cell carcinoma associated with a 3;8 translocation

We examined tumors from 2 patients with hereditary renal cell carcinoma associated with a 3;8 translocation. Tumors from both patients showed loss of alleles at loci on 3p. Cytogenetic and molecular genetic studies demonstrated that the tumors from these patients had a loss of the translocated, distal portion of 3p.

The translocation breakpoint in this disorder is located at 3p14.2. One idea about the pathogenesis of renal neoplasia in individuals with the 3;8 translocation is that the translocation breakpoint inactivated one copy of a renal cell carcinoma gene; renal tumors formed when the normal counterpart of this gene was lost from renal tubular cells. Our results suggest that this concept requires modification.

Sporadic renal cell carcinoma

Sixty sporadic renal cell carcinomas were examined for loss of alleles on the short arm of chromosome 3. Loss of heterozygosity was found in 88% of informative renal tumors. The frequency of loss of heterozygosity for loci on 3p was similar for clinical stage 1/2 and clinical stage 3/4. Loss of heterozygosity was found in clear cell, granular cell and sarcomatoid variants of renal cell carcinoma. Several renal tumors that did not show loss of alleles at 3p were shown not to represent renal cell carcinomas.

Loss of heterozygosity at loci on chromosomes 11, 13 and 17 were found in clinical stage 3,4 renal cell carcinomas but not in clinical stage 1,2 tumors. These results suggest that the changes on chromosomes 11, 13 and 17 represent genetic events that may be involved in tumor progression.

Small cell and non small cell lung carcinoma

Deletion mapping was performed in small cell and non small cell carcinoma of the lung. Three loci, DNF15S2, RAF1 and D3S18, were homozygous in all tumors in the SCLC panel. These loci, which are in regions 3p21 and 3p25, may be involved in the origin or evolution of SCLC.

In non SCLC the pattern of allele loss on 3p is different from SCLC, suggesting a difference in pathogenesis at the genetic level.

Isolation and characterization of new chromosome 3p probes.

Two thousand single copy human DNA fragments were isolated from a chromosome 3 library. The single copy DNA fragments were sorted into 3p and 3q locations. Eight hundred 3p fragments were assigned regional locations with a somatic cell hybrid panel. Four hundred single copy fragments from the distal half of 3p have been tested for polymorphism. About 100 of these fragments detect restriction fragment length polymorphisms. Several of these probes (LIB 45-82, LIB 2-47, LIB 12-69', LIB 11-31, LIB 38-96) have been mapped with the 40 families provided by CEPH.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08578-01 LIB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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)*

| | | | |
|---|---|---|---|
| PI: | B. Zbar | | |
| Other: | M. Lerman | Expert | NCI LIB |
| | F. Latif | Visiting Fellow | NCI LIB |
| | K. Tory | Consultant | NCI LIB |

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Cellular Immunity Section

INSTITUTE AND LOCATION

NCI-FCRDC, Frederick, MD 21701

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

A genetic map has been constructed for the distal portion of 3p. This map begins at DNF15S2 and extends to D3S22. The map is delineated by 9 polymorphic DNA markers. The distance covered by the map is about 75 centimorgans. About 100 new polymorphic DNA markers have been identified for this area.

Fifty-one polymorphism screening membranes were prepared. Seventeen sets of filter membranes were prepared with the parents of CEPH families. One hundred and nine single copy 3q probes were tested; 45 of these 3q probes were found to detect DNA polymorphisms. Nine 3p probes were tested for frequency of heterozygosity with the parents of CEPH families.



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

LABORATORY OF MATHEMATICAL BIOLOGY

SUMMARY

October 1, 1989 through September 30, 1990

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 along with experimental techniques of biochemistry, virology, and electron microscopy to study picornaviruses, adenoviruses and other virus-cell systems.

The availability of a large number of nucleotide and amino acid sequences enables detailed studies of a particular system as well as searches for general trends. Detailed studies can involve structural computations and the effect of single point mutations. Searches for general trends may involve comparisons of structures of related genes. General patterns can also be discerned in studies of sequences fulfilling analogous functions, such as promoters, taken from a variety of genes/organisms or by searches for overall sequence characteristics such as those required by genome packaging (Le, Currey, Konopka, Nussinov, Maizel and Owens).

New analytical tools for studies of proteins and nucleic acids have been developed and implemented (Barber, Le, Maizel, Nussinov, 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. 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.

Techniques are being developed and are leading to sensible three-dimensional models for perforin and apo-lipoproteins. Biochemical data will permit testing of these predicted models. This work is the beginning of what must become a more routine part of gene sequence analysis (Peitsch). Preliminary sequence/structure analyses have also been performed on oncogenes, DNA binding regions and reverse transcriptases (Barber).

Information Theory in Molecular Biology. Studies on the use of concepts of information theory in understanding molecular sequence patterns have advanced through analysis of experimental results showing that the patterns at T7 phage promoters, unlike several other prokaryotic recognition sites, have more sequence information than necessary for transcription (Schneider). Work is under way to provide a theoretical description of molecular machinery such as enzymes, ribosomes, etc., that can set bounds on theoretical capacity and hence aid in design of molecular devices.

Molecular Biology of Glycosyl Transferases. The structure-function relationship of b1-4galactosyltransferase (GT), its gene structure and expression during 3T3 cell growth (Qasba, Masibay and Boeggeman) is under investigation. Analysis of its gene structure showed that there is a family of sequences related to each other only within the 5' half of the b1-4galactosyltransferase gene. Exon 1 exists at least in four related copies, exons 2 and 3 each have three related copies in the genome, while exons 4,5 and 6 exist as unique sequences. Exon 1 which codes for the first 140 residues of the protein that contains the cytoplasmic amino terminal portion and the membrane anchoring region, the "stem" structure of the enzyme, is separated from the 2nd exon by at least 25 kb of intervening sequence and thus is far away from the rest of the sequences coding for the catalytic domain of the protein. The b1-4galactosyltransferase gene belongs to "immediate early" and "early" genes like c-fos, steroid receptors, c-jun, c-myc, etc. in that it is expressed early during the cell's transit from $G_0$ to S and expression is superinduced by protein biosynthesis inhibitors. The plasma membrane pools of the enzyme increase during 3T3 cell's transit from $G_0$ to S. The deletion constructs derived from the parent b1-4galactosyltransferase

expression vector that expresses the functional enzyme in COS-7 cells are being used to determine the regions of the protein involved in membrane, nucleotide sugar and acceptor binding.

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 development of ever larger atomic and 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 relatively 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 to investigate the binding of small peptides to larger proteins (Covell, Jernigan).

Molecular modeling has been proceeding in four areas: membrane proteins (Guy, Raghunathan and Peitsch), small peptides (Guy, Jiang, and Jernigan), DNA double helices (Jernigan, Nussinov 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, pardaxin, d lysin, and PGLa; these have assisted in understanding how they can lyse cells and form channels. Small peptide 2D NOE NMR data indicating close atoms have been combined with molecular calculations to yield models for several small peptides. DNA double helices exhibit some dependence on the base sequence; these details are being studied by investigating the sequence dependence of the DNA helix flexibility. Methods to calculate the induction of bends of specific shapes and curvatures are being developed. One question that is unresolved is whether or not the details of these conformations play a functional role in gene regulation. For DNA-protein interactions, these flexibilities and their asymmetries appear to play important roles.

Image Processing. Two areas of research are pursued in the Image Processing Section (Maizel): development of analytic methods to analyze two dimensional gels (Lemkin), and nucleic acid secondary structure studies (Shapiro). In the first area, the GELLAB-II software system is being developed, with concentration on increasing: resolution, gel image scanning, quantitation accuracy, results annotation capabilities and integration of X-window based user graphics windowing interface. A constant goal has been to simplify the user's role in performing the exploratory data analysis of a composite 2D gel database. Additional software was written toward achieving this goal. A major part of the effort has been in integrating the UNIX based GELLAB-II software with UNIX

workstation based X-windows interactive graphics for portability. A substantial effort is being made to document GELLAB-II and create tutorials to facilitate training new users.

GELLAB collaborative work (Lemkin) has continued with the groups of Dr. P. Sonderegger (U. Zurich) - axonal secreted proteins; Dr. E. Lester (U. Tenn.) - adult human leukemias; Dr. H. Bauer (Austrian Acad. Sci.) - blood brain barrier; Dr. P. Hunziker (U. Zurich) - Cd toxicity; Drs. R. Getzenberg and D. Coffey (J. Hopkins U.) - nuclear matrix protein control of gene expression; Drs. P. Rogan and J. Strathern (BRI/FCRDC) - 2D DNA gels for monitoring active transcription. 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.

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 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 structure problem domain involving various software/hardware complexes available at the FCRDC 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 is being ported to a SUN workstation from a SYMBOLICS 3675 to permit broader user access.

The system is being used to study the fine structural details of the HIV-1 <rev> responsive element (Shapiro, Dayton, Konings and Maizel), the structure of 4.5SRNA and its relationship to tRNA release (Shapiro and Brown), the structure of the 5' non-coding regions of poliovirus (Shapiro, Currey and Maizel) and its relationship to neurovirulence, and the structure of mast cell RNA and its relationship to mast cell differentiation (Shapiro and Stevens).

Simulation, Analysis and Modelling of Physiological Systems. Historically the first research area in the laboratory, continues through development of the simulation, analysis, and modeling (SAAM/CONSAM) computer programs (Zech, Greif). The development of a version of SAAM30 to run under the DOS operating system on personal computers that makes use of the Intel 80386 and 80486 central processor continues. 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. While SAAM30 runs quickly on these computers, it has required more than four megabytes of memory and a Weitek coprocessor. A new version of SAAM was developed to make use of the 80387 numeric (math) coprocessor, and increase the availability of computers on which investigators can make use of SAAM ten fold.

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 was made because of the number of other software writers which have also written to this standard. Writing to this popular output standard makes available, device drivers for more than 300

popular graphics devices. The PLOT command has been extended so that color, line-type and axis-pair can be specified. This version of CONSAM also includes a group of template graphs along with numerous other additions to the graphics in CONSAM, these changes make the CONSAM graphics suitable for publication if printed on a good laser printer.

The SAAM group participated in an international committee to contribute advice regarding the direction of kinetic analysis by serving as the chairman of the advisory committee for a resource in kinetic analysis. The SAAM group organized and participated in workshops on the topics of SAAM/CONSAM in kinetic analysis. A special workshop was again organized to discuss the kinetics of lipids and lipoproteins, 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 were sent to hundreds of investigators.

The SAAM project also carried out collaborative research efforts (Zech), involving large numbers of investigators, in the analysis of data in the fields of: 1) lipid and lipoprotein metabolism, 2) the testing of a hypothesis concerning human epinepherine metabolism, 3) the quantitative description of whole body metabolism and pharmokinetics of cancer preventive selenium compounds, and 4) the quantitative description of the metabolically significant vitamin A dynamics underlying homeostatic mechanisms that function to regulate the general physiological functions of growth and differentiation, reproduction, and vision and their relationships in cancer prevention. This effort also includes clinical duties and responsibilities for more than 30 lipid and lipoprotein turnover studies carried out in collaboration with the NHLBI. The SAAM project also carried out collaborative research on the chemical, pharmacological and behavioral aspects of caffeine and other drugs.

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 the 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. The

predictions have thus far been tested in vitro; during the next year the emphasis will shift to testing in a murine micrometastasis system in vivo.

In 1988 a serendipitous finding led members of the Section to identify a new molecular target for combinaton 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 will begin in the summer of 1990 in collaboration with groups at two other institutions.

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); 4) general analysis of combination chemotherapy in cancer and AIDS. Because no published algorithm or computer package was adequate for analysis of data on the antiviral effect of such drug combinations, a new approach was developed within the Section. It combines enzymology-based "pseudomolecular" models with new statistical techniques. The resulting computer program (COMBO) and concepts of interaction are proving useful for analysis of combination chemotherapy of both cancer and AIDS.

Membrane Structure and Function

The research goals in the Membrane Structure and Function Section (Blumenthal, Puri, Schoch, 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

The activities of the Membrane Biology Section (Pinto da Silva, Fujimoto, Pimenta and Ru-Long) are focused on the development and application of methods that combine freeze-fracture and colloidal gold cytochemistry. To this end, our new methods, "fracture-flip" and "simulcast", routinely provides views of the nanoanatomy of cell and membrane surfaces at close to macromolecular resolution. The results that we obtained with this new method far surpass in resolution those available from scanning electron microscopy. Some ongoing work involving methods previously developed in our laboratory is related to the study of surface capping of CD3, CD4, and HLA antigens in human lymphocytes (collaboration with Drs. M.R. Torrisi, A. Pavan, and Professor L. Frati, Dept. Experimental Medicine, Institute of General Pathology, Univ. of Rome School of Medicine). We have now modified fracture-flip to visualize and to label the inner surface of the plasma membrane of biomembranes and to observe human lymphocytes during ligand-induced capping of antigens.

To develop fracture-flip as a new anatomical and cytochemical tool, we used cells with highly differentiated cell surfaces as found in unicellular parasites and, also, in both vertebrate and invertebrate spermatozoa involved the establishment of collaborative research projects with researchers at the Laboratory of Parasite Diseases, NIAID (Drs. D. Sachs, T. Nash, D. Dwyer, A. Sher, R. P. Silva) and those at the Institute of Biophysics in Rio de Janeiro (Drs. W. de Sousa, T. Souto-Padron). The merging at the molecular level, biochemical, immunologic, ultrastructural, and cytochemical approaches is

particularly exciting. The work on the localization of specific proteins on the surface of spermatozoa involves collaboration with Drs. D. Nishioka and R. Ward (Dept. Biology, Georgetown University).

We are now developing a new method - "simulcast" that provides views of the inner apolar domains of membranes by freeze-fracture images contiguous with nanoanatomical views displayed in fracture-flip images of the same membrane. Our results show that this new approach can also be combined with immunogold cytochemistry. At the same time, we pursue the development of new methods to expose the cytoplasmic surface of plasma membranes. We have already successfully developed methods that involve Triton-x extraction of freeze-fractured specimens, but for the moment, these can only be applied to erythrocytes and to isolated organelles. To expose inner surfaces of eucaryotic cells, we must develop methods to dissociate and extract cytoplasm and organelles with minimum and/or controlled extraction of components of the membrane skeleton. At this date we have been able to observe microtubular skeletons at the inner surface of the plasmic membrane of parasitic cells.

Another component of our research -- membrane fusion/intercellular junctions-- has been pursued from an ultrastructural point of view. Applying fracture-flip methods to view the actual apical surface of membranes in stretched toad bladder epithelia (P. Pinto da Silva, and S. Ru-Long) should allow us to observe lateral surface views of the tight junction. Theoretical work on the structure of the junction and permeability properties of epithelia is being pursued. Information pertinent to membrane fusion may eventually become available in fracture-flip immunogold labeling studies involving binding and/or assembly of viral particles. These are now being started by Drs. Pavan and Torrisi in Rome using cell cultures infected with Sindbis and Epstein-Barr virus.

With the discovery of fracture-flip this Section has entered a new period where many interesting problems can be approached and/or reapproached. This is due to the dramatic increase in resolution (about two orders of magnitude) which we routinely obtain in extended surface views of membranes. The research efforts of this Section are now to explore some of these new opportunities. It must be understood that underlying the apparent diversity of experimental models is our search of the unifying concepts for the nanoanatomy, topochemistry, and dynamics of biological membranes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08300-18 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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<br>Detailed from OD, NHLBI | LMMB, NCI |

Other Professional Personnel:

| | | |
|---|---|---|
| Peter C. Greif, M.D. | Computer Programmer Analyst | LMMB, NCI |
| Kevin C. Lewis, Ph.D. | IRTA Staff Fellow | LMMB, NCI |

COOPERATING UNITS *(if any)*

Dr. Ray Boston, Murdoch Univ., Australia; Dr. Charles Schwartz, Medical Gollege of Virginia, Richmond, VA; Dr. Waldo R. Fisher, Univ. of FL, Gainesville, FL; Drs. H. Bryan Brewer, Daniel Rader, Carlo Gabelli, Alexander Mann, Juergan Schaefer, of

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.25 | 1.25 | 0.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Continuing the development of a computer system (SAAM) for the simulation, analysis, and modeling of bio-kinetic systems, we added software to implement additional functional relationships between the parameters as dictated by the suffocated class of compartmental models which the biomedical community calls on SAAM to solve. 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. 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. While SAAM30 runs quickly on these computers, it has required more than four megabytes of memory and a Weitek coprocessor. A new version of SAAM was developed which makes use of the 80387 numeric (math) coprocessor, increasing the availability of computers on which investigators can make use of SAAM ten fold.

Interactive SAAM, CONSAM, 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). It is this feature that makes the CONSAM output available on a broad range of graphics hardware. The PLOT command in CONSAM has been extended so that color, line-type and axis-pair can be specified. The line-types have been extended and line-types 10 through 19 now invoke fill patterns. Along with numerous other additions to the graphics in CONSAM, these changes make the CONSAM graphics suitable for publication if printed on a 300 dpi laser printer. A multicompartmental model of free and esterified cholesterol metabolism in VLDL, IDL, LDL, and HDL has been developed to quantitate pathways of HDL esterified cholesterol movement. This is a very significant pathway because of the involvement of reverse cholesterol transport in the pathophysiology of atherosclerosos and the involvement of the inhibition of cellular cholesterol synthesis in the fernesylation of ras proteins.

Cooperating Units (Continued):

Molecular Diseases Branch, NIHLB; 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; Dr. Barbara Howard, Medlantic Research Foundation, Washington D.C.; Drs. Oscar Lenaris & Jeffery Halter, U of Michigan; Dr. Ba-Bie Teng & Allen Sniderman, Royal Victoria Hospital, Montreal, Quebec; Dr. Gilbert Thompson, Hammersmith Hospital, London, England; Dr. David M. Foster, University of Washington, Seattle, WA. and Dr Ahmed Kissebah, University of Wisconson; Dr. Michael H. Green, The Pennsylvania State University. Dr's Danial Nixon, and Richard Costlow, Nutrition Labratory, DCPC, NCI. Gene Barnett, Biometric Research Institute, Arlington, VA.

Project Description #1:

Project #1 The development of mathematical and computer tools for the simulation of and analysis of bio-kinetic data and the implementation of these tools within the framework of SAAM and CONSAM.

Major Findings:

(1) Computational Procedures: The Library command was added to the SAAM environment. The library utility permits the organization and storage of information in a treelike hierarchy designed to facilitate modeling investigations. The arrangement of the library is similar to that of an operating system directory tree, except that the management of the tree as well as the data stored at the nodes of the tree are available from within SAAM/CONSAM. The entire library is stored as a single binary file in the operating system directory from which SAAM/CONSAM was activated. Multiple libraries can be created and used one at a time through use of the file command. Each level in the branching library hierarchy may be regarded as an area of modeling application. An area or node may contain various models, data collections of observed data, command histories, text files, and/or other information pertinent to a study. The library of information is managed by a set of commands which can be listed using the dictionary command. The library commands are as follows:

```
                    GETTING STARTED:
DICTionary -------- Display list of commands
EXIT -------------- Exit from the library subsystem.
                  WHERE AM I?
CURRent ----------- Display current library and node.
PATH -------------- Display path to current node.
TREE -------------- Recursive list of subtree.
```

```
                    WHAT IS AT THIS NODE
LIST ---------------- List contents of current node
DETAiled ----------- Detailed description at this node
                 MOVING AROUND THE LIBRARY:
TOP ---------------- Go to top node (always named top)
AREA --------------- Move up to the proceeding node.
"Node name" -------- Move to the named node.
                    UPDATING THE LIBRARY
INSTall ------------Install the current deck at this node.
REMOve -------------Remove the contents of this node.
               RETRIEVING MODELS FROM THE LIBRARY
LOAD ---------------- Replace deck with node contents
APPEnd ------------- Append node to current deck
                    BUILDING THE LIBRARY
CREAte ------------- Create a new node.
MODIfy ------------- Modify a description of a node.
```

Function dependency of parameters within SAAM/CONSAM provide a mechanism for varying the effective value of a parameter during the course of a model solution. This capability permits the investigation of time dependent or nonlinear dynamic systems. It is essential in the development of feedback control models of physiological models which are not in the steady state. The implementation of function dependencies employs a variety of mathematical operations to modify the value of a parameter during solution. These operations are specified by means of operation code identifiers. Prior to the current enhancements there were nine operation codes for specifying function dependence of parameters. Five new function dependencies have been implemented in the current version of SAAM/CONSAM to facilitate investigation of special types of system behavior. Each of these new operations is related to the condition where the value of the driving function crosses zero either from above or below. The old and new function codes are listed below:

| | Dependency | Special Code | Interpretation* |
|---|---|---|---|
| 1 | addition | + | X'=X+Z |
| 2 | subtraction | - | X'=X-Z |
| 3 | multiplication | * | X'=X*Z |
| 4 | division | / | X'=X/Z |
| 5 | power | 5 | X'=X**Z |
| 6 | exponential | 6 | X'=EXP(X*Z) |
| 7 | | 7 | X'=if Z>0 then X<br>elseif Z=0 then X<br>else 0 |
| 8 | | 8 | X'=if Z>0 then X*Z<br>else 0 |
| 9 | | 9 | X'=if Z>0 then X/Z<br>elseif Z=0 then 1.0E12<br>else 0 |
| 10 | First crossing from above | A | X'=if Z>0 then Z<br>else 0 |

| | | | |
|---|---|---|---|
| 11 | Boxing Function | B | X'=if Z>0 then 1<br>elseif Z<0 then -1<br>else 0 |
| 12 | Complement 10 | C | X'=if Z<0 then 0<br>else Z |
| 13 | Time of last Crossing | D | X'such that Z(X',0)=0<br>if Z never crosses X'=0 |
| 14 | Time of first Crossing | E | X'such that Z(X',0)=0<br>if Z never crosses X'=0 |

*where X is the value of a parameter prior to the operation
X' is the value of a parameter after the operation
Z is the function which modifies X. Z is either a G-function (complex state variable) or F a state variable.

The latest copy of SAAM and CONSAM (version 30.1 4/26/90) for execution under the DOS 4.01 operating system uses either a 80387 (SAAM387) or 1167 (SAAM1167) numeric (math) coprocessor and 5 Mbytes of memory. We have been running test versions of SAAM 30 on the 80386/1167 under DOS since May' 89 and on the 80386/80387 since October '89. We have found several problems for which we have devised solutions in the 4/26/90 version of SAAM. CONSAM has been running with enhanced graphics software since late in the fall of 1989 and we have detected and corrected several problems specific to CONSAM. There was a problem with the FILE INPUt command in the DOS version in that it did not always reset to the default. This was fixed in the 4/26/90 DOS version. While the new graphics in CONSAM work well, the interface with DOS has not been as robust as we would like this will be improved in later version this year.

The SAAM program running under the DOS operating system fits in less than 2 Mbytes of extended memory which means that it can easily be run on a machine with 4 Mbytes of memory, with some extra room for file buffers and other dynamic allocations of memory made by the operating system. There should even be ample room for "SHADOWRAM" within a 4 Mbytes budget if your machine has this feature. Starting with the 1990 versions we have been forced to use some of the capabilities of DOS introduced in version 3.3 and fixed in DOS 4.01 The software will no longer run under DOS 3.2 or earlier versions. We suggest running in DOS 4.01.

New versions of the Fortran and C compilers for 1990 create larger versions of the executable image, therefore CONSAM starting with the version of 4/26/90 requires about 3.2 Mbytes of extended memory. The entire CONSAM30 fits in memory using just over 3.2 Mbytes of extended memory while keeping the "realmode" 640 Kbytes of memory available for use with memory management, graphic drivers, terminate and stay resident programs, and the SYST command. This presents a problem for machines which use "SHADOW RAM" because most of these do not allow access to the memory between 640 Kbytes and 1 Mbyte, and this memory can not be used by CONSAM. This may also present a problem for other 386's depending on how much of the memory between 640K and 1 Mbyte is available. The problem is exacerbated by the fact that on most of the 80386 computers which use "SHADOW RAM" the extra memory consumed by "SHADOW RAM" is not made available to programs even if the "SHADOW RAM" is disengaged or shut off. This means that

the present version of CONSAM, when run on a machine with "SHADOW RAM", needs more than 4 Mbytes. We are suggesting 8 Mbytes as the code tends to grow as updates and new capabilities are added. However, we have run this version of CONSAM on one 80386 computer with 6 Mbytes of RAM, under DOS 4.01, with the "SHADOW RAM" turned on. Others have reported that the CONSAM program functions well on a machine with 5 Mbytes of memory with the "SHADOW RAM in place. We further understand from the compiler supplier that the next version of the compiler will produce even larger executable images, meaning that the size of both SAAM and CONSAM will grow. All things considered, one needs 5 Mbytes of memory to run CONSAM.

We find the new 80386/80387 or 80386/1167 versions to be very fast compared to the old VAX 11/780 and even fast compared to our VAX 8350 and 8650. While there is an example of a very complicated SAAM deck which runs no faster on a 33 MHz. 386/387 than on our VAX 8600, this is the exception and not the rule. Others have reported that a version of SAAM run on a 386SX/387SX computer with 5 Mbytes of memory ran with a speed about the same as a VAX 11/750. The major increase in speed comes from three sources. First, DOS is not a virtual operating system, so the entire copy of SAAM/CONSAM is in memory. Second, DOS is a minimal operating system and therefore has little overhead compared to, say, UNIX. Third, many more operations are performed in double precision in this version of CONSAM.

Changes have been made to optimize for the numeric (math) coprocessor, be it Weitek 1167/3167, or the 80387. Testing and optimization with the newer Cyrix CX83D87 has not yet begun, and we anticipate even newer experimental numeric (math) coprocessors will also become available soon. This increase in precision makes a special difference when SAAM/CONSAM is required to fit data. Not only is this version slightly more accurate (testing not yet complete), but it takes less time to converge towards a best estimate of a rate constant.

SWCON is a version of CONSAM which makes use of a utility called SWAPDOS. SWAPDOS is a "shareware" memory management utility which parks CONSAM on the disk in response to the SYSTem or EDIT commands. This makes almost 500 K bytes of real mode memory available for use by other programs run under these commands. CONSAM makes use of the Mark and Release utilities in its memory management. When using the Mark and Release utility less than 100 K Bytes of real mode memory is available for use following the SYSTem or EDIT commands. This is not enough memory to run many popular programs from within CONSAM. Our solution to this problem is to provide a version of CONSAM called SWCON using SWAPDOS. We have found SWAPDOS to be a better environment than Mark and Release as it provides the capability to run much additional DOS software from within CONSAM, for example Word Perfect.

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 was made because of the number of other software writers which have also written to this standard. It is this feature of the 80386 DOS version of CONSAM which requires graphic driver software. It is also this feature that makes the CONSAM output available on a broad range of graphics hardware.

The user many have copies of GSS driver software mounted on their hard disks as a part of other software. One of the most popular programs with GSS drivers, which we find on many systems, is SIGMA PLOT by Jandel Scientific; others less frequently found are Sigma-Scan by Jandel, JAVA by Jandel, Systat by Systat, Inc., and Statgraphics by STSC, Inc. This feature facilitates the interaction of CONSAM with these programs. Writing to this popular output standard makes available, from GSS directly and other software houses indirectly (e.g. Programmers Paradise), device drivers for more than 300 popular graphics devices. This is the same virtual device interface used by Harvard Graphics, MICROCADAM CORNERSTONE, PalmPerfect, DB Graphics, CADwrite, AT&T Truevision, Calcomp, Concept Technologies/QMS, Control Systems, Galagraph, Imagraph, IBM, Matrox, Microfield, Marrow Designs, NCR, Number Nine Computer, Olivetti, Paradise, Pixelworks, QMS, Sigma Designs, Sony, Tektronics, Texas Instruments, Vectrix, Vermont Microsystems, Verticom, Wyse, and Zenith. Device drivers are available for several makes of EGA, SuperEGA, VGA, SuperVGA, 8514/A, MGX-1000, Artist Publisher, Artist Designer, DGIS, MVA-1024, TGIS, and ATLAS standard display devices and additional output devices too numerous to list. In addition GSS is continually adding new drivers as new, better, higher resolution, less expensive display and output devices (printers, plotters, film recorders, and other hard copy devices) become available. This arrangement also makes available many fonts so that the resolution one views on the simple EGA monitor in 8 colors can be reproduced in high resolution on a 300 dpi printer or in 256 colors on a color printer. There are or will soon be drivers for all popular printer standards including PostScript, PCL and CaPSL. We have included a more complete list of the drivers available from GSS as a separate enclosure.

One of the nice features of having the ability to change drivers is that one can select a driver which enables the user to print to a file for subsequent use by another program, for example, one of the popular word processors. Three of the hardcopy device drivers with this option are the "Computer Graphics Metafile" the "Postscript", and the "HPGL" drivers. Our experiences using the HPGL driver and Word Perfect 5.0 have been excellent. This word processor was used to make the example included at the end of the letter. Because HPGL was intended to drive a plotter only the stroke fonts are well interpreted by the word processor when it reads HPGL files. While it may not look as nice on your High Resolution Monitor, this convenient capability is made even more convenient in this version of CONSAM386 by setting the default font to a stroke font for use with the HPGL driver.

The PLOT command has been extended so that color, line-type and axis-pair can be specified. The line-types have been extended and line-types 10 through 19 now invoke fill patterns. Along with numerous other additions to the graphics in CONSAM, these changes make the CONSAM graphics suitable for publication if printed on a 300 dpi laser printer.

This version of CONSAM also includes a group of template graphs with graph names g1_1, g1_2, g2_2, g3_1, g2_3, g3_3, g1_4, g2_4, g3_4, g4_4. These template can be used for example by typing "PLOT G1_4 Q(1)". The template can not be changed; however, the user can create a specific graph-name by copying the parameters of the template into a new name.

All these changes are documented in the new manual pages for the PLOT and SETUp commands. These manual pages are available by typing DICT PLOT or DICT SETU while in CONSAM on the 80386 computer.

In addition, this new CONSAM 386 graphics package can easily read any outside file and plot the first column along the x-axis, the second column along the y-axis and produce error bars (up, down, or both) from the third column. This file can be plotted before or after an existing CONSAM plot. The instructions for this use of the graphics package are given in the manual pages for the TOGGle command, and can be viewed by typing "DICT_TOGG" from CONSAM.

If two or three device drivers are used (we routinely use two, one for display, a second for output), the initial 640_Kbytes memory space is somewhat full depending on the size of the drivers (The FX-80 driver is one of the largest, probably because so much has to be done by the software and so little by the hardware). Having two graphics device drivers solves the problem of hardcopy output, as this output is available with a single key stroke.

Additional capabilities to specify the x-axis and y-axis have been included in this version of CONSAM 386. For example, it is now possible to plot the errors against the observed and/or calculated values, a capability long overdo according to statisticians. If this is not the best designation of axes, it is easy to reverse the axes and plot the observed values or calculated values against the errors. Help is available by typing DICT DTYPE (short for data-type) from within CONSAM.

Probably the most important change to this version of CONSAM is the inclusion of a DICT command extension which allows the display (while running CONSAM) of the manual page for many (and soon all) CONSAM commands (i.e. DICT PLOT), symbols (i.e. DICT ynot) expressions (i.e. DICT DTYPE), files (i.e. DICT FORT.3), Buffers (i.e. DICT EDITBUF). This could also be extended to chapters from the SAAM and CONSAM manuals, and tutorials; however, this latter capability is not yet coordinated with the CONSAM library capabilities. These manual pages, etc. are included in a sub-directory in ASCII format so that they can be added to, deleted, or modified to the taste of the individual user. With a little thought this can even be accomplished with the CONSAM editor, but any editor you have on your system will work. This was a very small change to CONSAM and we expect many users will send in improved manual pages and, better yet, "how to" advice which could be included in the dictionary of SAAM and CONSAM information. This approach has a drawback in that one is restricted to names which satisfy the rules for naming DOS files (i.e. the symbol y- will be found in a file called ynot and * will be found in a file called star). The present plan is to also include a parallel HELP command in the next version with a one page summary of each manual page.

Testing of this 386 version of SAAM/CONSAM has been performed using AT&T, Olivetti, Intel, Gateway, and Northgate 386 computers, under DOS version 4.01. All have been run with more than 4 Mbytes of memory. By the time this report is final, we may have completed testing of CONSAM386 version on Compaq, IBM, Dell and other models of 386 machines; however, we have NOT yet tested this software

on any 80386SX computers. Others have run the software on a HP Vectra 386 with 8 Mbytes of memory and a HP 386SX with 5 Mbytes of memory with little difficulty. Another group has run the previous version of this software on a Toshieba 5100, but they are experiencing some difficulties with the graphical interface.

(2) SAAM Workshops, Distribution, & Newsletter: In the past year we have been involved in several workshops. A second NATO workshop is planed for September 1990. A two day meeting with workshops and tutorials was conducted in conjunction with the Resource for Kinetic Analysis. Approximately 45 participants were in attendance. Approximately 50 investigators attended the eighted annual workshop to discuss Lipid and Lipoprotein Kinetics. Demand for this meeting is increasing because this is the principal opportunity to discuss compartmental modeling of lipoproteins and lipids. The PC version of SAAM/CONSAM were announced at this meeting.

Almost 100 copies of the SAAM/CONSAM software have been provided to the scientific community over the past 12 months in an effort to establish other centers in the collaborative effort, as pointed out by the scientific counselors as necessary. This involves combining and confronting theorist and experimentalist with topics which can profit from the application of computer simulation and computation and further serves to obtain the best experimental data for analysis an inclusion in data bases, such as the lipoprotein and selenium data bases. To this end we plan to continue to distribute these programs as necessary in the scientific community.

Project Description #2:

Project #2 Application of SAAM and CONSAM to the Simulation and Analysis of Bio-kinetic Data. So that the Bio-Kinetic data collected will be applicable to compartmental analysis, this effort includes chairmanship of the Radioactive Research Drug Committee where all tracer studies come under review for scientific merit. This effort also includes 800 hours of patient contact and primary responsibility for more than 30 lipid and lipoprotein turnover studies carried out in collaboration with the NHLBI.

Major Findings:

(1) In collaboration with Dr. Schwartz, a multicompartmental model of free cholesterol and esterified cholesterol metabolism in VLDL, IDL, LDL, and HDL has been developed to quantitate pathways of HDL esterified cholesterol movement. This is a very significant pathway because of the involvement of reverse cholesterol transport in the pathophysiology of atherosclerosos. In addition the enzymes involved in the synthesis and inhibition of cholesterol and its esterification modulate the action of farnesyl Pyrophosphate on several proteins, including ras proteins.

Five fasting normo-lipidemic subjects with intact interhepatic circulation received strategic combinations of two of the following; autologous HDL or LDL labeled with &3H-Esterified Cholesterol, &3H-Free Cholesterol, &1&4C cholesterol, or &1&4C mevalonic acid. A metabolic model was developed using free

and esterified cholesterol mass, and free and esterified &3H and &1&4C radioactivity in VLDL, IDL, LDL, HDL, RBC's and bile samples collected over a three day period. Eighty percent of the cholesterol esterification occurred in the HDL fraction, 3.2&%0.5 &mMol/min. HDL-cholesterol ester subsequently transferred to VLDL, 3.0&%0.8 &mmol/min, and IDL, 2.0&%0.7&mmol/min. Exchange and transport of esterified cholesterol occurred between HDL and LDL, with 4.1&%0.7 &mmol/min from HDL to LDL and 6.1&%1/0&mmol/min from LDL to HDL. In contrast HDL cholesterol ester transfer to all tissues was only .12&%.05&mmol/min. Cholesterol ester transfer to tissues, mainly hepatic from the beta lipoproteins, IDL and LDL was 2.6&%0.9 and 1.4&%0.5 &mmol/min, respectively. The uptake of HDL cholesterol ester by tissues is small, with 97 percent of the cholesterol ester uptake from beta lipoproteins.

(2) In collaboration with Dr. Fisher and Dr. Stacpoole detailed kinetic studies were performed in subjects using an endogenous 3H-leucine tracer a precursor of nascent apolipoprotein B. Subjects were studied before and after restabilzation on lovastatin (a drug important in RAS modulation), 40 mg/day in divided doses and following a diet low in fat. High carbohydrate, low fat diets decrease plasma low-density lipoprotein cholesterol and apolipoprotein B mass in normal subjects and patients with familial hypercholesterolemia. To further investigate the mechanisms of these effects on cholesterol metabolism, 4 normal, 4 heterozygous familial hypercholesterolemic, and one homozygous familial hypercholesterolemic subjects were studied on a basal (45% carbohydrate, 40% fat) diet and during continues nasogastric infusion of Vivinex (90% carbohydrate, 1% fat). The change in total cholesterol, LDL-cholesterol, HDL cholesterol, VLDL-cholesterol and plasma triglycerides for normals were -27%, -39%, -32%, +59%, and +54%. and for familial hypercholesterolemic subjects, -26%, -36%, -30%, +82%, and +76%. Fecal sterol balance measurements demonstrated a 24% decrease in whole body cholesterol systhesis in normals, from 8.4&%4.4 to 6.4&%1.3 mg/kg/day and in familial hypercholesterolemic subjects a 58% decrease from 11.4&%5.6 to 4.8&%1.7 mg/kg/day. ApoB kinetic studies were performed using a &3H-leucine on basal and Vivonex regimens. A compartmental model was used to estimate the metabolic rates. High carbohydrate feeding resulted in an increased production but a prolonged delay in the secretion of triglycerides enriched VLDL. This reflects the increased intracellular assemblage time for more triglyceride rich lipoproteins. Total apoB synthesis was not altered on the low fat diet. A decrease in the residence time of the LDL-apoB was the primary determinate in the decrease in LDL-cholesterol concentrations following the low fat diet. This low fat, high carbohydrate, diet results in a fall of LDL concentration, suppression of cholesterol synthesis, an increase in the clearance of LDL, an increase in the cellular assemblage of VLDL, and an increased production of larger VLDL triglyceride rich particles which reside in the hepatocyte longer.

(3) 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; we are investigating selenium kinetics. Little is currently known about the kinetics of selenium in humans. The Selenium Pharmacokinetics Study a joint study between NCI and USDA was designed to estimate basic pharmacokinetic parameters for two prototype forms of selenium: sodium selenite (inorganic) and selenomethionine (organic).

Further, there was interest in whether these parameters would vary if either form was administered in a fasting or a non-fastig state as selenium is thought to be a cancer preventative in the proper dose and form.

The metabolism of selenium appears much more complex than was originally thought when the study was designed. This has led to kinetic modeling. The main study was preceded by a pilot study. Six subjects were each given a single, oral dose of 200 micrograms of sodium selenite. The cold stable isotope, &7&4Se, was used as a label for the dose. These six sets of data have been used to realize a model for selenium metabolism. The model developed describes the kinetics of sodium selenite metabolism in humans, based on plasma, urine, and fecal samples obtained from six subjects over a four week period following a single oral 200 &mg dose of the enriched stable isotope tracer $^{74}Se$. The model describes absorption distributed along the GI tract and enterohepatic recirculation. The model includes four kinetically distinct plasma components, a subsystem consisting of the liver and pancreas, and a slowly-turning-over tissue pool. For the six subjects, the ranges of mean residence times for the four plasma components are respectively: 0.2-1.1 hrs., 3-8 hrs., 9-42 hrs., and 200-285 hrs.; for the hepato-pancreatic subsystem, 4-41 days; and for the tissue pool, 115-285 days. Approximately 84% of the administered dose was absorbed and after 12 days about 65% remained in the body. The model predicts that, after 90 days, about 35% would be retained, primarily in the tissues. Separating Se metabolism into several distinct kinetic components is a first step in identifying the efficacious, nutritious, and toxic forms of the element. The techniques of compartmental modeling give us the power to make a separation between the tracer remaining from the first study and the tracer given in the second. By retaining label in the appropriate compartments in our model, we were able to adjust for the carryover effect in estimates for the second study.

In a portion of the main study sixteen subjects were each given labeled selenite. Subjects were on a controlled diet for three days prior to dosing, and twelve days thereafter. This allowed us to determine their total intake of selenium, and helped assure that they would be in steady state. A split unit design was chosen for the study. Each person received a single dose in both fasting and non-fasting nutritional states. This design was chosen to allow precise measurement of any differences resulting from fasting state, while minimizing the number of subjects required. Data analysis has centered around estimation of those parameters necessary to make decisions about size and frequency of dosing. Our analysis up to now has been based on extending the pilot study model to include these data.

There appears to be a difference in the two most rapid plasma components. In a crossover study in which each subject serves as his own control, there is always the possibility of carryover. In other words, the possibility of tracer from the first study influencing the second study. Our first task was to detect and remove carryover effects, if any. A comparison of the plasma data for each subject showed that the tail of the plasma curve for the second study was higher than that for the first study. This was true regardless of fasting order. To account for this carryover effect, we used the model to simulate the amount of

tracer remaining in the body after 90 days. We estimate that about 40 % of the first dose remained at the time the second was given. Most of this was in the slowly-turning-over tissues.

A pilot model for the analysis of the kinetics organic selenium, selenomethionine has been developed using the first two studies. This model will be used to compare the kinetics of organic selenium compounds and its modulation by fasting and nonfasting status of the study subjects. These models will allow us to make population estimates of the various pharmacokinetic parameters. Finally, these estimates will be compared using analysis of variance techniques. Both main effects and interactions of form selenium and fasting state will be investigated. Using kinetic modeling techniques, we have separated the metabolism of Se into multiple components. If, as we have suggested, the kinetically distinct plasma components represent chemically different forms of Se, each may have a specific, probably different, mechanism of action in relation to nutritional requirements, toxicity, and cancer prevention. If Se is an effective cancer prevention agent, its efficacy may not be uniform throughout the body; the amount and form reaching various organs or tissues may be of considerable research interest. Studies can be planned to identify those particular forms that show the greatest potential beneficial effect, and their particular kinetics further characterized.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08303-18 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Membrane Fusion Mediated by Viral Spike Glycoproteins.

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


Robert Blumenthal, Ph.D., Chief, Membrane Structure & Function Sect.,LMMB, NCI,

Other Professional Personnel:

| | | |
|---|---|---|
| Anu Puri, Ph.D. | Visiting Associate | LMMB, NCI |
| Michael Clague Ph.D. | Visiting Fellow | LMMB, NCI |
| Christian Schoch Ph.D. | Visiting Fellow | LMMB, NCI |
| Dimiter Dimitrov Ph.D. | Visiting Scientist | LMMB, NCI |


COOPERATING UNITS *(if any)*

Dr. Joel Lowy, NHBLI; Dr. Michel Ollivon, CNRS, France; Dr. Abraham Loyter, Hebrew University, Israel; Dr. Joshua Zimmerberg, NIDDK; Dr Yi-der Chen, NIDDK; Dr F. Booy, NIDDK; Dr R.W. Doms, NIAID, Dr S.J. Morris, UMKC, Kansas City.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Structure & Function Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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


The 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 encelope protein of HIV, the G protein of Vesicular Stomatitis Virus (VSV), the HN and F proteins ofSendai 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.





PHS 6040 (Rev. 1/84)

GPO 914-918

## Project Description

Major Findings:

1. We have designed a flow chamber, which allows rapid switching of the buffer solution around cells attached to a microscope cover-slip during microscopical observation We have performed single cell fusion quantitative measurements with a photomultiplier tube (PMT) mounted onto the camera port of the microscope. A carefully centered pinhole fitted between the PMT and the objective limited the field monitored by the PMT to a circular area with diameter of ca. 40 ÊM. The use of this chamber has made it possible for us to record fusion events in single cell pairs.

2. We examined the transient properties of previously observed fusion intermediates (Sarkar et al, J Cell Biol 109:113-122 (1990) by testing whether the fusion induced by lowering the pH can be arrested by bringing the pH back to neutral. By switching the pH back and forth between acid and neutral in the GP4F-RBC system, we could show that the t1/2 for commitment of HA to the fusogenic state is 6-10s whereas the time lags are 2-4-fold larger. Reacidification from neutrality, following failure to fuse, leads to fusion with the same lag time and time-course. Thus, the lag time is independent of history of exposure to pH and there are no partial intermediates within the "maturation" process which can take place in the absence of full triggering of fusion.

3. In order to study the role of target membrane in viral fusion we chose the erythrocyte membrane whose phospholipid arrangement can readily be modified. The phospholipids of normal erythrocytes are arranged asymmetrically across the plasma membrane; phosphatidylcholine (PC) and sphingomyelin are predominantly on the outer surface, whereas others such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) are predominantly restricted to the inner leaflet. However, erythrocytes can be lysed and resealed under conditions where the asymmetric distribution of phospholipids is lost or retained. Erythrocyte ghosts with a lipid-symmetric phospholipid bilayer distribution are susceptible to fusion with VSV However, conversion of phosphatidylserine (a negatively- charged lipid) in the lipid-symmetric ghost membrane to phosphatidylethanolamine by means of the enzyme phosphatidylserine decarboxylase did not alter the target membrane's susceptibility to VSV fusion.

4. We correlated the susceptibility to fusion with increased mobility of phospholipids in the bilayer of the target as monitored by electron spin resonance. The results indicate that the susceptibility to VSV fusion is not dependent on any particular phospholipid, but rather related to packing characteristics of the target membrane.

5. VSV fusion was equally effective before and after PS-depletion of eryhtrocyte ghost membranes. Moreover, liposomes consisting of PC with cis-unsaturated fatty acyl chains were equally effective targets as thos consisting of PS with the same fatty acyl composition. This indicates that the charge on the target membrane does not play a significant role. However,

lowering the ionic strength of the medium resulted in a somewhat longer delay in the onset of dequenching and a slower rate of dequenching [8]. The result indicates that electrostatics between components on the viral membrane might play a role in the overall fusion process.

6. Membrane fusion can also be brought about by addition of agents that cause dehydration (e.g. polyethyleneglycol, (PEG)). However, we observed the same kinetics of fluorescence dequenching in virus-cell as well as cell-cell fusion in presence and absence of PEG.

7. We have studied the disposition of a synthetic peptide corresponding to the sequence of the amino terminus of the HA2 subunit of influenza virus hemagglutinin with artificial lipid membranes using a battery of fluorescence techniques. We measured tryptophan fluorescence as a function of depth dependent quenchers, and also recorded decay associated spectra under various conditions. We observed no gross positional change of the tryptophan residue with respect to the bilayer center as a function of pH.

8. Various mutants of HA have been constructed by site-directed mutagenesis that introduced single, nonconservative amino acid changes in the fusion peptide [34]. Cell biological assays indicated different fusion phenotypes: substitution of glutamic acid for the glycine residue at the amino-terminus of HA2 abolished all fusion activity, but the protein could still undergo a conformational change to the low pH form, albeit at a slower rate. This substitution presumably stabilized the neutral conformation of the protein. In contrast, substitution of glutamic acid for the glycine residue at position 4 in HA2 accelerated the rate and raised the threshold pH at which the conformational change and fusion occurred, presumably by destabilizing the neutral conformation of the protein. We have obtained SV40-HA recombinant virus vectors from Dr Mary-Jane Gething and studied the fusogenic activity of HA proteins altered in the fusion peptide sequence, after expression in Simian cells using our spectrofluorometric assay. The kinetics of fusion induced by the altered HA proteins followed very well the pattern of fusogenic activity measured by cell biological techniques.

9. We have correlated density of viral envelope proteins with fusion kinetics, specifically lag times. The surface density can be modulated either by expression on the surface or the amount of cleavage of the precursor to the active form. Analysis of the results might lead to conclusions concerning the number of viral envelope proteins participating in the fusion process.

Publications:

Sarkar DP, Morris SJ, Eidelman O, Zimmerberg J, Blumenthal R. Initial stages of influenza hemagglutinin-induced cell fusion monitored simultaneously by two fluorescent events: cytoplasmic continuity and lipid mixing. J Cell Biol 1989; 109:113-122.

Sarkar DP, Blumenthal R. The role of the target membrane structure in fusion with sendai virus. Membrane Biochemistry 1989;7: 231-247.

Blumenthal R. Reconstituted virus envelopes as tools for cellular engineering. In: Verna R, Blumenthal R, Frati L, eds. Bioengeneered Molecules: Basic and Clinical Aspects, Advances in Experimental Medicine, Serono Symposia Series, New York: Raven Press, 1989; 1:46-56.

Chen Y, Blumenthal R. Use of Self-quenching Fluorophores in the study of membrane fusion: Effect of slow probe redistribution. Biophysical Chem, 1989;34:283-292.

Clague MJ, Schoch C, Zech L, Blumenthal R. Gating kinetics of pH-activated membrane fusion of vesicular stomatitis virus with cells: stopped flow measurements by dequenching of octadecylrhodamine fluorescence. Biochemistry 1990; 29:1303-1308.

Harris HW, Hendler JS, Blumenthal R. Toad urinary bladder vesicles containing particle aggregates possess very high water permeability. Am J Physiol 1990;258 (Renal Fluid Electrolyte Physiol. 27:)F237-243.

Lowy RJ, Sarkar DP, Chen Y, Blumenthal R. Single influenza virus-cell fusion events observed by fluorescent video microscopy. Proc Natl Acad Sci U S A 1990; 87:1850-1854.

Herrmann A, Clague MJ, Puri A, Morris SJ, Blumenthal R, Grimaldi S. Effect of erythrocyte transbilayer phospholipid distribution on fusion with vesicular stomatitis virus. Biochemistry, 1990;29:4054-4058.

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, in press.

Walter A, Eidelman O, Ollivon M, Blumenthal R. Functional reconstitution of viral envelopes. In: Wilschut J, Hoekstra D. eds. Cellular Membrane Fusion: Fundumental Mechanisms and Applications of Membrane Fusion Techniques. New York:Marcel Dekker, Inc., 1990, in press.

Description of AIDS Research:

We use recombinant vaccinia virus containing the gene for the envelope glycoprotein of HIV obtained from Dr B. Moss. It has been demonstrated that cell-surface expression of this protein, in the absence of other HIV structural or regulatory proteins, is sufficient to induce CD4-dependent cell fusion. We examine the question of target cell specificity using lipid membranes with and without incorporated CD4. As the biological targets we use membrane preparations from CD4-expressing cell lines (e.g. CEM, H-9, Jurkat). We also use the erythrocyte membrane whose phospholipid arrangement can readily be modified. Phospholipid specificity can be assessed by changing bilayer phospholipid distributions. We incorporate CD4 into erythrocyte membranes by a brief low pH treatment. Alternatively it is possible to implant CD4 into erythrocytes by coreconstitution with Sendai envelope proteins. The basic approaches towards the elucidation of mechanism(s) of HIV-mediated membrane fusion are similar to the specific research goals we have formulated to study mechanisms of fusion induced by other enveloped viruses. In the allosteric model we considered the proton as the ligand mediating the conformational change of the viral protein which triggers the fusion reaction. That conceptual framework was developed in the context of extensive experimental studies in our laboratory with VSV and influenza virus whose mode of entry is pH-dependent. However, the HIV envelope fuses directly with the plasma membrane at neutral pH. In such case we have proposed that the "ligand" that induces the conformational change in the viral spike glycoprotein might be some portion of the cell surface receptor for the viral protein. The most likely candidate to fill that function for the HIV envelope protein is the CD4 receptor protein or some portion of that molecule. Efficient expression systems have been described in which a recombinant, soluble form of CD4 (sCD4) is secreted into tissue culture supernatants. The sCD4, which retains the structural and biological properties of intact CD4, will be used as the "ligand" in our studies on conformational changes and fusogenic activity of the HIV envelope protein. Initial steps of membrane fusion are monitored on single cells and cell populations using spectrofluorometry and quantitative video microscopy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 08320-15 LMMB

PERIOD COVERED: October 1, 1989 to September 30, 1990

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. | Theoretical Physical Chemist | LMMB, NCI |
| Other Professional Personnel: | | |
| Shou-ping Jiang | Visiting Scientist | LMMB, NCI |
| H. Robert Guy, Ph.D. | Sr. Staff Fellow | 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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 1.3 | 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.)

The solution conformations of two tachykinins, substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$), physalaemin (pGln-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-$NH_2$), senktide(Asp-Phe-Phe-Gly-Leu-Met$NH_2$) and septide (Asp-Phe-Phe-Pro-Leu-Met-$NH_2$) have been studied here with a combination of NMR 2-dimensional NOE's and semi-empirical energy calculations. The number of possible conformations is extremely large, and requires a large computational 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.

Specific treatments of solvation of biopolymers were derived. They suggest the importance of short water bridges in stabilizing both intra- and inter-molecular conformations. In this sense, folded proteins could be stabilized if they fit especially well into the partially disordered lattice of waters.

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 (SP) 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:

Ben-Naim A. Solvent effects on protein association and protein folding. Biopolymers 1990;29:567-596.

Ben-Naim A, Ting K-L, Jernigan RL. Solvation thermodynamics of biopolymers I. Separation of the volume and surface interactions with estimates of the volume and surface interactions. Biopolymers 1989;28:1309-1325.

Ben-Naim A, Ting K-L, Jernigan RL. Solvation thermodynamics of biopolymers II. Correlations between functional groups. Biopolymers 1989;28:1327-1337.

Ben-Naim A, Ting K-L, Jernigan RL. Solvent effect on binding thermodynamics of biopolymers. Biopolymers 1990;29:901-919.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 08335-14 LMMB

PERIOD COVERED: October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
"Targeting" Liposomes for Selective Interaction with Specific Cells and Tissues

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

John N. Weinstein, M.D., Ph.D. Chief, Theoretical Immunology Section, LMMB, NCI

COOPERATING UNITS (if any) Drs. Sharon M. and Larry M. Wahl, NIDR; Drs. Mika Popovic and Suzanne Gartner, LTCB, DCE; R. J. Parker, Office of Director, DCE

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Theoretical Immunology Section

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

| TOTAL MAN-YEARS: | PROFESSIONAL: 2.0 | 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.)

We have studied three conceptually different ways of "targeting" liposomes:

(1) Antibody-mediated targeting. We found that antibody-bearing liposomes bind in large numbers to cells which bear the appropriate antigen. However, the bound liposomes are internalized only if endocytosis is possible. Upon endocytosis, liposome-entrapped methotrexate (MTX) can escape from the endocytic apparatus and bind to cytoplasmic dihydrofolate reductase, inhibiting growth of the cell. In the course of these studies, we developed the first heterobifunctional method for coupling antibody to liposomes. Current studies are directed toward HIV-infected cells.

(2) Physical targeting. We designed "temperature-sensitive" liposomes, which break down and selectively release an entrapped drug in vivo at temperatures achievable by local hyperthermia. These liposomes selectively deliver MTX or cis platinum to mouse tumors in vivo and inhibit their growth.

(3) Compartmental targeting. We have demonstrated the delivery of liposomes and entrapped drug to lymph nodes after subcutaneous and intraperitoneal, injection and have determined cellular sites of localization. These studies have been extended to antibody-bearing liposomes. These strategies are being applied to problems in the therapy of cancer and AIDS. With respect to AIDS, we have formulated liposomes containing anti-viral drugs (dideoxynucleoside triphosphates) which do not ordinarily enter cells but which can be carried into monocyte/macrophages by the liposomes. Alternatively antibody is attached to the liposomes by our previously developed methods for delivery to targeted cell types.

Active research discontinued in 1989 - 1990.



PHS 6040 (Rev. 1/84)

GPO 814-818

Project Description:

Active research on this project was discontinued in 1988 - 1989.

Bibliography:

Saebeni J, Wahl SM, Wahl LM, Gartner S, Popovic M, Parker R, Black C, and Weinstein JN: Inhibition of HIV-1 in monocyte/macrophage cultures by 2', 3'-dideoxycytidine-5'-triphosphate, free and in liposomes, AIDS and Human Retroviruses, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08341-12 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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, |
| Other Professional Personnel: | | |
| H. Robert Guy, Ph.D. | Senior Staff Fellow | LMMB, NCI |
| Kai-Li Ting, Ph.D. | Computer Programmer | LMMB, NCI |
| Vellarkad Viswanadhan | Biotechnology Fellow | LMMB, NCI |
| Benjamiin Denckla | Summer Student | LMMB, NCI |

COOPERATING UNITS *(if any)*

Drs. J. Segrest & Anantharamaiah, U. Alabama, Birmingham; Pat Elwood, MB, NCI

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

☐ (a1) Minors

☐ (a2) Interviews

xx

B

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


Statistical mechanical algorithms (HAL, HALP, HALCO) were devised for evaluating amphipathic helical structures and more general structure- function relationships in proteins and peptides. These computational approaches are being used to define issues of structure and possible immunogenicity with respect to HLA antigens and to the envelope polyprotein of HIV.

Also developed was a new joint prediction approach to protein secondary structure. The method combines neural network, information theory, sequence homology, hydrophobicity, loop potential, and amphipathicity criteria (including HAL). We have applied it to structure predictions on the human and bovine folate binding proteins, which are important in the cellular handling of folates and antitumor drugs such as methotrexate.

Quantitative structure/activity relationship (QSAR) studies are being done on a set of 20 inhibitors of nucleoside transport into cells. In parallel, a molecular mechanics study is being done on analogues of the unusual nucleoside transport inhibitor dipyridamole. Our group has found that dipyridamole (and at least one other nucleoside transport inhibitor) potentiates the activity of azidothymidine against HIV-1 replication in cultured monocyte/macrophages and T-lymphocytes (see report # Z01 CB 08392-02 LMMB). Animal studies are in progress and clinical trials are being planned, in parallel with continuing attempts to understand the mechanisms of action and molecular structure/function relationships.

Project Description

Major Findings:

(1) Development of the HAL (Helical Amphipathicity Locator) algorithm and computer program set for analysis of structure/function relationships in proteins. Related packages further developed during the last year include HALP, HALPSTAT, HALCOMP, COPHAL and HALCO. (12) Application of these programs to the problem of finding T-cell antigentic peptides and other structural features of the HIV envelope protein. (13) Predicted peptides have been synthesized and have undergone physical chemical and immunological testing.

(2) Use of HAL to determine binding sites on proteins that interact with suramin. The predictions were used by collaborators for rational design of next-generation suramin analogues.

(3) Development of a new joint prediction approach to protein secondary structure. The method combines neural network, information theory, sequence homology, hydrophobicity, loop potential, and amphipathicity criteria (including HAL). We have applied it to structure predictions on the human and bovine folate binding proteins, which are important in the cellular handling of folates and antitumor drugs such as methotrexate.

(4) Quantitative structure - activity study of certain synthetic nucleotide inhibitors of nucleotide transport in animal cells.

(5) Conformational analysis of DPM and related analogs using the MM2(87) molecular mechanics package.

Publications:

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, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08359-09 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Monoclonal Antibodies in the Lymphatics for Diagnosis and Therapy of Tumors

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

John N. Weinstein, M.D., Ph.D. Chief, Theoretical Immunology Section LMMB, NCI

COOPERATING UNITS *(if any)* Dr. A. Keenan, Dr. S.M. Larson, LHM, CC; Dr. R. Parker, Dr. S. Sieber, DCCP; Dr. L. Liotta, Dr. G. Bryant, LP, DCBD; Dr. M. Lotze, Dr. R. Rosenberg, SB, DCT; Dr. J. Mulshine, NCI-NMOB, DCT; Dr CDV Black, ROB, DCT

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☐ (a1) Minors

☐ (a2) Interviews

B

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

We have defined a new approach to the use of monoclonal antibodies for diagnosis and therapy of tumor in lymph nodes: delivery to the nodes via lymphatic vessels after subcutaneous injection. To establish a firm pharmacokinetic basis for this approach, we first studied antibodies to normal cell types in the mouse lymph node. In vitro binding characteristics were combined with in vivo pharmacological parameters to develop a quantitative understanding of the delivery process using the SAAM computer modeling system. Armed with that background information, we then demonstrated and analyzed specific uptake in lymph node metastases of a guinea pig tumor. The approach was extended to include endoscopic techniques for reaching lymph node groups not accessible by subcutaneous injection. Imaging studies were followed up with attempts at therapy. For diagnosis of early metastatic tumor in the nodes, the lymphatic route can provide higher sensitivity, lower background, lower systemic toxicity, and faster localization than the intravenous route. It will also minimize the problem of cross-reactivity with antigen present on normal tissues. This technique has been applied to detection of lymph node metastases in clinical stage II malignant melanoma and cutaneous T-cell lymphoma (CTCL) (with very good results). Similar protocols have been approved for breast carcinoma, small cell lung carcinoma, and non-small cell lung carcinoma. Our studies of CTCL produced the most efficient antigen-specific imaging yet achieved in humans by any technique. The longer term aim is to understand the pharmacology of monoclonal antibodies and other ligands in order to develop criteria for rational molecular design of biological antitumor agents. This work on "immunolymphoscintigraphy" has been followed up clinically by others, who recently published encouraging results on diagnosis of axillary metastases of breast cancer.

Active research on this project was discontinued in 1988-9 except for publications and patents.

Project Description:

Active research on this porject was disontinued in 1988 - 89.

Publications:

Weinstein JN. Antibody lymphoscintigraphy. In: Goldenberg DM, ed. Cancer imaging with radiolabeled antibodies, in press.

Patents:

Mulshine J and Weinstein JN. US Patent 07/133,978: Treatment or diagnosis by endoscopic administration into the lymphatics, February 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08363-08 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Protein Modelling

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

| | | |
|---|---|---|
| H. Robert Guy, Ph.D. | Senior Staff Fellow | LMMB, NCI |
| Other Professional Personnel: | | |
| G. Raghunathan, Ph.D. | Visiting Fellow | LMMB, NCI |
| S. Durrell, Ph.D. | IRTA Fellow | LMMB, NCI |
| G. Boheim, Ph.D | Guest Researcher | U. of Bochum |

COOPERATING UNITS *(if any)*

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| 3.0 | 3.0 | 0 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The primary goal of this project is to develop methods to predict structures of membrane proteins from their sequences and available experimental data and to use these methods to develop structural models of specific membrane proteins. We have developed a hierarchical approach to modeling membrane proteins. The initial phase produces relatively imprecise models that require little computational time. Subsequent phases are increasingly precise and require increasing amounts of computation to calculate and minimize the energies of the structures. The models we have developed can be classified into three general structural motifs: (1) structures for which all transmembrane segments are α helices, (2) structures for which all transmembrane segments are β strands, and structures for which both α helices and β strands are in the transmembrane region.

The simplest structures we have been analyzing are a series of peptides (melittin, 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 that we are currently analyzing are voltage activated channels (sodium, calcium, and potassium channels), transmitter-activated channels (nicotinic acetylcholine, $GABA_a$, and glycine receptors), receptors that activate G proteins (adrenergic, muscarinic acetylcholine, serotonin, dopamine, substance K, and opsin receptors), gap junction channels, and a superfamily of proteins that include synexin.

PROJECT DESCRIPTION

Major Findings:

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 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. We are collaborating with Harvey Pollard's group to test this model. Working with Franco Conti, we have developed a similar model for voltage-gated sodium, calcium, and potassium channels. In these models an eight stranded antiparallel β barrel is surrounded by eight α helices which are surrounded by sixteen α helices. Several predictions of this model have been confirmed by mutagenesis experiments. We are collaborating with Franco Conti and Walter Stümer to test other aspects of these models.

Little is known about how proteins insert into membranes. On the basis of our modeling we proposed a new mechanism for insertion of amphipathic structures. In this model amphipathic peptides or amphipathic portions of proteins assemble on one surface of a membrane to form 'raft-like' structures that are hydrophobic on one surface and hydrophilic on the opposite surface. The protein 'rafts' displace the lipid monolayer on one surface. If monomers are α helices that pack in an antiparallel fashion, as proposed for δ lysin, then the 'rafts' are rectangular and insertion occurs when two rafts meet and then fold across the second lipid monolayer. If monomers pack in a parallel fashion, as proposed for pardaxin, then the 'rafts' are circular and insertion occurs when residues in the center of the 'raft' cross the remaining portion of the membrane.

Publications:

Guy HR. Models of voltage- and transmitter-activated channels based on their amino acid sequences. In: Pasternak CA, ed. Monovalent cations in biological systems. CRC Press, 1990;31-58.

Lazarovici P, Primor N, Gennaro J, Fox J, Shai Y, Lelkes PI, Caratsch CG, Raghunathan G, Guy HR, Shih YL, Edwards C. Origin, chemistry and mechanisms of action of pardaxin; a repellent, presynaptic excitatory, ionophore polypeptide. In: Hall S. (Ed) Marine toxins: origin, structures, and pharmacology. American Chemical Society, 348-364.

Guy HR. Molecular models for voltage activated channels. In: Hondeghem L, ed. Molecular and cellular mechanisms of antiarrhythmic agents. Futura Publishing Co. Inc., 1989;155-178.

Raghunathan G, Seetharamulu P, Brooks B, Guy HR. Models of δ hemolysin membrane channels and crystal structures. Proteins, in press.

Guy HR, Conti F. Pursuing the structure and function of voltage-gated channels. Trends in Neuroscience, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08365-08 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Structural Properties of T-cell Antigenic Sites

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

| | | |
|---|---|---|
| Robert L. Jernigan, Ph.D. | Theoretical Physical Chemist | LMMB, NCI |
| Other Professional Personnel: | | |
| Hanah Margalit, Ph.D. | Visiting Associate | LMMB, NCI |

COOPERATING UNITS (if any) Jay A. Berzofsky, Metabolism Branch, DCBDC, NCI; James L. Cornette, Department of Mathematics, Iowa State University; Charles DeLisi, Mount Sinai School of Medicine; David G. Covell, ASCL, PRI, FCRDC.

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| 0.1 | 0.1 | 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.)


Intrinsic sequence features are important for T-cell recognition. T lymphocytes (T cells) recognize only a small number of segments (antigenic sites) of a foreign protein, and only when the segment is presented on the surface of an antigen presenting cell in association with a major histocompatibility (MHC) molecule. Previously, by statistical methods we found properties of the known antigenic sites that distinguish them from the rest of the protein. The property that was strongly correlated with T-cell antigenicity was α-helical amphipathicity, and we developed a predictive algorithm, based mainly on this property. We are currently interested in studying the sequence features that determine the MHC restriction. We hope to incorporate those properties into the predictive algorithm, so that in addition to prediction of immunodominance it will identify the MHC restriction of a peptide. The ability to better predict T-cell sites from the primary sequence will be important for the design of synthetic and recombinant vaccines.

Our approach has been to simulate the complex of antigen-MHC class II molecule, based on the known structure and published coordinates of the class I molecule. By repeating the analysis for all antigens that are restricted to a specific MHC, we can look for consistent rules, and then test our hypothesis by simulating the same antigens with other MHC types for which they are known not to be restricted. Recently, we have also applied a new method, developed for protein folding problems, to generate all possible conformations of an amino acid sequence in the limited space of the binding region.

PROJECT DESCRIPTION

Major Findings:

As derived earlier from studies of immunodominant sites in proteins, the following properties correlate with a peptide being a T cell immunodominant site: the ability to fold into an α-helix, the resulting helix being amphipathic, having COOH-terminal lysines, and not being segmentally amphipathic. Variant peptide studies allow another type of statistical evaluation, and preliminary results confirm the role of α-helical amphipathicity as a correlate of peptide antigenicity. In addition to the properties that make a site immunodominant, we are studying the sequence features that determine the MHC restriction. For this analysis we use two approaches. Both of them use the known three dimensional structure of class I molecule to model the structure of class II molecule, using sequence alignment methods. The structure contains two α helices with a β sheet floor, forming a cleft within which the peptide is believed to interact with the MHC molecule. We are looking for the best placement of the peptide in this cleft, trying to identify peptide and MHC residues that are crucial for the interaction. By the first approach we model the peptide as an α helix. There are many possible conformations for complexes formed between the two α helices and an antigenic α helix. The likelihood of each conformation is evaluated so that the most favorable ones can be determined. By the second approach no specific structure is assumed for the peptide but its structure is a result of the simulation. A set of points on a cubic lattice is used both to restrict the position of the peptide to the binding region, as well as for the generation of all of its conformations consistent with this constraint. One residue of the peptide is tethered to the protein, in this case, based upon hydrophobicity patches of the protein. The large MHC protein is taken to be rigid. Typically, a few million conformations of the peptide are generated. Subsequently, the energies of all these conformations are evaluated in terms of residue-specific, pairwise contact energies that favor non-bonded, hydrophobic interactions. The results give some indication of the relative influence of the intra-molecular peptide energy and the inter-molecular binding energy. The simulations are done for each Ia type and the data base of its known antigens separately. From these models, specific sequence features that determine the MHC restriction can be studied and it is hoped that the properties determining immunodominance of antigenic sites can be understood at a basic level. Understanding the biochemistry of these structures will be valuable for the design of synthetic or recombinant vaccines.

Several methods are now available for predicting immunodominant T cell sites: they have commercial importance as predictors of possible peptide vaccines. A statistical evaluation of these schemes has three objectives: to encourage the further collection of unbiased data, to determine the best method, and to have a criterion by which to judge future schemes.

Publications:

Cornette JL, Margalit H, DeLisi C, Berzofsky JA. Identification of T cell epitopes and use in the construction of synthetic vaccines. Methods in Enzymology 1989;178:611-634.

Margalit H, DeLisi C, Berzofsky JA. Computer predictions of T cell epitopes. In: Woodrow GC, Levine MM, Eds. The molecular approach to new and improved vaccines. New York: Marcel Dekker, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 08366-07 LMMB

PERIOD COVERED: October 1, 1989 to September 30, 1990

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

The Percolation of Monoclonal Antibodies into Tumors.

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

John N. Weinstein,M.D., Ph.D. Chief,Theoretical Immunology Sect. LMMB, NCI

Other Professional Personnel:

Kenji Fujimori, M.D. Visiting Fellow LMMB, NCI

COOPERATING UNITS (if any)

Dr. L. Liotta, LP, DCBD; Dr. John Fletcher, LAS, DCRT; Drs. David Coloher and Jeffrey Schlorn, LTIB, DCBD; Dr. John Cook, ROB, DCT

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.4 | 1.4 | 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.)

Before a monoclonal antibody (or other biological ligand) can label or kill a tumor cell, it must first reach that cell. For portions of a tumor far from the nearest blood vessel or other source of antibody, access may be limited by the rate at which the molecule can "percolate" through the extracellular space. We are investigating the spatial and temporal profiles of immunoglobulin (Ig) distribution generated by diffusion and convection through tumors, taking into account the possibilities of (a) saturable specific binding to cells, (b) nonsaturable, nonspecific binding, and (c) metabolic degradation. We first developed theoretical models that splice together the global pharmacology and the microscopic percolation process. Significant predictions thus far include the following: (1) The diffusion coefficient and/or hydraulic conductivity may limit flux of antitumor Ig through tumors. (2) The flux of non-binding control Ig is much less likely to be limited by diffusion or convection. Nonspecific Ig's penetrate more deeply and more quickly into the tumor. (3) Even with saturable binding (but not metabolism), the "C times T" exposure of tumor cells to antibody will be the same throughout the mass. (4) Metabolism will decrease the relative "C times T" exposure of cells farther from the source. This may be a major barrier to effective treatment of solid tumors with ligand molecules. (5) Most interesting, antibodies with low affinity may be preferable at a given dose to those with high affinity for some therapeutic applications.

We plan to test predictions of the model using micrometastases of human melanoma in nude mice. The distribution of antibody will be determined by fluorescence techniques and autoradiography. Concepts arising from this study are being applied to the design of clinical studies with monoclonal antibodies.

In addition to the investigations of immunoglobulin and other ligands (e.g.,soluble CD4) as administered agents, we are considering the physiology of endogenous molecular species including the lymphokines and growth factors.



PHS 6040 (Rev 1/84) GPO 914-818

Major Findings:

(1) For physiologically reasonable ranges of parameters, the diffusion coefficient and/or hydraulic conductivity may limit flux of antitumor antibodies through tumors; (2) The flux of non-binding control antibody is much less likely to be limited by the rate of diffusion or convection through the tumor. Such antibodies are predicted to penetrate more deeply and more quickly into the tumor; (3) In the presence of saturable binding but not metabolism, the "C times T" exposure of tumor cells to antibody will be the same throughout the mass. However, the period of exposure will be later for cells farther from the source of antibody; (4) Metabolism will decrease the relative CxT exposure of cells farther from the source. This may be a major barrier to effective treatment of solid tumors with ligand molecules; (5) Antibodies of low affinity may be preferable to those of high affinity at a given dose for some purposes, e.g. in therapy with alpha-emitting conjugates or toxin conjugates. (6) Quantitative, but not qualitative, changes in predicted behavior are seen as the geometry changes from Cartesian to cylindrical (for cords of tumor cells surrounding a central blood vessel) to spherical (for flux into a nodule of cells); (7) the characteristics for IgG, F(ab'), and Fab can been compared; (8) Bivalent binding can be, simulated; (9) the global pharmacology can be integrated with the percolation calculations; (10) the computer program package developed ("PERC") is broadly capable of handling various ligands of differing valence. (11) the "PERC-RAD" program was developed to calculate the radiation dose distributions resulting from spatial concentration distributions determined by PERC results. Calculations predict surprisingly great inhomogeneity for modules such as those of nodular lymphoma treated by I-131 immunoconjugates.

Publications:

Fujimori K, Covell DG, Fletcher JE, Weinstein JN. A modeling analysis of IgG, F(ab') and Fab in tumors. Cancer Research, 1989;49:5656-5663.

Weinstein JN. Antibody lymphoscintigraphy. In: Goldenberg DM, ed. Cancer imaging with radiolabeled antibodies, in press.

Weinstein JN, Fujimori K. Antibody-mediated drug delivery. In: Poste, ed. Protein Design and the Development of New Therapeutics and Vaccines, in press.

Weinstein JN, Fujimori K. Predictive pharmacology of monoclonal antibodies. Cancer Therapy: New Trends, in press.

Fujimori K, Covell DG, Fletcher JE, and Weinstein JN: A modeling analysis of monoclonal antibody percolation through tumors: A binding site barrier, J Nucl Med, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08367-07 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Selective Cytotoxicity in the Lymphatics

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

John N. Weinstein, M.D., Ph.D. Chief, Theoretical Immunology Section LMMB, NCI

COOPERATING UNITS (if any) Dr. Christopher D.V. Black, ROB, DCT, NCI; Drs. R.J. Parker and S.M. Sieber., OD, DCE, NCI; Dr. O.A. Gansow and R.W. Atcher., COP, DCT, NCI; Drs. R.A. Kroczek and E.M. Shevach., LI, NIAID

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

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NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS. | PROFESSIONAL. | OTHER. |
|---|---|---|
| 0.0 | 0.0 | 0.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Following subcutaneous injection, radiolabeled monoclonal antibodies bind efficiently to normal and tumor target cells in the lymph nodes (Project #Z01 CB 08359-03 LMMB). This finding prompted us to attempt specific therapy using monoclonal antibody conjugates.

An immunotoxin made from the A-chain of ricin coupled to an anti-mouse MHC antibody has proved to be highly toxic for lymphoid cells and similar results have been found with ricin A-chain conjugated to monoclonal antibodies against subsets of mouse lymphocytes in vitro. We have also been able to augment the effects of these toxins by the action of certain drugs which are known to affect cell biological processes. These studies have led to a new hypothesis for the cell biological pathway (the "neutral bypass") by which toxin molecules enter the cytoplasm from antibody conjugates to kill a cell.

A further type of immunoconjugate for specific cell killing in vivo consists of a radioactive compound chelated to an antibody. We have demonstrated selective ablation of lymph node B lymphocytes in mice injected subcutaneously with an anti-murine B cell antibody labeled with the alpha particle emitter 212Bismuth. The relative potency of this conjugate for B cells in vivo was 10-fold higher than for T cells taken from the same nodes.

In order to assess the effects of antibodies and antibody conjugates in vivo, we have established two models of lymph node T cell activation. In one, the stimulus is the plant lectin concanavalin A administered into the footpad; the other stimulus is allogeneic cells. Both of these stimuli induce T cells to express receptors for IL-2. The concanavalin A model is susceptible to the inhibitory effects of cyclosporin A whereas the allogeneic model is not.

Active research discontinued in 1988.

Project Description:

Major Findings:

Inactive in 1989 - 90 except for one manuscript plus granting of patent (see below).

Active research on this project ended in 1988.

Bibliography:

Weinstein JN. Antibody lymphoscintigraphy. In: Goldenbnert DM, ed. Cancer imaging with radiolabeled antibodies. In press.

Patents:

Mulshine J and Weinstein JN. US Patent 07/133,978: Treatment or diagnosis by endoscopic administration into the lymphatics, February 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08370-07 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Interactions in Globular Proteins and Protein Folding

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

| | | |
|---|---|---|
| Robert Jernigan, Ph.D. | Theoretical Physical Chemist | LMMB, NCI |
| Other Professional Personnel: | | |
| Kai-Li Ting, Ph.D. | Computer Programmer | LMMB, NCI |
| Peter Greif, M.D. | Computer Prog. Anal. | LMMB, NCI |

COOPERATING UNITS *(if any)* David Covell, ASCL, PRI, FCRDC.

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|---|---|---|
| 0.8 | 0.8 | 0.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

A novel approach has been taken to the problem of protein folding that examines the complete range of folded topologies accessible in the compact state of globular proteins. The procedure is to generate all conformations, with volume exclusion, upon a lattice in a space restricted to the individual protein's known compact conformational space. This approach affords an enormous reduction in the number of conformations that must be considered for the complete problem. Five proteins have been treated using this method; avian pancreatic polypeptide (36 residues), crambin (46 residues), rubredoxin (52 residues), pancreatic trypsin inhibitor (58 residues), and neurotoxin (62 residues). All conformations generated are evaluated in terms of residue-specific, pairwise contact energies that favor non-bonded, hydrophobic interactions. Native structures for these proteins are always found within the best 3% of all conformers generated. Additional methods are being developed to further narrow the choice of the best among the good conformers. One approach superimposes all atoms onto a lattice conformation, and refines this structure with conventional molecular dynamics and energy minimization. For the test case of one low energy lattice conformation for crambin, an all-atom structure was built that was within 1.46 a RMS deviation from the native crystal structure. These methods are simple and general and can be used to determine most favorable overall packing arrangements for the folding of specific amino acid sequences within a restricted space. Other aspects being considered include: combining the folding calculations with sequence homologies and investigating hydrophobic cores, binding, choices of overall shapes and the relationship between good packing and secondary structures.

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 dense 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 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.

Publication:

Jernigan RL, Margalit H, Covell DG. Coarse graining conformations: A peptide binding example. In press: Theoretical biochemistry and molecular biology; A computational approach. Schenectedy, New York: Adenine Press.

Jernigan RL, Davies D, Scheraga H. Experimental and theoretical protein folding. J Biomol Str and Dyn 1989;6:1039-1043.

Covell DG, Jernigan RL. Conformations of folded proteins in restricted spaces. Biochemistry 1990;29:3287-3294.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08371-07 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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. | Theoretical Physical Chemist | LMMB, NCI |
| Other Professional Personnel: | | |
| Gopalan Raghunathan, Ph.D. | Visiting Fellow | LMMB, NCI |

COOPERATING UNITS *(if any)* Dr. Ruth Nussinov, Institute of Molecular Medicine, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; Dr. Jacob Mazur, Gary W. Smythers, and Dr. David Wang, Program Resources, Inc., Frederick, MD; Howard Nash, LMB, NIMH; Akinori Sarai, RIKEN Institute.

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 1.5 | 0.1 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Conformational analysis of DNA shows that the origin of the B-form double helix can be substantially attributed to the atomic charge pattern in the base pairs. The pattern favors specific helical stacking of the various base pairs. The base pairs alone, without the sugar-phosphate backbone have a strong tendency to be helical. The backbone appears to play a relatively passive role in determining the helix form of DNA. Both electrostatic and van der Waals interactions play a role, but electrostatic interactions are particularly important in A-form DNA.

Sequence studies of bases flanking homooligomer tracts indicate that there are strong biases: usually preferred are bases complementary or identical to the run. A or T runs are preferentially flanked by A's or T's whereas G or C runs are preferentially flanked by G's or C's.

The asymmetries of conformational fluctuations were calculated. These directional preferences were used to a construct specific sequence model 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.



PHS 6040 (Rev. 1/84) GPO 914-910

PROJECT DESCRIPTION

Major Findings:

We have studied the sequence dependence of DNA conformational transition between B- and A-forms. The role of intramolecular interactions between base pairs, without backbone, has been examined for the conformational transition between B- and A-form helices. The base pairs themselves usually have intrinsic conformational preferences for B- or A-form. Calculation of all ten possible base steps shows that the base combinations, CC (or GG), GC, AT, and TA, have tendencies to assume A-conformation. Results show that it is particularly easy to slide along the long axis of the base pair for these steps, with AT and CC showing especially flat energies. However, a preference for B- or A-conformation depends on the electrostatic energy parameters, in particular, on dielectric and shielding constants; A conformation is preferred for low dielectric constant or low shielding. Both A and B conformations are stabilized mainly by electrostatic interactions between 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 fluctuation.

Publication:

Mazur J, Sarai A, Jernigan RL. Sequence dependence of the B-A conformational transition of DNA. Biopolymers 1989;28:1223-1233.

Sarai A, Mazur J, Nussinov R, Jernigan RL. Sequence dependence of DNA conformational flexibility. Biochemistry 1989;28:7842-7849.

Nussinov R, Sarai A, Smythers GW, Jernigan RL. Distinct patterns in homooligomer tract sequence context in prokaryotic and eukaryotic DNA. Biochim Biophys Acta 1989;1008:329-338.

Nussinov R, Sarai A, Smythers GW, Wang D, Jernigan RL. Strong patterns in homooligomer tracts: occurrences in non-coding and in potential regulatory sites in eukaryotic genomes. J Biomol Str Dyn 1989;7:707-722.

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. New York: Adenine Press, in press.

Raghunathan G, Jernigan RL, Ting K-L, Sarai A. Solvation effects on the sequence variability of DNA double helical conformations. J Biomol Str Dyn, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08374-06 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Membrane Fusion: Structure, Topology and Dynamics; Cell Junctions

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

Pedro Pinto da Silva, Ph.D. Chief, Membrane Biology Sect. LMMB, NCI

COOPERATING UNITS *(if any)* A. Aguas, Institute of Experimental Cytology, University of Porto, Portugal; K. Fujimoto, Dept. of Anatomy, University of Kyoto School of Medicine.

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. | PROFESSIONAL. | OTHER. |
|---|---|---|
| 0.2 | 0.2 | 0.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Freeze-fracture electron microscopy of the zonula occludens (tight junction) has been confined to illustrate anastomosing network that characterizes lateral aspects of the junctions, but has failed to reveal apical aspects of the juxta-luminal (first) tight junction strand. The juxta-luminal strand is always deeply set in a narrow indentation formed by the adjoining epithelial cells. In this study we distended toad urinary bladders to attenuate this indentation in order to obtain freeze-fracture apical views (both exoplasmic and protoplasmic) of the juxta-luminal elements of the tight junction. Our results demonstrate transcellular continuity of the apical exoplasmic membrane halves of neighboring epithelial cells at the junctional site. In addition, they show the existence of intramembrane bridging elements alongside intramembrane tight junction strands. Our results support the linear-membrane-micellar model of the tight junction (Pinto da Silva & Kachar, 1982). We used the acrosome reaction of boar sperm cells to study the dynamics of surface transmembrane glycoproteins (TMG) during secretion. The acrosome reaction is the $Ca^{2+}$-dependent fusion of a large cytoplasmic vesicle (the acrosome) with the overlaying segment of the plasma membrane (acrosomal cap) that leads to the release of the acrosomal enzymes. We found that TMG move in the direction of either one of two opposite poles, proximal and distal, of the cap. This bimodal movement of the TMG reorganizes the acrosomal cap into three domains: the first two, the apical rim and the equator, are membrane domains where the integral proteins are directed to, accumulated, and form small aggregates; the third, comprises the extensive in-between area from where TMG were displaced. The topography of the new membrane domains of the acrosomal cap is now coincident with that of the unaltered domains of the acrosomal membrane underneath. The mirroring of the acrosomal membrane by the plasma membrane is followed by fusion between the two membranes.





GPO 614-918

PROJECT DESCRIPTION

Major Findings:

The zonula occludens (tight junction or T.J.) is the transcellular intermembrane specialization responsible for the structural integrity of epithelia and for the regulation of transepithelial permeability. The structure and topology of this junction are the subject of a great deal of research, with tow theories being tested: one views the T.J. as a linear, anastomosing array of trancellular integral membrane protein rivets; the other, presented by our laboratory, views the T.J. as an instance of membrane fusion in a process that involving the participation of both membrane lipids and integral membrane proteins. In our view, formation of the T.J. involves the establishment of a structural transcellular topological continuity between the exoplasmic halves of the plasma membrane of the adjoining epithelial cells at both apical and basoletaral domains. To prove that this continuity exists at the apical domain we stretched toad bladder epithelic to greatly alternate the apical though that is formed at the junction. In this way we avoided deflection of the fracture plane from the plasma membrane at the junctional though. Our results show that a structural and topological continuity exists between the exoplasmic apical leaflets of adjacent cells. In addition we observed what appear to represent lateral views of transcellular protein units that, we believe have a role in structural integrity of the epithelium.

We are now developing a new theory to explain the modulation of transepithelial permeability. In addition, we are now starting the observation of apical surface views of the tight junction as revealed by our method of fracture-flip.

In other work the acrosome reaction was induced in spermatozoa according to the method of Green (1978), as adapted for boar sperm by Russel and coworkers (1979). The sperm samples were incubated at 37°C in HBSS containing 2 mM $CaCl_2$ and 10 μM A23187 ionophore (stock solution in DMSO). Other samples were used as controls of the calcium effect; they were incubated in the same medium in absence of $CaCl_2$ (HBSS + A23187). Sperm aliquots were also incubated in absence of the ionophore (HBSS + $CaCl_2$). The reaction was arrested at 1, 5, 10 and 60 minutes by glutaraldehyde fixation (1.5% in HBSS, pH 7.4, for 60 min at 4°C). The percentage of sperm cells that underwent the acrosome reaction was evaluated by Normarski, phase and bright-field light microscopy. We showed that incubation of boar spermatozoa in 2 mM $Ca^{2+}$, in presence of 10 μM A23187 ionophore, triggers a global displacement of the large IMP of the sperm-head plasma membrane. Our "fracture-label" studies identified these sperm IMP as TMG that bind the lectin wheat-germ agglutinin (WGA) (Aguas & Pinto da Silva, 1983). Therefore, the lateral movement of large IMP reported here reflects translational displacements of TMG. We found that this translational displacement of TMG involves migration over unusually large distances (up to 2 μM), occurs with 1-5 minutes after $Ca^{2+}$ addition to the medium, and ends when the glycoproteins are accumulated at two opposite poles (rim and equator) of the sperm-head surface. As in absence of $Ca^{2+}$, the displacement of IMP does not occur, we conclude that $Ca^{2+}$ is associated to the translational displacement of TMG in the area of the sperm-head surface located over the acrosomal vesicle

(acrosomal-cap). The new location of TMG leads to the bimodal (transmembrane protein-rich/protein-poor) organization of the entire acrosomal cap into just three large, new domains. The resulting topography of the $Ca^{2+}$-induced domains on the sperm plasma-membrane coincides with that of the stable, structural domains of underlying outer acrosomal membrane.

Our results strengthen the evidence on the importance of WGA-binding glycoproteins of the sperm surface on the modulation of acrosome reaction. Previous studies by Vacquier's group (Lopo & Vacquier, 1980; Podell & Vacquier, 1984; Podell et al., 1984; Trimmer et al., 1985) showed that WGA blocks the influx of $Ca^{2+}$ in echinoderm sperm and, in consequence, the acrosome reaction. Biochemical characterization of the WGA receptors of sperm plasma membranes identified an integral 210 KD glycoprotein (Podell et al., 1984). Antibodies against this WGA-binding glycoprotein inhibit the $Ca^{2+}$ influx and the acrosome reaction (Trimmer et al., 1985; Trimmer et al., 1987). Because the bimodal TMG displacement occurs before any membrane fusion events, we postulate that the WGA-binding TMG, on changing their planar topology, may be involved in the formation and/or activation of the sperm-surface $Ca^{2+}$-channels as required for the acrosome reaction to occur. The inhibitory effects of both WGA and specific antibodies on the acrosome reaction would, therefore, be a consequence of the restricted mobility of the TMG upon crosslinking by the ligands.

Publications:

Aguas A, Pinto da Silva P. Bi-modal re-distribution of surface transmembrane glycoproteins during $Ca^{++}$-dependent secretion (acrosome reaction) in boar spermatozoa. J Cell Sci 1989;93:467-479.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08376-06 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Fracture-label: Identification and Partition Transmembrane Proteins

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

Pedro Pinto da Silva, Ph.D. Chief, Membrane Biology Sect. LMMB, NCI

COOPERATING UNITS *(if any)* M.R. Torrisi, A. Pavan, P. Mancini, Institute of General Pathology, Univ. of Rome School of Medicine, Rome Italy.

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: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.1 | 0.1 | 0.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

In order to focus the activities of the Membrane Biology Section experimental work on Fracture-label has been suspended and the project terminated. The principal investigator published two invited reviews describing his pioneering work on the splitting of biological membranes and differential partitioning of integral membrane proteins.

PROJECT DESCRIPTION

Major Findings:

Experimental work has been suspended; please see Publications.

Publications:

Pinto da Silva P. Freeze-fracture images: 1967-1971. In: Hui SW. ed. Freeze-fracture studies of membranes. Boca Raton: CRC Press Inc, 1989;1-9.

Torrisi MR, Mancini P, Pinto da Silva P. Cytochemical access to plasma and intercellular membranes of freeze-fractured hepatocytes and salivary gland cells. In: Motta P. ed. Ultrastructural pathology. Italy, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER: Z01 CB 08380-06 LMMB

PERIOD COVERED: October 1, 1989 to September 30, 1990

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:

| | | |
|---|---|---|
| Devjani Chatterjee, Ph.D. | Visiting Associate | LMMB, NCI |
| John Owens | Computer Specialist | LMMB, NCI |
| Ruth Nussinov, Ph.D. | Consultant | LMMB, NCI |
| Lewis Lipkin, M.D. | Chief, Image Processing Section | 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

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| 4.5 | 3.5 | 1 |

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Viral systems are used to model the complex macromolecular processes and organization of normal, infected and transformed cells. Computers are used to study the nucleic acid and protein sequences that embody the information necessary for these systems to function.

HIV and related viruses present challenging problems in virus-cell molecular biology and disease. Gene and protein sequences of these viruses are comparatively studied for relationships among them and to other known and hypothetical proteins. Secondary structures of the RNAs are found to vary in correlation with pathological and sequence differences.

Foot-and-mouth diseases 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 analyses of proteins and nucleic acids have been developed and implemented in conjunction with techniques of biochemistry, virology, and electron microscopy on sequences of picornaviruses, adenoviruses, human immunodeficiency viruses. Graphic representations revealing homology, and reverse complementarity are coupled with numerical methods to aid the prediction of secondary structure, splicing, promoters, and recombination in nucleic acid molecules. Computer programs are developed locally and elsewhere for application on Cray XMP, VAX and graphic workstations to perform sequence analysis and structure predictions. Methods to assess the significance of predictions use Monte Carlo simulations, evolutionary comparisons and biochemical data. Roles for genes and proteins are deduced by comparison with databases of sequences of known function and structure.

Project Description:

Other Professional Personnel (Continued):

| | | |
|---|---|---|
| Anne Barber, M.D. | Sr. Staff Fellow | LMMB, NCI |
| Bruce Shapiro, Ph.D. | Computer Specialist | LMMB, NCI |
| Manuel Peitsch, Ph.D. | Visiting Fellow | LMMB, NCI |
| Shu-Yun Le, M.S. | Visiting Fellow | LMMB, NCI |

Major Findings:

Multiple sequence alignment for the detection of signals: Multiple sequences for related genes provide a powerful data set for detection of structural entities that are involved in recognition by enzymes or regulatory elements, and for understanding common structural motifs. A dynamic program, ANA, for creating alignments of hundreds of sequences, extracting the features and assessing the statistical significance of such alignments has been developed on a Silicon graphics workstation using the C language. Extensions to this program permit rapid assessment of the probability of chance occurrence for a given feature using a method based on binomial probability theory. A similar but non-dynamic program has been developed from the existing FEATUR program in pascal on the VAX computer. Using these programs a number of observations of consensus signals in the vicinity of splice sites, promoter sites and other items contained in the GENBANK "feature" table have been compiled. Analogous programs have been developed for use with amino acid sequences. They are used to find common structural domains between HIV proteins and known families of enzymes such as G-proteins and reverse transcriptases and structural proteins, other viral sequences and potential antigenic sites. A new program "MOTIFN" was developed to find 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. Some sequencing laboratories feel that with the growing automation of the sequence determination process, the limiting step will be analysis of the sequence data by computer unless superior methods are available. There will be a continuing need for methods to compare sequences both more easily and more thoroughly. Knowledge bases of experience with the meaning and interpretation of results will also be needed. A variety of methods are used in the laboratory. One of the most powerful of these uses the Cray XMP to search for regions of local homology with the thorough program, SEQHP, or the more rapid SEQFP/SEQFT programs of Kanehisa, and then to uses the SEQDP program to assess the non-randomness of the matches. These have now been combined and modified to produce output suitable for direct input into relational database programs such as FRAMIS or INGRES. 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-liproteins, portions of reverse transcriptase and portion of CD-4 antigens using similarity search (DFASTP, SEQFDP, SEQHDP) molecular mechanical (CHARRM, AMBER) and molecular graphics programs (MOGLI, MIDAS, GEMM). In some cases these results are stimulating new insight into the functions of proteins derived from gene sequencing.

Detection of potential RNA secondary structure: RNA and DNA structure are of increasing concern as the innate catalytic activity, and other functional properties of polynucleotides are being recognized and more experience with protein-nucleic acid interactions is accumulated. Evidence from simple systems suggest that there is a hierarchical precedence to the determination of single-stranded polynucleotide structure in the order that primary sequence determines regions of helical base pairing and stacking that pack into 3-dimensional structures. Thus it is necessary to make reasonable predictions of the secondary structure in preparation to predicting the full structure. 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 the 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, in press.

Hausheer F, Barnett G, Colvin M, Maizel JV. Analysis and prospective design of drugs for the treatment of cancer and AIDS by supercomputer. Presentation at ICS 1988 Third Intl Conference on Supercomputing and Second World Supercomputing Exhibition. Boston: Intl Conference on Supercomputing, 1988;201-206.

Konopka AK, Chatterjee D. Distance analysis and sequence properties of functional domains in nucleic acids and proteins. Gene Anal Techn. In press.

Le S-Y, Chen K-H, Braun MJ, Gonda MA, Maizel JV. Stability of RNA stem loop structure and distribution of non-random structure in the human immunodeficiency virus (HIV-1). Nucleic Acid Res 1988;16:5153-5168.

Le S-Y, Chen K-H, Nussinov R, Maizel JV. An improved secondary structure computation method and its application to intervening sequences in the human alpha-like globin mRNA precursors. Comp Appl in the Biosc 1988;4(3):337-344.

Le S-Y, Maizel JV. A method for assessing the statistical significance of RNA folding. J of Theor Biol, in press.

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.

Maizel JV. Supercomputing in biomedical research: Applications to sequence analysis and molecular biology. Proc of the 2nd Intl Symp, Chicago, IL: Cray Research, 1988;81-95.

Maizel JV. Supercomputing in molecular biology: Applications to sequence analysis. IEEE Eng in Med @& Biol Mag 1988;7:27-30.

Malim MH, Bohnlein S, Fenrick R, Le S-Y, Maizel JV, Cullen BR. Functional comparison of the rev trans-activators encoded by different primate immunodeficiency virus species. PNAS, in press.

Malim MH, Hauber J, Le S-Y, Maizel JV, Cullen BR. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature 1989;338:254-257.

Nakata K, Kanehisa M, Maizel JV. Discriminant analysis of promoter regions in E. Coli sequences. CABIOS 1988;4:367-371.

Nakata K, Maizel JV, Jr.: Prediction of DNA-binding protein by discriminant analysis. Computer Applications in the Biosciences, in press.

Nussinov R, Barber AM, Maizel JV. The distributions of nucleotides near bacterial transcription initiation and termination sites show distinct signals that may affect DNA geometry. J Mol Evol 1987;26:187-197.

Owens J, Chatterjee D, Nussinov R, Konopka AK, Maizel JV. A fixed-point alignment technique for detection of recurrent and common sequence motifs associated with biological feature. Comp Appl in the Biosci 1988;4:73-79.

Rivera VM, Welsh JD, Maizel JV. Comparative sequence analysis of the 5' noncoding region of the enteriviruses and rhinoviruses. Virology 1988;165:42-50.

Steven AC, Trus BL, Maizel JV, Unser M, Parry DAD, Wall JS, Hainfeld JF, Studier FW. Molecular substructure of a viral receptor-recognition and protein - The GP17 tail-fiber of bacteriophage-T7. J Mol Biol 1988;200:351-365.

Sunderman FW, Barber AM. Finger-loops, oncogens, and metals. Ann Clin Lab Sci 1988;18(4):267-288.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08381-07 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Computer Aided Two-Dimensional Electrophoretic Gel Analysis (GELLAB)

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


Peter F. Lemkin, Ph.D. Computer Specialist IPS, LMMB, NCI

Other Professional Personnel:

Robert W. Ashmore Scientific Applications Analyst, PRI/FCRDC


COOPERATING UNITS (if any) Dr. E.Lester (Tenn. Ctr. Hlth. Serv.); Dr. P.Sonderegger, T.Hale (Biochem. Inst. Zurich); Dr. M.Alley (NCI/FCRDC); Dr. H.Bauer, Dr. A.Amberger, (Austr. Acad. Sci., Salzberg); Dr. T.Krekling (Agr. Norway); Dr. J.Myrick (CDC/Atlanta); Dr. P.Rogan (ABL/FCRDC); Mr. R.Getzenburg, Dr. D.Coffey (J. Hopkins).

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Image Processing Section

INSTITUTE AND LOCATION

NCI/Frederick Cancer Research and Development Center, Frederick, MD 21701

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.0 | 2.0 | 0 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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


Continuing development of GELLAB-II software system. It is 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 now runs under UNIX on workstations and the CONVEX supercomputer and uses X-windows for portable interactive graphics.

Substantive applications include: analyzing sets of lymphocyte gels and long term cell lines from human hematological malignancies to identify potential marker proteins for possible microsequencing (Lester); axonal proteins synthesized during axonal regeneration (Sonderegger); cadmium toxicity of epithelial cells (Hunziker); cerebral cell morphology control (Bauer and Amberger); assess stability of long term tumor cell lines (Alley); 2D DNA gels for identifying differentially expressed genes (Rogan and Strathern); Transcription control in prostrate cell lines (Getzenberg and Choffey). GELLAB-II has been exported to the U. of Zurich (Hale), CDC/Atlanta (Myrick), U. Norway (Krekling). Additional changes to facilitate continued collaboration and exporting of enhanced versions of GELLAB has been actively pursued.

PROJECT DESCRIPTION

Major Findings:

Continued work on applications and stabilization of GELLAB-II 2-dimensional gel electrophoresis analysis system. It now runs on UNIX workstations and the CONVEX supercomputers. The recent work has also integrated more of the programs resulting in a smoother user interface.

2D gel scan data is sometimes available from other laboratories. We have modified the GELLAB-II software to easily and consistently handle increased gel image size and higher resolution necessary for today's higher resolution gels. The general image conversion program, PPXCVT, was extended so that images from additional scanners (Molecular Dynamics, Kendrick Labs, Bioimage, etc.) could be converted to the format required by GELLAB-II - facilitating use of GELLAB for use by other laboratories which do not have our scanner. It converts other laboratory formats to our standard image format.

The GELLAB-II gel annotation database spot query facility was enhanced to make random spot queries for quantitative data easier. This interactive program can be used to both define and search spot annotation data which may be both textual and computational. It will be used both for record keeping of data from the current experiment as well as information about specific proteins from other laboratories.

Additional GELLAB C-code library procedures were written to support GELLAB programs. This includes a new GELLAB-II X-windows X11 library which helps enforce a consistent windowing user interface making it easier to learn GELLAB.

The 25+ GELLAB-II programs (about 140,000 lines of code) were fine tuned to simplify the user interface and some use X-windows. The composite gel database program CGELP2 has added functionality to integrate it better with many of the other programs so that generate of derived gel images is smoother. Additional effort was made in simplifying the code for eventual maintenance by others. Enhancements were made to facilitate ease of use as well as flexibility of analysis. The calibration facility was extended to incorporate different types of pIe and MW standards and extrapolate these with spot position.

The AUTOPAIR program has stabilized so that it is reliably pairing spots using only a one or a few landmarks (vs the 20 previously used). We have evaluated different sets of gels using the old method of spot pairing using the CMPCL2 and AUTOPAIR and the results seem to indicate that AUTOPAIR can be used - saving user time and giving more accurate results.

As increased 2D gel image resolution is required with recent improvements in 2D gel production (over 2000 spots/gel on standard size gels), our CCD camera can capture this higher resolution. Major changes in many modules of GELLAB-II were made to handle this increased resolution for variable size images.

Additional work was done in optimizing the spot quantitation program so as to minimize computation times for increased resolution. This is important as

computation times increase with the square of the image size. The SG2GII program was enhanced using larger Gaussian smoothing and other filters as well as other enhancements which let us handle higher resolution gels faster with more accuracy.

This year, Robert Ashmore (PRI/FCRDC) has concentrated on enhancements to the spot segmentation program to use it on higher resolution gels and the automatic spot pairing program (to pair spots between two gels).

In order to maximize the interfacibility of GELLAB-II with primary data from other 2D gel systems (both academic and commercial systems), some changes were made to the fundamental data structures to allow us to input paired spot CGELP2 data from non-GELLAB sources. This would also allow us to use the statistics and search record-keeping part of GELLAB on other types of biomedical data.

To ease learning GELLAB using the exported software in other laboratories, demonstration program scripts and tutorials for new users were improved. This has been iterated and is being optimized as we gain experience with what "information" is missing when learning the system outside of our lab.

A book on GELLAB-II methodology and usage has been prepared. It is intended to be used primarily as a reference for the GELLAB-II system. As such, it contains material which includes a general introduction to gel analysis methods, tutorials on introductory and advanced methods for computer assisted analysis of 2D gels, and a discussion of exploratory data analysis techniques as applied to gel analysis.

A Portable Picture System - Xpix:

Work has continued on Xpix, a portable menu driven image processing system for X-windows which currently runs on SUN, microVAX, etc. workstations under UNIX. Xpix is used with GELLAB-II to interactively view, enhance, process, etc. gel images. Xpix has additional capability which makes it suitable for analyzing 1D gels as well as other types of images. It was designed so that new functionality can be added through external UNIX programs rather than in making time consuming and more difficult changes to the program itself. Additional functionality has been added to Xpix to make it more powerful as well as easier to use. It has been rewritten since last year so as to take advantage of the X11 GELLAB X-window library. This library was then used for adding a consistent windowing image interface to other GELLAB programs.

Collaborations:

A number of collaborations are continuing and some new ones were started: Mike Alley (DTP/NCI/FCRDC) has been 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 (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 been studying blood-brain barrier using brain capillary epithelial cells. Peter Hunziber (U. Zurich) - Cadmium toxicity on adapted and unadapted cell lines of Metallothionein-like proteins. Robert

Getzenberg and Donald Choffey (J. Hopkins U.) has been studying the alteration of nuclear matrix proteins and its determination of tissue specific gene expression. Peter Sonderegger (U. Zurich) continue the collaboration on studies of axonal proteins to include the identification of axonally secreted proteins from new neuronal populations. Peter Rogan and Jeff Strathern (ABL/FCRDC) 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 in order to handle this new type of data.

We have exported GELLAB-II to: Biochemistry Institute at the Univ. of Zurich (Sonderegger and Hale); Dr. James Myrick (CDC/Atlanta) who is using it with his BioImage system; the laboratory of Dr. Trygrv Krekling (Agr. Univ. Norway) and are collaborating on enhancements.

Publications:

Lemkin PK. The GELLAB-II 2D gel analysis system. LMMB, NCI/FCRDC: Image Processing Section, 1989; 697.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08382-07 LMM

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Computer Analysis of Nucleic Acid Structure

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

| | | |
|---|---|---|
| Bruce A. Shapiro, Ph.D. | Computer Specialist | LMMB, NCI |
| Other Professional Personnel: | | |
| Danielle Konings, Ph.D. | Visiting Fellow | LMMB, NCI |
| Kathleen Currey, M.D. | Guest Researcher | LMMB, NCI |
| Ruth Nussinov, Ph.D. | Consultant | LMMB, NCI |
| Jacob V. Maizel, Jr., Ph.D. | Chief, Lab. of Math. Biol. | NCI |

COOPERATING UNITS *(if any)* Andrew Dayton, Ph.D., LIR, IDIR; Melvin Pomerantz, Ph.D., Research Staff Member, IBM, Watson Research Center; Stanley Brown, Ph.D., ABL; Richard Stevens, Ph.D., Associate Professor, Harvard Medical School; Sarah Lesher, Applications Analyst, PRI; Wojcieok Kasprzak, Consultant, PRI.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Image Processing Section

INSTITUTE AND LOCATION

Frederick Cancer Research and Development Center, Frederick, MD 21701

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.7 | 2.0 | 0.7 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

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 analysizing RNA conformations from various perspectives. This has involved the development of new algorithms to explore secondary and tertiary structural motifs. This has made available an experimental computer workbench to allow a researcher to interactively pursue various areas of interest concerning RNA structure in a coherent way. This includes both the use of facilities to explore in detail multiple structures as well as individual RNA structures. This system permits queries of relationships that exist in the RNA secondary and tertiary structure problem domain involving various software/hardware complexes available at the FCRF and elsewhere. A large portion of the system has been ported to a SUN thus allowing broader access.

Algorithms have been developed that involve RNA secondary and tertiary structure analysis specifically the measurement and visualization of structural similarity from global and contextual viewpoints. A fast algorithm has been developed to compare RNA structures using the concept of levels of abstraction. This permits the definition and searching of structures from loose to a more restricted specifications. A research effort is underway using the system to examine the potential effects of tertiary RNA interactions.

The system is being used to study the fine structural details of the HIV-1 rev responsive element (RRE), the structure of 4.5SRNA, the structure of the 5' non-coding regions of poliovirus and the structure of mast cell RNA.

Exploratory work has begun utilizing an scanning tunneling microscope to visualize the fine structure of molecules, specifically RNA, DNA and proteins.

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 computer. One of the objects of this research is to make available an experimental computer workbench to allow a researcher to interactively pursue various areas of interest concerning RNA secondary 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 Facility 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 FCRF network. This includes the SUN'S, SILICON GRAPHICS, a CONVEX, VAXES and the CRAY. These algorithms are invoked from the SYMBOLICS utilizing one common mouse and window system that reduces the users need to know the various software/hardware complexes. The user has the interactive capability to fold literally hundreds of structures and to cluster these structures to determine which are similar and which are not as well as which substructures are similar and which are not (see 1,2,4). 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 (Wojcieok Kasprzak and Sarah Lesher, PRI) thus allowing broader access to the research community. The SUN version of the system is at the point now where we are almost ready for testing by selected individuals.

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. New comparative studies are underway involving the rev responsive element (RRE) of HIV-1 RNA, 4.5SRNA, the 5' non-coding region poliovirus RNA, mast cell RNA.

Computer and biological experiments are being 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 (with Dr. Andrew Dayton, Dr. Daniel Konings and Dr. Jacob Maizel).

Computer and biological experiments are underway to determine the structure of 4.5SRNA and its relationship to ribosomal function an tRNA release. It has been shown that similar secondary structures may be derived from sequences of clones complementing 4.5SRNA of E. coli even though sequence homology is not that strong. This work is being done in collaboration with Dr. Stanley Brown.

Work has continued in collaboration with Dr. Kathleen 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 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 Dr. Shuyun Le, Dr. Jih-H Chen, Dr. Ruth Nussinov and Dr. Jacob Maizel (6).

Collaborative computer and biological experiments have just begun to determine if there exists any structure/function relationships in mast cell RNA. It has been found that a small number of genes are expressed during differentiation when the cell is exposed to IL-3 and then to fibroblasts. We are pursuing the possibility that RNA structure may be significant in this regard. This work is being done in collaboration with Dr. Richard Stevens.

A phase of the work utilizing a new tree comparison algorithm to do flexible and accurate comparisons of RNA secondary structures has been completed (see 4). This included the ability to determine precisely where differences reside between to 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. The algorithm was utilized in the research done in (1). The algorithm appears to be useful as an aid in phylogenetic studies of RNA secondary structures.

In applying the above algorithm to suboptimal foldings of an RNA sequence it has been found that out of several hundred suboptimal structures of a given RNA sequence there are just a few specific morphologies that contain a fairly large number of similar structures. Further exploration of this phenomenon is now in progress. This work is currently being conducted in collaboration with Dr. Kathleen Currey and Dr. Andreas Konopka.

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. The reduces the number of locations that must be examined in the folding matrix. This work has been done in collaboration with Dr. Ruth Nussinov, Dr. Shuyun Le, and Dr. Jacob Maizel (5).

Exploratory work has begun in an attempt to visualize the rev responsive element of HIV-1 using a scanning tunnelling microscope. This microscope has the potential resolution of less than 10 angstroms 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. This work is being done in collaboration with Drs. Melvin Pomerantz, Andrew Dayton, Danielle Konings and Jacob Maizel.

Publications:

Margalit H, Shapiro BA, Oppenheim AB, Maizel JV Jr. Detection of common motifs in RNA secondary structures. Nucleic Acids Res 1989;12(12):4829-4845.

Le S-Y, Owens J, Nussinov R, Chen J-H, Shapiro B, Maizel JV Jr. RNA secondary structures: comparison and determination of frequently recurring substructures. CABIOS 1989;5(3):205-210.

Chen J-H, Le S-Y, Shapiro B, Currey KM, Maizel JV Jr. A computational procedure for assessing the significance of RNA secondary structure. CABIOS 1990;6(1):7-18.

Shapiro BA, Zhang K. Comparing multiple RNA secondary structures using tree comparisons. In press CABIOS, 1990.

Nussinov R, Shapiro B, Le S, Maizel JV Jr. Speeding up the dynamic algorithm for planar RNA folding. In press Mathematical Biosciences, 1990.

Le S, Shapiro B, Chen J, Nussinov R, Maizel JV Jr. Potential RNA pseudoknots downstream of the frameshift sites of retroviruses. Submitted Nucleic Acids Research, 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08386-04 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Gene structure of β(1-4)galactosyltransferase

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

| | | |
|---|---|---|
| Pradman K. Qasba, Ph.D. | Research Chemist | LMMB, NCI, |

Other Professional Personnel:

| | | |
|---|---|---|
| Elizabeth Boeggeman Ph.D. | IRTA Fellow | LMMB, NCI |
| Arni Masibay Ph.D. | IRTA Fellow | LMMB, NCI |

COOPERATING UNITS *(if any)*

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| 1.2 | 1.2 | 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.)*

Sequence analysis of several cDNA clones of β1,4galactosyltransferase from our laboratory has shown that there exist multiple mRNA's for galactosyltransferase which are most likely generated from the multiple copies of the gene and by utilizing the multiple polyadenylation signals present in the gene. We studies the gene structure of galactosyltransferase in relation to the primary structure of the protein. The structural analysis of the gene has shown that it has six exons and a multiple related copies of only the 5' half of the gene. Exon 1 has at least four related copies, exon 2 and 3 each have three related copies in the genome, while exons 4, 5 and 6 exist as unique sequences. Exon 1 codes for the first 140 residues of the protein that contain the cytoplasmic amino terminal and the membrane anchoring portions of the protein and is separated from the second exon by more than 25 kb of intervening sequence. Our results show that the region of the protein involved in membrane anchoring and targeting is coded by a portion of the gene which is far away from the rest of the sequences coding for the catalytic domain of the protein. Our results also suggest the possibility that the surface and Golgi galactosyltransferases might be generated by utilizing different copies of exon 1 sequences or by alternate splicing or both.

Project Description

Major Findings:

Gene Structure of β(1-4)Galtactosyltransferase:

**Gene Organization of bovine β1-4galtactosyltransferase: Variable copy numbers of various Exons:**

Since the transcripts of β1-4galactosyltransferase under go complex processing, and also several membrane bound and secretory forms of the enzyme are known to exist, we investigated the gene organization and copy number of β1-4galactosyl-transferase.

A bovine λ-genomic library has been screened using different restriction fragments of the cDNA clone as hybridization probe. We have isolated several λ-genomic clones carrying different exon-intron sequences of β1-4galactosyl-transferase. The nucleotide sequence of the cDNA region in the λ-genomic clones was determined by dideoxy chain termination method using 20mer synthetic oligonucleotides, corresponding to the cDNA sequences, as the sequencing primers. The gene contains 6 exons. The length of 5 introns varies and the size decreases as the exon number increases. In relation to the structural topology of the enzyme, the following information about the gene structure has been obtained.

1) Exon 1, which contains about 180 nucleotides of 5'-noncoding sequence and the coding sequence for the first 141 residues of the protein, is separated from 2nd exon by at least 25 kb of intervening sequence. Until now we have not been able to isolate any overlapping clones between exon 1 and 2. The region of the protein coded by this exon contains the cytoplasmic amino terminal portion and the membrane anchoring region, the "stem" structure of the enzyme. The low molecular weight secreted form of β1-4galactosyltransferase does not contain the first 105 residues of the precursor protein but is still enzymatically active. These results show that the region of the protein involved in membrane anchoring and targeting is coded by a portion of the gene which is far away from the rest of the sequences coding for the catalytic domain of the protein.

2) Exon 2, which codes for 78/79 residues of the protein (residues 142 to 220), is separated from exon 3 by at least 13 kb intervening sequence. Here again we have not been able to isolate any overlapping clones between exon 2 and 3.

3) Exons 3, 4 and 5, coding for 61-62, 41-42 and 33-34 residues of protein, respectively, are separated by relatively short intervening sequences. Third, fourth and fifth intervening sequences are about 6, 2, and 0.5 kb long, respectively.

4) Sequence analyses of Class II cDNA clones showed that polyadenylated RNA contain either only exon 4 sequence or exons 4, 5 and 6 sequences. In mouse species also some of the aberrant cDNA clones have been isolated which either

contain only exon 4 sequence, or a nucleotide sequence inserted between exons 2 and 3 followed by deletion of exon 5. The 5' flanking sequences of these aberrant molecules contain multiple inverted copies of Alu- equivalent sequences and polyadenylation sites.

5) Exon 6 is 2865 nucleotides long. It is the longest exon which codes for the 43-44 COOH-terminal residues of the protein and contains 2737 nucleotides of 3'noncoding sequence. Near the beginning of this exon there is an Alu-equivalent sequence. Within this exon there are potential polyadenylation sites which are utilized in different organs to generate mRNA which lack distal 3'noncoding sequences.

6) There are multiple copies of the exons 1-3 in the bovine genome. The genomic DNA was digested with the restriction enzymes whose recognition sequences flanked the exons and the DNA was then analyzed by Southern blotting. Exon sequences were amplified by PCR method and used as hybridization probes. The results of the Southern blot analyses show that there are at least three to four related copies of exon 1, three related copies of exon 2, two related copies of exon 3. For exons 4, 5 and 6 there seem to be only single copies. Whether these related copies of exons 1, 2, or 3 represent pseudoexons, or related genes, or repeats of the same exons remain to be determined. (Similar situation exists in the mouse and human genome (personal communication from Dr. Narimatsu)).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08387-03 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Fracture-flip: Nanoanatomy of Cell Surfaces

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

| | | |
|---|---|---|
| Pedro Pinto da Silva, Ph.D. | Chief, Membrane Biology Sect. | LMMB, NCI |
| Other Professional Personnel: | | |
| P.F. Pimenta, Ph.D. | Visiting Fellow | LMMB, NCI |
| S. Ru-Long, M.D. | Visiting Fellow | LMMB, NCI |
| H. Wang-Ying, M.D. | Visiting Fellow | LMMB, NCI |

COOPERATING UNITS *(if any)* Drs. M.R.Torrisi, A.Pavan, L.Fratti, P.Mancini, G.Lucania (Inst. of Patologia Generale, U.Rome Sch.Med. Rome, Italy); Drs. D.Nishioka, R.Ward, (Dept.Bio., Georgetown U.); Drs. D.Sachs, R.P.Silva(Lab Parasitic Diseases, NIH); Drs. W.deSousa, T.Souto-Padrone(Inst. of Biophys);Fed.Univ. Rio deJaneiro, Brasil.

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☐ (a1) Minors

☐ (a2) Interviews

B

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

This is the project where the brunt of the research effort of the Membrane Biology Section is applied. The research effort involves: 1) the application of Fracture-flip to the study of the nanoanatomy and topochemistry of biological membranes; and 2) the development of new methods derived from fracture-flip. Description of projects in parasitology, virology, cellular immunology, reproductive biology and cell biology is given under "Major finding". A new method "simulcast" has just been discovered. It permits the observations of the hydrophobic interior and actual surfaces of one single membrane.



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

PROJECT DESCRIPTION

Major Findings:

Fracture-flip is a method discovered in our laboratory that uses existing electron microscopy equipment to reveal macromolecular resolution views of cell and membrane surfaces. For fracture-flip cells are freeze-fractured and carbon (but not platinum) is evaporated onto the fractured face. The specimens are thawed, washed repeatedly in distilled water and the carbon replicas (with the exoplasmic halves of membranes attached) are then turned "upside-down" ("flipped"). The actual surfaces of membranes (from the exoplasmic halves of the membrane) are now on top. After air-drying, they can be imaged at high resolution by evaporation of platinum. Over the past year we have concentrated most of the activity of the Membrane Biology Section in the application of fracture-flip to study the nanoanatomy and topochemistry of membrane surfaces and to develop new methods based on fracture-flip to study the protoplasmic surfaces of membranes, to increase the resolution of the images, and to produce images of both the freeze-fracture morphology and surface nanoanatomy of contiguous areas in a single membrane (See "Simulcast" below).

The potential application of Fracture-flip are truly extraordinary and we have barely started to explore some of them. Briefly we have used fracture flip to: 1) Produce the first ultrastructural differentiation of the infective phase of Leishman major. 2) Describe the nanoanatomy and lectin topochemistry of trypomastigotes, amastigotes and epimastigotes of Trypanosoma cruzi (in particular, we have produced the first high resolution views of the cytostomes, a structure that had evaded high resolution scanning electron microscopy). 3) Provided what is, by far, the highest resolution, and the best images, of the process of surface capping of CD4 and HLA antigens in human thymocytes. 4) Shown the distribution of specific surface sites on the surface of sea urchin sperm as revealed by the use of monoclonal antibody immonogold cytochemistry. Other projects currently underway include: 1) the observation of the cell surfaces of neutrophyls during chemotaxis and the study of the redistribution, penetration and disposal of surface antigens and receptors during cell migration.

Other projects include: a. nanoanatomy and surface topochemistry of H-9 cells infected with HTLV-III (B); b. continuation of the topochemical mapping of specific epitopes on sea urchin sperm membranes as revealed by immunogold labeling; c. budding of Epstein-Barr virus on the inner membrane of the nuclear envelopes; d. budding of sindbis virus from infected baby hamster kidney cells in culture.

Among the projects that aim at the further development of fracture flip we can refer: 1) Development of "Simulcast" a new method that allows the simultaneous observation of fracture-faces and membrane surfaces in a single membrane. We have just sent for publication the description of this method as well as the first results it has produced. 2) Search for methods to digest away cytoplasmic components in order to apply fracture-flip to the observation of

the membrane skeletons of plasma membranes. To this end we are using Herpetomonas (a plant parasite), Leishmania, rat hepatocytes and human lymphocytes. Fixed cells are freeze-fractured and carbon casts made. The specimens are then thawed and exposed to a sequence of detergents and tryptic enzymes. Among the projects that we want to pursue is the study of membrane skeletal ultrastructure and dynamics during capping of CD4 and of HLA antigens. Our initial results with Herpetomonas show the inner surface of the plasma membrane as lined by parallel arrays of microtubules. Once our method is developed we plan to continue it with "simulcast" (see above) and complementary replication. This way it will be possible to provide, for the first time, views of the apolar interior of the membrane (as revealed by the exoplasmic and protoplasmic fracture faces) and of the inner and outer surfaces of the same membrane. Of course, we will combine this with immonogold cytochemistry.

Our recent work has given us clear leadership in the development of methods to study the ultrastructure (or, as we coined it, the nanoanatomy) of cell and membrane surfaces as well as in the combination of these methods with colloidal gold immunocytochemistry. In particular we have by-passed a whole field of research - high resolution scanning electron microscopy - by producing images that are better by about two orders of magnitude. We have done this without having to develop or acquiring a single piece of equipment: our work is the outcome of the phylosophy that orients the work of our section since 1975 - namely, the placing of the first priority on thinking and creativity, rather than in the never ending acquisition of equipment, or the quick following of fashions and fads. Our lab is now the front runner in a new scientific approach to the structure and dynamics of biological membranes. The pace and quality of the latest results assure us that our leadership position should remain unchallenged for some time to come.

Publications:

Fujimoto K, Pinto da Silva P. The surfaces of nuclear envelope membranes as revealed by fracture-flip. Eur J Cell Biol 1989;50:390-397.

Fujimoto K, Pinto da Silva P: Fracture-flip/Trition X-100: a new method to study the ultrastructure and topochemistry of the cytoplasmic surfaces of biomembranes. J Histochem Cytochem, accepted pending revisions.

Pimenta PF, da Silva RP, Sacks DL, Pinto da Silva P. Cell surface nanoanatomy of Leishmania major as revealed by fracture-flip. A surface network of 44 nm fusiform filaments identifies infective developmental stage promastigotes. Eur J Cell Biol 1989;48:180-190.

Pimenta PF, de Sousa W, Souto-Padron T, Pinto da Silva P. The cell surface of Trypanosoma cruzi: a fracture-flip, replica-staining label-fracture study. Eur J Cell Biol 1989;50:263-271.

Pinto da Silva P, Andersson-Forsman C, Fujimoto K. Fracture-flip: nanoanatomy and topochemistry of cell surfaces. In: Motta P. ed. Cells and tissues: a three dimensional approach by modern techniques in microscopy. New York: Alan R. Liss, 1989;49-56.

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 as revealed by immunogold fracture-flip. J Cell Sci, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08388-03 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Expression of β(1-4)galactosyltransferase in growing 3T3 cells

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


Pradman K. Qasba, Ph.D., Research Chemist LMMB, NCI

Other Professional Personnel:

Arni Masibay Ph.D. IRTA Fellow LMMB, NCI

Elizabeth Boeggeman Ph.D. IRTA Fellow LMMB, NCI


COOPERATING UNITS *(if any)*

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☐ (a1) Minors

☐ (a2) Interviews

B

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


The β1,4galactosyltransferase enzyme is localized in the trans Golgi where glycoproteins get galactosylated. This enzyme has also been found at the cell surface where it acts as cell-adhesion molecule, mediating intercellular adhesion by binding to terminal N-acetylglucosamine residues on lactosaminoglycans substrates, there by bridging cells together, and as a high affinity receptor for the laminin long arm. The cell surface and Golgi transferase seem to be differentially regulated. We monitored the level of galactosyltransferase mRNA and protein during the 3T3 cell-cycle. The level of mRNA increases 4 to 5 fold after 2 h of serum-stimulation of quiescent 3T3 cells. Protein biosynthesis inhibitors like cycloheximide and anisomycin superinduce galactosyltransferase mRNA expression. The levels of galactosyltransferase protein, as measured by immunoprecipitation of $^{35}S$-labeled protein and galactosyltransferase activity, begins to increase by 2-3 h and reaches a peak 9 h after quiescent cells are stimulated to proliferate. The observed increase in the activity is to a large extent associated with the plasma membrane fraction. The results show that: a) galactosyltransferase mRNA induction parallels the expression of a set of "early" genes during the $G_1$ phase of the cell-cycle which do not require de novo protein biosynthesis and b) during 3T3 cell growth specific activity of the enzyme increases substantially on the cell surface.

Project Description

**Expression and Regulation of β1-4galactosyltransferase during 3T3 cell Growth:**

Glycosyltransferases in general and β1-4galactosyltransferase in particular have been postulated to be involved in cell-cell interactions and in intercellular adhesion -- a fundamental biological property of cells which play a major role in a variety of physiological processes including morphological differentiation, cell development, and metastases. β1-4galactosyltransferase, besides being a Golgi enzyme, has been shown to be also associated with the plasma membrane. Recently it has been shown that during differentiation of embryonal carcinoma cells the pool of Golgi enzyme increases without any effect on the membrane galactosyl-transferase pool. We have investigated the regulation of β1-4galactosyltransferase expression when resting 3T3 cells are stimulated to proliferate.

Major Findings:

1) The β1-4galactosyltransferase 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 β1-4galactosyltransferase mRNA expression occurs early during the cell's transit from $G_o$ to S, and does not require prior protein biosynthesis. Protein biosynthesis inhibitors like cycloheximide and anisomycin superinduce β1-4galactosyltransferase mRNA expression.

2) The β1-4galactosyltransferase protein levels and activity increase on about the same time scale as the mRNA. The majority of the this observed increase in the galactosyltransferase activity is associated with the plasma membrane fraction. In contrast to the increase in Golgi pool during differentiation of embryonal carcinoma cells, the plasma membrane pool of β1-4 galactosyltransferase increases in 3T3 cells during their transit from $G_o$ to S.

Our results suggest that β1-4galactosyltransferase 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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08389-03 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Structure-Function Relationship of β(1-4)galactosyltransferase

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


Pradman K. Qasba, Ph.D. Research Chemist LMMB, NCI,

Other Professional Personnel:

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Elizabeth Boeggeman Ph.D. IRTA Fellow LMMB, NCI


COOPERATING UNITS *(if any)*

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☐ (a1) Minors

☐ (a2) Interviews

B

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


Using β1,4galactosyltransferase and its modifier protein α-lactalbumin as a model system for the studies on the structure-function relationship of proteins, continued this year. We reported for the first time that the cDNA sequence of bovine β1-4galactosyltransferase (constructed from a partial cDNA clone and a genomic fragment) engineered in an Okayama-Berg vector, upon transfection of COS-7 cells, codes for an enzymatically active galactosyltransferase. The expressed galactosyltransferase activity is modulated by α-lactalbumin to change the acceptor specificity to glucose, to synthesize lactose. These results show that the expressed galactosyltransferase protein is fully functional and that the binding sites for UDP-galactose, N-acetylglucosamine, glucose and α-lactalbumin are all intact and operative. We have now generated many deletion constructs from the parent β1,4galactosyl-transferase expression vector to determine the regions of the protein which are involved in membrane, nucleotide sugar and acceptor bindings. In addition we are altering the parent construct in such a way that the expression of the cDNA sequences have a potential to produce either soluble and secreted forms or membrane anchored forms of the protein.

## Project Description

Objective:

To determine a "functional" region or a unit of a Protein.

Using the methodology of the recombinant DNA technology, we are developing general methods to produce the normal and altered proteins. Mammalian and prokaryotic expression vectors are engineered in such a way that the same vector can be used to produce protein in quantities enough to test its function, and as well to produce the engineered protein in large quantities for determining various physical properties and X-ray crystallographic studies.

Major Findings:

Expression of bovine β1,4galactosyltransferase 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 promotor. The resulting vectors, pLsGT and pLasGT contain galactosyltransferase cDNA in the sense and antisense orientation.

Upon transfection of Cos-7 cells with transfected with pLsGT, pLasGT, $pSV_2neo$ DNA or mock transfected there is about 12 fold increase in the activity of the extracts assayed in the presence of N-acetylglucosamine (NAG) as an acceptor substrate only from pLsGT transfected cells compared to the extracts from pLasGT- a vector carrying galactosyltransferase cDNA in an antisense orientation -, $pSV_2neo$ DNA or mock transfected cells. The increase in activity is absolutely dependent on the added exogenous acceptor substrate. The expressed GT protein in pLsGT transfected COS-7 cells can be modulated by bovine a-Lactalbumin to accept glucose as a substrate to produce lactose. These results show that the bovine GT protein expressed transiently in COS-7 cells and coded by the cDNA is fully functional and that the binding sites for UDP-galactose, NAG, glucose and a-lactalbumin are all intact and operational.

The enzyme in the transfected cells from the sense galactosyltransferase cDNA is also precipitated by the polyclonal antibody raised in rabbits against SDS-PAGE purified bovine milk galactosyltransferase. The immunopellets obtained by the precipitation of the antigen-antibody complex with the second antibody, showed galactosyltransferase enzymatic activity.

A mouse monoclonal antibody directed against a fifteen residue long synthetic peptide, corresponding to the amino terminal region of the cDNA encoded galactosyltransferase protein, binds the expressed bovine galactosyltransferase and the immunoprecipitates exhibit galactosyl-transferase enzymatic activity.

Deletion constructs of β1-4 galactosyltransferase:

Based on the general topological information of glycosyltransferases and the gene structure, we have generated many deletion constructs from the parent β1,4galactosyltransferase expression vector. We have deleted the DNA sequences which code for the potential membrane anchoring domain of the galactosyltransferase protein and the residues coding for the portion of the protein which is absent from the secreted protein to study the effects on the transport and secretion of the protein. These constructs are being used for the localization of these and other altered galactosyltransferases in the cell and for the assignment of various functional and interacting domains of the protein.

Publications:

Masibay AS, Qasba P K. Expression of Bovine Beta (1, 4)galactosyltransferase cDNA in COS-7 Cells. Proc Nat Acad Sci 1989; 86: 5733-5737.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08390-02 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Analysis of HIV Assay Kinetics

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

John L. Spouge, M.D. Ph.D. Visiting Scientist LMMB, NCI

Other Personnel:

Peter Nara VCS, NCI, Frederick Cancer Research Facility

COOPERATING UNITS *(if any)* Scott Layne, T-7, Theoretical Division, Los Alamos National Laboratory; Micah Dembo, T-10, Theoretical Division, Los Alamos National Laboratory

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☐ (a1) Minors xx

☐ (a2) Interviews

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

HIV assays have not yet been standardized, and a comparison between assays, although important, is not yet dependable. For example, a series of five papers in Nature evaluating soluble CD4 (sCD4) as a blocker of HIV infectivity were divided two and three, for and against the efficacy of the blocking properties of sCD4. With these results in mind, we developed a theory to account for the hidden variables influencing the outcome of the experiments.

In our model, four unknown parameters characterize the virus under assay conditions: shedding and inactivation rates, an infection rate, and the association constant for gp120-sCD4 interaction. These parameters can be determined by experiments following certain protocols. Peter Nara is currently carrying out some of these protocols at FCRF, and the results show that viral infectivity assays and physical methods produce similar estimates of the gp120-sCD4 association constant. This is a considerable saving of experimental effort since the viral infectivity assays are much less laborious than the physical methods. Further experiments characterizing various viral strains and their tropism for different cell lines are planned.

PROJECT DESCRIPTION

Major Findings:

No additional work was performed on this project.

Publications:

Layne SP, Spouge JL, Dembo M. Quantifying the infectivity of HIV. PNAS 1989;86:4644-4648.

Spouge JL, Dembo M, Layne SP. Analytic results for a quantifying HIV infectivity. Bull Math Biol 1989;51:715-730.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08391-02 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Improving Sequence Alignment Algorithms

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

John L. Spouge, M.D., Ph.D. Visiting Scientist LMMB, NCI

COOPERATING UNITS *(if any)*

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☐ (a1) Minors

☐ (a2) Interviews

B

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

Because of the increased size of databases, computer processing of sequence data requires efficient algorithms. Probably the most frequently used algorithms are variants of the Needleman-Wunsch algorithm, used to align and compare two sequences. Comparison of more than two sequences is a problem of current interest, but this is even more computationally expensive.

A theory relating alignment efficiencies and inequalities directly was used to develop several "new" algorithms, which were then tested empirically on a mini-computer. One of these was shown to have a clear superiority in both time and memory requirements and is the fastest known algorithm for aligning two sequences under Needleman-Wunsch single indel weight penalties. Similar new algorithms allowing multiple indel penalties (which are biologically more realistic) were also tested and one was shown to be the best alignment algorithm under multiple indel weights currently available. The theory is also applicable to multiple sequence alignment.





GPO 914-918

PROJECT DESCRIPTION

Major Findings:

No additional work was performed on this project.

Publications:

Spouge JL. Speeding up dynamic programming algorithms for finding optimal lattice paths. SIAM J Appl Math 1989;49:1552-1566.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08392-02 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Combination Chemotherapy of AIDS and Cancer

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

| | | |
|---|---|---|
| John N. Weinstein, M.D., Ph.D., | Senior Investigator | LMMB, NCI |
| Other Professional Personnel: | | |
| Janos Szebeni, M.D. | Visiting Associate | LMMB, NCI |
| Vellarkad N. Viswanadhan | Biotechnology Fellow | LMMB, NCI |
| Shaila Patel | Howard Hughes Scholar | LMMB, NCI |

COOPERATING UNITS (if any)

LTCB, DCBD, NCI; LMI, NIDR; O.D., DCE, NCI; Dept of Pathology, Uniformed Services Univ. of Health Sci.; P.R.I., NCI/FCRF

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.4 | 1.4 | 1.0 |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

xx

B

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

We recently developed a new approach to combination chemotherapy of AIDS. Dipyridamole (DPM) is widely used as an oral agent for cardiovascular indications because of its platelet antiaggregant and coronary vasodilation activities. We recently found that DPM potentiates the activity of azidothymidine (AZT) and other dideoxynucleosides against human immunodeficiency virus (HIV-1) in cultured human monocyte/macrophages and stimulated T-lymphocytes. In cultured human lymphoblastoid cells, DPM potentiates the antiviral activity, and it also protects the cells from AZT's cytotoxic effects. DPM does not potentiate the cytotoxic effect of AZT on human bone marrow progenitor cells in vitro. These findings suggest that DPM might increase the therapeutic index of AZT and, perhaps, other dideoxynucleoside agents in vivo. However, the in vitro results cannot be used to predict clinical efficacy or safety. AZT/DPM has been approved for study by the AIDS Clinical Trial Group. Clinical trials are expected to start in the summer of 1990, in conjunction with collaborators. Other aspects under study:

1. Mechanism: DPM blocks cellular uptake of physiological nucleosides but not of AZT. The potentiation of AZT may thus result, in part, from decreased influx of the nucleosides that compete with AZT for viral reverse transcriptase.

2. Molecular structure: A structure for DPM has been computed from the crystallography. Quantitative structure-activity relationships (3D-QSAR) are being studied to predict which features of nucleoside transport inhibiting molecules are required for activity.

3. Analysis of combination chemotherapy: Because no published algorithm or computer package was adequate for analysis of our data on the antiviral effect of drug combinations, we have developed a new approach. The computer program package, called COMBO, will be useful in the context of cancer as well as AIDS.





GPO 914-916

Project Description

Major Findings:

1. Dipyridamole (DPM) appear to inhibit 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 also protects those cells against the cytotoxic effects of AZT. Thus, the "in vitro therapeutic index" for those cells is greatly increased.

4. DPM does not potentiate the toxicity of AZT for human bone marrow progenitor cells in a CFU 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.

7. DPM has been reported to induce interferon, but we find no such induction in our studies. We do find, however, 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 algorithm has been developed (in collaboration with Dr. B. Bunow, Civilized Software, Inc.) for analysis of data on drug synergy and antagonism. The method starts with a series of expressions based on enzyme kinetic models

and heuristic principles. A program package called "COMBO" was developed to perform the following tasks, operating in the MLAB computing environment: (i) globally fit each model by least squares regression with constant weights; (ii) use the results in a windowing technique to generate updated weights; (iii) repeat regression with the updated weights; (iv) iterate the previous two steps; (v) use a combination of normal theory and Monte Carlo techniques to determine confidence limits on the fitting parameters; (vi) construct a set of derived parameters that express various aspects of synergy, potentiation, and antagonism; (vii) calculate confidence limits on those constructed parameters; (vii) produce graphical displays of the error model, the residuals of each fit, and the contours of equipotent drug effect.

Bibliography:

Betageri GV, Szebeni J, Hung K, Wahl LM, Corcoran M, Weinstein JN. Effect of dipyridamole on transport and phosphorylation of thymidine and azidothymidine in human monocyte/macrophages. Biochemical Pharmacology, in press.

Bunow B, Weinstein JN. Combo: A new approach to the analysis of drug combinations in vitro. Annals NY Acad Sci, in press.

Szebeni J, Wahl SM, Schinazi RF, Popovic M, Gartner S, Wahl LM, Weislow OS, Betageri G, Fine RL, Dahlberg JE, Hunter E, Weinstein JN. Dipyridamole potentiates the activity of zidovudine and other dideoxynucleosides against HIV-1 in cultured cells. Annals N.Y. Acad.Sci., in press.

Szebeni J, Wahl SM, Wahl LM, Gartner S, Popovic M, Parker R, Black C, Weinstein JN. Inhibition of HIV-1 in monocyte/macrophage cultures by 2',3'-dideoxycytidine-5'-triphosphate, free and in liposomes. AIDS and Human Retroviruses, in press.

Viswanadhan VN, Ghose AK, Szebeni J, Weinstein JN. Mapping the binding site of the nucleoside transporter protein: A 3D-QSAR study. Biochem Biophys Acta, in press.

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, in press.

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, in press.

Patents:

Chemotherapeutic composition for AIDS. Application filed.

New antiretroviral agents and delivery system for the same. Application filed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08394-02 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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. S. Karlin, Stanford University, Dept. Mathematics; Dr. R. Macgregor, AT&T Bell Laboratories, Biophysics.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

Frederick Cancer Research and Development Center, Frederick, MD 21701

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 0.75 | 0.75 |

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☐ (a1) Minors

☐ (a2) Interviews

B

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

The general long term goal of this project is to unambiguously measure the information content of nucleotide sequences and to use these measurements to classify new (i.e. previously unknown) sequences. Unfortunately the general problem of evaluating information content (or measuring 'meaning') is one of the most challenging intellectual puzzles of the century and has generated more questions than answers thus far. For this reason our general aim had to be reduced to the following list of more specific goals:

1) Generate large collections of functionally equivalent sequences (i.e. playing the same biological role) that will be suitable for statistical analysis.

2) Prepare the software to statistically analyses large collections of sequences.

3) Perform computational experiments to determine sequence characteristics that are unambiguously associated with various biological functions.

4) Test the validity of determined sequence characteristics by using them to map functional domains in indiscriminantly sequenced nucleic acids.

PROJECT DESCRIPTION

Major Findings:

The vast majority of sequence research studies reported to date were devoted to characterization of contiguous sequence fragments that occur more frequently than other contiguous motifs. On the other hand, substantial experimental evidence indicates that specific functions of nucleic acids fragments are rarely associated with single, contiguous motifs. The patterns most often associated with a biological function are non-contiguous and consist of several short oligonucleotides separated by gaps of variable length and composition. For this reason we focused primarily on extensive statistical analyses of occurrence of non-contiguous patterns in large collections of functionally equivalent sequences. It has also been necessary to re-examine the existing approaches to analysis of contiguous patterns in order to generate a unified methodology to test hypotheses of the kind: Pattern P occurs in every sequence from collection C.

The following are the most important results obtained from the analysis of large collections of functionally equivalent sequences:

1) Several non-contiguous patterns (like two base periodicity or clustering of dinucleotides) are 'intron specific' and do not occur in exons or non-alu repeated DNA sequences.

2) Exons and bacterial genes also display periodic non-contiguous patterns but the period is three bases and not two as in introns.

3) Introns might be the hot-spots for illegitimate recombination since several similar non-contiguous patterns have been observed in the sequences of eukaryotic non-homologous recombination.

4) Compositional complexity (measured by Shannon entropy over mononucleotide frequencies) of intron sequences is generally lower than in exons.

We applied the results listed above to map functional domains in nucleic acid sequences. The Complexity Charts method resulting from this project does not require a priori knowledge of any biological properties involved. In particular we do not need to know anything about the existence of the genetic code to detect genes coding for proteins (including variable immunoglobulin regions) as well as to locate introns within them. Various non-coding regions (such as spacers, upstream and downstream regions of genes) can be clearly identified as well.

Significance:

Progress in nucleic acids sequencing techniques will lead to large amounts of poorly annotated sequence data. For this reason reliable computational tools to map putative domains will be essential for planning future sequencing strategies and for understanding already existing sequence data. Sequence characteristics resulting from our studies are indeed strongly associated with

those functions that they represent. This is a reason why we believe that mapping procedures based on those characteristics should be developed.

On the other hand, collections of sequences generated during this project as well as our program CLEANER are now available for other sequence researchers. This might help to reduce the number of published artifacts that are due to incorrect samples of studied sequences.

Publications:

Konopka AK, Owens J. Non-Contiguous patterns and compositional complexity of nucleic acid sequences. In: Bell G, Marr T. eds Computers and DNA. Addison-Wesley Longman, 1989;147-155.

Konopka AK, Owens J. Complexity charts can be used to map functional domains in DNA. Gene Anal Techn Appl 1990;7:35-38.

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, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08395-02 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Somatic Cell Non-homologous Recombination

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

Andreas K. Konopka, Ph.D. Visiting Scientist LMMB, NCI

Other Professional Personnel:

Jacob V. Maizel, Jr., Ph.D. Chief, LMMB LMMB, NCI

COOPERATING UNITS *(if any)* Dr. A. Sarai, RIKEN Life Science Center, Ibaraki, Japan; Dr. T. Jovin, Max-Planck Institute, Goettingen, FRG

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INSTITUTE AND LOCATION

Frederick Cancer Research and Development Center, Frederick, MD 21701

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.5 | 0.5 | 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.)*

Unusual DNA rearrangements that require no extended sequence similarity (of parental molecules) can cause severe genetic alterations of chromosomal fragments in somatic cells. Thus far about 600 parental sequences for this kind of crossing-over (called non-homologous or illegitimate recombination) are known. It is expected that their number will increase with the rate 50-100 per year. The goals of this project are:

1) Create a database of parental sequences for illegitimate recombination.

2) Analyze such a database for the statistically significant occurrence of sequence and structure patterns.

3) Provide simple tools for experimental molecular biologists to decide if newly sequenced DNA fragments contain potential recombination 'hot-spots'.

4) On the basis of sequence features found in our database postulate and study structures of possible intermediates for illegitimate recombination.

PROJECT DESCRIPTION

Major Findings:

Studies of sites stored in our database have shown that:

1) Topoisomerase I cleavage sites and runs of purines are major sequence features in the close vicinity of the breakpoint.

2) Those parental sequences that contain runs of five or more contiguous purines are associated with at least one Topo I cleavage site.

This and other evidences suggest that unusual (non-B) DNA structures might serve as intermediate in many instances of non-homologous recombination. Thus far we proposed that:

3) Local homopurine (homopyrimidine) tracks in two parental DNA molecules (double stranded) can form a tetraplex in which purines and pyrimidines remain mutually antiparallel but two purine strands (as well as two pyrimidine strands) are parallel to each other (Konopka and Sarai, 1990).

In this setting, formation of the four stranded structure would not require sequence similarity of parental DNA molecules because the basis for recognition would be purine - purine hydrogen bonding instead of Watson - Crick type base pairing.

Significance:

Deletions,inversions,transpositions,amplifications and other alterations of eukaryotic chromosomal fragments often result from non-homologous forms of recombination.

Two general models to explain the phenomenon of non-homologous recombination have been in use since the pioneering work of Franklin (1967). According to the first model, slipped strand mispairing (staggered loop formation) can cause bypassing of short DNA regions and thereby a deletion of those (bypassed) fragments. The second proposal concerns 'malfunctioning' of enzymes whose normal function is to catalyze a coordinate breakage and rejoining of phosphodiester bonds in DNA. One possible result of such an error could be recombining sequences that share little or no sequence similarity.

Both above models have been proposed with the assumption that illegitimate recombination events are rare and 'haphazard' (Franklin, 1967) and that their occurrence is a result of a 'mistake' or an 'error' in an otherwise 'normally' functioning system. Today our estimate of the frequency and 'randomness' of non-homologous recombination is different than it was in the late sixties. It is no longer believed that illegitimate crossing over is 'haphazard'. Although in prokaryotic systems this kind of recombination indeed occurs very rarely compared to homologous crossing over, it seems to be quite a frequent event in eukaryotic somatic cells.

Our model (if proven correct) provides an exhaustive explanation why recombination is possible without an extensive sequence similarity. It also implies that illegitimate recombination events do not need to be infrequent.

Publications:

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, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 CB 08396-02 LMMB

PERIOD COVERED

October 1, 1989 to September 30, 1990

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 |

COOPERATING UNITS *(if any)* John Spouge, NLM (Bethesda); Peter Basser, DRS, BEIB (Bethesda); Joe Mack, ABL, Crystallography Laboratory (Frederick); Peter Rogan, ABL, Laboratory of Eukaryotic Gene Expression (Frederick).

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|---|---|---|
| 1.0 | 0.0 | 0.6 |

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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 goal of this project is to find a fundamental theory of how macromolecules are able to act or interact precisely. The work done on information theory by Claude Shannon 40 years ago provides a well formulated mathematical foundation to study communications and computer operations. To apply this mathematics to molecular biology first requires a careful definition of what we are talking about. The "molecular machines" found in living things are able to operate precisely by choosing particular states from an array of possible states. This is similar to a teletype choosing a character to print from all possible characters. From this simple concept, a theory of molecular machines is being constructed which parallels Shannon's information theory. Because the two theories are so closely related, one can apply Shannon's theorems to molecular biology. One result is that the precision of molecular interactions can now be explained in terms of communications codes.

The project has three major components: theory, computer analysis and genetic engineering experiments. The theory guides the construction of computer programs for analysis of DNA and RNA binding sites, the experiments provide data to be analyzed by programs in light of the theory. A project on human splice junctions has produced a striking picture of how the spliceosome may have evolved. We are proceeding to set up the laboratory to analyze at least 1000 synthetic bacteriophage T7 promoters variants.

PROJECT DESCRIPTION

Major Findings:

The first half of the theory of molecular machines was completed. Four papers are planned, two have been submitted and two more are almost ready to be submitted (they should be in review by September 1990). The abstracts for the two submitted papers are:

Channel capacity of molecular machines, Abstract. Like macroscopic machines, molecular-sized machines are limited by their material components, their design, and their use of power. One of these limits is the maximum number of states that a machine can choose from. The logarithm to the base 2 of the number of states is defined to be the number of bits of information that the machine could "gain" during its operation. The maximum possible information gain is a function of the energy that a molecular machine dissipates into the surrounding medium $P_y$, the thermal noise energy which disturbs the machine $N_y$ and the number of independently moving parts involved in the operation ($d_{space}$): $C_y = d_{space} \log_2 \left( \frac{P_y + N_y}{N_y} \right)$ bits per operation. This "machine capacity" is closely related to Shannon's channel capacity for communications systems.

An important theorem that Shannon proved for communication channels also applies to molecular machines. With regard to molecular machines, the theorem states that if the amount of information which a machine gains is less than or equal to $C_y$, then the error rate (frequency of failure) can be made arbitrarily small by using a sufficiently complex coding of the molecular machine's operation. Thus the capacity of a molecular machine is sharply limited by the dissipation and the thermal noise, but the machine failure rate can be reduced to whatever low level may be required for the organism to survive.

Energy dissipation from molecular machines, Abstract. Single molecules perform a variety of tasks in cells, from replicating, controlling and translating the genetic material to sensing the outside environment. These operations all require that specific actions take place. In a sense, each molecule must make tiny decisions. To make a decision, each "molecular machine" must dissipate an energy $P_y$ in the presence of thermal noise $N_y$. The number of binary decisions that can be made by a machine which has $d_{space}$ independently moving parts is the "machine capacity" $C_y = d_{space} \log_2 \left( \frac{P_y + N_y}{N_y} \right)$. This formula is closely related to Shannon's channel capacity for communications systems, $C = W \log_2 \left( \frac{P + N}{N} \right)$.

This paper shows that the minimum amount of energy that a molecular machine must dissipate in order to gain one bit of information is $\varepsilon_{min} = k_B T \ell n(2)$ Joules per bit. This equation is derived in two distinct ways. The first derivation

begins with the Second Law of thermodynamics, which shows that the statement that there is a minimum energy dissipation is a restatement of the Second Law of thermodynamics. The second derivation begins with the machine capacity formula, which shows that the machine capacity is also related to the Second Law of thermodynamics.

One of Shannon's theorems for communications channels is that as long as the channel capacity is not exceeded, the error rate may be made as small as desired by a sufficiently involved coding. This result also applies to the dissipation formula for molecular machines. So there is a *precise* upper bound on the number of choices a molecular machine can make for a given amount of energy loss. This result will be important for the design and construction of molecular computers.

Splice junction analysis: project description. Splice junction analysis was performed in collaboration with R. Michael Stephens. We analyzed the sequences at the 5' and 3' ends of about 1800 human introns to characterize the patterns that are available to the spliceosome. We discovered that most (90%) of the information of splice junctions is on the intron side. We propose that this feature evolved to allow more flexibility on the coding regions. We observed that the information patterns match "bind-and-chew" or footprint data quite closely. A particularly striking position is at -3 on the 3' end of the intron. This location contains less than 0.005 bits of information and is accessible to hydroxyl radical attack. We propose that the base at this position is exposed to the solvent. We also noticed that the consensus for the two sites is similar, but with highly divergent emphasis. The 5' pattern is (roughly) cag|GT, while the 3' side is (roughly) CAG|gt. We propose that the two "hands" that grab the 5' and 3' sides evolved from a common ancestor, with a pattern (roughly) like CAG|GT. On the 5' side this pattern was shifted during evolution to reside on the right (inside the intron), while on the 3' side this pattern was shifted during evolution to reside on the left (also inside the intron). A basic prediction from this hypothesis is that the two "hands" should have homology. We have found evidence that the two spliceosome components responsible for binding (the U1/U2 and U4/U5/U6 snRNPs) do indeed have homologies. During the summer, we will submit this result in a short paper.

Sequence logos: project description. The description of the consensus binding sites given above is very crude. Every position in a binding site should have a different "emphasis". This can be defined precisely as the information content of the pattern. While working on the splice junctions, we discovered a graphical method to display the sequences. The figure demonstrates the method for ribosome binding sites in E. coli. The height of each stack of letters is the information content. The stack is divided up into portions proportional to the frequency of each base. The bases are printed in these proportions, with the most common base on top. These "binding site logos" are described in a paper to be submitted in May 1990.

Aids research: Construction of Sequence Logos for retroviral integrases for comparison to the HIV integrase, in collaboration with Joe Mack (2% of effort).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CB 08907-07 OD

PERIOD COVERED

October 1, 1989 to September 30, 1990

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

Regulation of Immune Response to Tumor Cells and Alloantigens

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

| | | | |
|---|---|---|---|
| PI: | C.C. Ting | Senior Investigator | OD DCBDC NCI |
| Others: | M.E. Hargrove | Microbiologist | OD DCBDC NCI |

COOPERATING UNITS (if any)

LAB/BRANCH

Office of the Director, DCBDC

SECTION

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NCI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS | PROFESSIONAL | OTHER |
|---|---|---|
| 2.0 | 2.0 | |

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

1. Mechanism of lymphocyte-mediated cytolysis. A model has been developed in this laboratory to determine lymphocyte-mediated fast lysis or slow lysis. Anti-T cell receptor antibody αCD3-induced activated killer cells which were maintained under the constant stimulation by αCD3 ($CD3\text{-}AK^{+}$) mediated fast lysis, and the activated killer cells which were maintained in IL-2 after initial activation ($CD3\text{-}AK^{-}$) mediated slow lysis. These two sets of killer cells were functionally different but phenotypically identical. The slow lysis model allows us to analyze the lytic mechanism in a step-wise manner. Through this approach we found that an activation phase preceded the lytic phase in the lytic reaction. In addition to the first signal (antigen, PMA etc.), second signal is also required in the activation phase. Cytokines such as IL-2 and TNF can serve as these additional signals. Activation by IL-2 is early and is PKC-dependent, and activation by TNF is late and is PKC-independent. After activation, cytolytic factor(s) is released in active form to complete the lytic process.

Glutathione (GSH), a tripeptide which regulates the binding, internalization and utilization of IL-2 also regulates the IL-2 dependent slow lysis.

2. Immunotherapy of tumor growth with CD3-AK cells. The in vitro antitumor activity of activated killer cells (CD3-AK) paralleled their in vitro cytolytic activity. A methylcholanthrene-induced sarcoma T-92 which was susceptible to the killing by both CD3-AK or LAK cells was also susceptible in vivo. Appropriate manipulation of the in vitro anti-tumor activity of these killer cells may help us to design proper strategy for the in vivo immunotherapy of tumor growth.



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

Major Findings:

I. Regulation and Control of Immune Response.

Mechanism of lymphocyte-mediated cytolysis:

1). Subsets of activated cytolytic lymphocytes that mediated fast or slow lysis. In the last decade, a great deal of effort has been made to study the mechanisms of lymphocyte-mediated cytolysis. Most studies have dealt with fast lysis which was measured by a 24h short-term $^{51}Cr$ release assay. Therefore, it was difficult to determine some of the lytic mechanisms in a step-wise manner in this short period of time. We have developed a system with two sets of functionally different but phenotypically identical lymphocytes which can mediate fast lysis (2-4h assay) or slow lysis (16-20h assay). Antibody against the CD3 complex of T cell receptor induced the generation of activated killer cells (CD3-AK). The killer cells that were maintained under the constant activation by αCD3 ($CD3\text{-}AK^+$) mediated fast lysis, and the killer cells that were maintained in IL-2 alone after initial activation ($CD3\text{-}AK^-$) mediated slow lysis. These two sets of killers were interconvertible, depending on the mode of activation.

2). Role of cytokines in fast and slow lysis. Exogenous IL-2 was required to maintain the in vitro growth of both $CD3\text{-}AK^+$ and $CD3\text{-}AK^-$ cells. However, in the effector phase of lytic reaction, IL-2 was required in slow lysis but not in fast lysis. TNFα synergized with IL-2 to mediate slow lysis. These cytokines were required to further activate the "slow killers" ($CD3\text{-}AL^-$); they were not the cytotoxic factors per se. After entering the lytic phase, the cytotoxic factor(s) was released in active form to complete the lytic reactions.

3). Glutathione (GSH) regulation of CD3-AK-mediated lysis. GSH, a tripeptide, has been shown to regulate the binding, internalization and utilization of IL-2 by T cells. In our system, we found that the cytolytic activity of $CD3\text{-}AK^-$ cells which was IL-2-dependent was subjected to the regulation by GSH, whereas the cytolytic activity of $CD3\text{-}AK^+$ cells which was largely IL-2 independent was not strictly regulated by GSH.

II. Tumor Immunology.

In vivo antitumor activity of CD3-AK and LAK cells:

We found a direct correlation between in vitro and in vivo antitumor activity of CD3-AK and LAK cells. Mastocytoma P815 was susceptible to CD3-AK but not to LAK, and the reverse was true for melanoma JB/MS. A methylcholanthrene-induced sarcoma T-92 was susceptible to the killing by both CD3-AK and LAK cells and tumor neutralization experiments showed that they were also effective in preventing in vivo growth of T-92. Experiments are undertaken to determine the effect of CD3-AK and LAK cells on the pulmonary metastasis of T-92. These studies should provide us with information to select appropriate killer cells for immunotherapy of tumor growth.

Proposed Course of Research:

1. Mechanism of lymphocyte-mediated cytolysis to further characterize the roles of different cytokines in the regulation of lytic reactions.

2. To determine the effect of various biological response modifier reagents (such as PMA, GSH etc.) on the regulation of slow lysis.

3. To determine the potential use of CD3-AK in immunotherapy of cancer.

Publications:

Yun YS, Hargrove ME, and Ting CC. In vivo antitumor activity of the anti-CD3-induced activated killer cells. Cancer Res 1989;49:4770-4774.

Ting CC, Hargrove ME, and Henrich P. Anti-CD3 antibody-induced activated killer cells. Subsets of killer cells that mediated fast or slow lytic reactions. Immunological Invest 1990; in press.

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 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.

Project Officer: Thomas A. Waldmann, M.D.
Program: Cancer Biology Resource
Technical Review Group: Ad Hoc Technical Review Group
FY: 1990 Funds: $251,773 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; maintence of wild mouse colony.

Approach: Maintain a closed conventional colony of inbred and congenic strains of mice, suitable for maintaining mice for long term plasmacytoma induction experiments. Develop BALB/c congenic strains carrying plasmacytomagenesis resistance (PCT-R) genes. Carry out procedures for identifying markers used in the construction of congenic strains. Maintain colonies of pedigreed wild ascites, tissues, high molecular weight DNA, pedigreed breeders to qualified investigators and collaborators.

Progress: Contractor has carried out basic plasmacytoma induction experiments using pristane alone in various BALB/c.DBA/2 congenic C x S RI, (BALB/c x DBA/2)$F_1$, N1 and BALB/cAn.BALB/cJ congenic strains. 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 identy 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. Through Joan Renner, Hazleton Laboratories has reorganized and revitalized the contract. In addition to the above described work, the contractor functions as the major animal resource for the Laboratory of Genetics.

Significance to Cancer Research: Provides essential support for the study of plasmacytomagenesis (carcinogenesis) with the specific goal of determining the genetic basis of susceptibility to tumor induction by mineral oil. Supplies essential biological material for investigators studying the biology of neoplastic plasma cells, tumor immunology, the genetics of immunoglobulins, and immunoglobulin synthesis.

Project Officer: Dr. Michael Potter, Dr. Beverly Mock
Program: Immunology Support
Technical Review Group: Intramural Support Contract Proposal Review Committee
FY 90 Funds: $907,354 B

CONTRACT RESEARCH SUMMARY

Title: Maintenance and Development of Inbred and Congenic Resistant Mouse Strains

Principal Investigator: Mr. David Bernard
Performing Organization: Hazleton Laboratories America, Inc.
City and State: Rockville, MD

Contract Number: NO1-CB-71091
Starting Date: 2/1/87 Expiration Date: 1/31/93

Goal: To maintain a colony of inbred pedigreed strains of mice which are needed to support ongoing NCI intramural research in transplantation immunology.

Approach: The contractor maintained a colony of approximately 40 special inbred and congenic resistant strains of mice by pedigreed brother-sister mating. Quality control testing was carried out at each generation by cytotoxicity typing of breeders from each strain. Alloantisera were raised between mouse strains to assist in this quality control typing, and sera and animals were shipped by the contractor to collaborating investigators at NIH and elsewhere.

Progress: The contractor maintained all inbred and congenic resistant strains of mice in excellent condition. Breeding of each strain and of hybrid strains, recordkeeping, and quality control testing were all highly satisfactory. The backcrossing 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 Officer: Dr. David H. Sachs
Program: Immunology Resource
Technical Review Group: Intramural Support Contract Proposal Review Committee
FY 90 Funds: $500,000 B

CONTRACT RESEARCH SUMMARY

Title: Maintains an Animal Holding Facility and Provides Attendance Research Services

Principal Investigator: Ms. Leanne DeNenno

Performing Organization: Bioqual, Inc.
City and State: Rockville, MD

Contract Number: NO1-CB-85607
Starting Date: 11/01/87 Expiration Date: 10/31/93

Goal: Maintain colonies of inbred mice (11,000 animals), inbred rats (500 animals), hamsters (50 animals), and rabbits (40 animals) 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 Immunology and Experimental Immunology Branches, NCI.

Approach: Colonies of mice, rats, and rabbits are to be housed and fed according to National Research Council standards. Technical manipulations and breeding are to be carried out as directed by the project officer.

Progress: This contract represents predominantly a facility for holding experimental animals in support of the research of the Immunology and Experimental Immunology Branches. As such, the contractor has maintained a colony of up to 11,000 mice, 500 rats, 50 hamsters, and 40 rabbits. Up to 1500 mice are housed in microisolators in a separate room with access to a cesium irradiator. Breedings of certain strains of mice and rats for experimental needs have also been performed when such animals have not been available commercially. Frozen samples of sera and cells are stored in freezers of appropriate temperatures are transferred to NIH as required.

Performance on this contract has been highly satisfactory. The animal colonies have been established and are being maintained according to National Research Council standards. Animal health has, in general, been excellent. Protocols have been carried out in a satisfactory fashion. Recordkeeping and transferring of animals to and from the NIH Campus have all been satisfactory. Maintenance of frozen products in appropriate freezers has been satisfactory.

Significance to Cancer Research: This animal colony is necessary in support of intramural research programs in the Immunology and Experimental Immunology Branches of NCI. Many of these programs are concerned with the immune response to cancer.

Projects Officers: Dr. David H. Sachs and Dr. John R. Wunderlich
Program: Immunology Resource
Technical Review Group: Intramural Support Contract Subcommittee A
FY 90 Funds: 778,651 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: NO1-CB-8-5608
Starting Date: 9/30/88 Expiration Date: 9/29/93

Goal: Perform a variety of in vivo experiments in mice (up to a colony of 3600 animals) that cannot be performed on NIH campus as designated by the Project Officer. These experiments are to be performed in support of intramural research programs in the Experimental Immunology Branch, NCI.

Approach: Experiments are to be performed involving the transfer of normal and neoplastic cells, infection with virus, inoculations of combinations of cells and virus, irradiation with $\gamma$-rays, preparation of radiation chimeric mice, thymus transplants and embryo transfers. Protocols and details of experiments are to be carried as directed by the Project Officer.

Progress: A number of experiments involving virus infection, lymphocyte transfer, and combinations of the above have been performed in several mouse strains to investigate the synergistic effects of cytomegalovirus and graft-vs-host reaction. Approximately 4500 mice were received into the contract facility during the year, and the facility contained approximately 2400 mice at any give time. Two preparations each of cytomegalovirus and influenza virus were made. Approximately 670 radiation chimeras, 1300 i.p. inoculations, 700 i.v. injections, 800 tail bleeds, 60 thymus grafts, 30 virus plaque assays, 40 lymphocyte preparations, and 100 ELISA tests for cytomegalovirus antibodies were performed during FY 1990. The mice have been delivered to the Experimental Immunology Branch laboratories as requested. Although other aspects of the contract are progressing, the embryo transfer and transgenic part of the contract continues to be a problem, with little or no evidence of progress.

Significance to Cancer Research: This experimental mouse facility is required to support the intramural research programs of the Experimental Immunology Branch of NCI in that it provides research that cannot be performed on the NIH campus due to animal restrictions and use of infectious agents in NIH animal colonies. All of the protocols used in the facility related to a variety of tumors, genetic manipulation of hemotopoietic cells, immunological resistance to syngeneic tumor development, models of immune deficiency, and reconstitution of the immune system.

Project Officer: Dr. Gene Shearer
Program: Immunology Resource
Technical Review Groups: Intramural Support Contract Subcommittee A
FY 90 Funds: $454,112 EST.

B

CONTRACT RESEARCH SUMMARY

Title: Feral Mouse Breeding Colony

Principal Investigator: Dr. Joan Renner and Ms. Evelyn Hogg
Performing Organization: Hazelton Laboratories America, Inc.
City and State: Rockville, MD

Contract Number: NO1-CB-95621
Starting Date: 12/01/88 Expiration Date: 11/30/91

Goal: Introduction of mammary tumors with biological (hormones and mouse mammary tumor virus, MMTV) and chemical carcinogens in various feral strains of Mus musculus and other species of Mus. Breeding of congenic strains of feral M. musculus that contain specific genetically transmitted MMTV genomes.

Approach: Maintain a closed pedigreed colony of 1,000 feral and inbred mice. The colony is composed of approximately 700 mice that are held long-term (2 years) for tumor development and 300 mice as a breed nucleus. The breeding nucleus is composed of four pedigreed outbred colonies of feral mice having unique characteristics that are pertinent to the study of mouse mammary tumorigenesis. They are: Czech II $V^+$ mice (Mus musculus musculus), Czech II V+ mice, and MS (M. spretus) mice. In addition, a limited breeding nucleus of the high-incidence C3H/OuJ and GR inbred mouse strains and the low-incidence BALB/c $\pi$ inbred strains are maintained. BALB/c mice foster-nursed on MS, and Czech II $V^+$ are also maintained for development of mammary tumors. GR X CZECHII $V^-$ backcross mice have also been bred to develop a CZECHII $V^-$ congenic line containing the MTV-2 endogenous MMTV provirus.

Progress: The contractor maintains the feral mouse colony in excellent condition, and carries out the breeding program, quality control, and maintenance of records in a highly satisfactory manner. Approximately 30 or more mammary tumors were obtained from each of the BALB/cfC3H, CZECHII $V^-$ fC3H, C3H, GR, CZECHII mouse strains. Other sublines of BALB/c mice foster-nursed on M. spretus, and CZECHII $V^+$ have been developed. MTV-2 has been introduced into the CZECHII $V^-$ genetic background and are at backcross N3. Twenty-eight hyperplastic outgrowth lines from CZECHII $V^+$ mice are being carried to determine the frequency of mammary tumor development. Single cell suspensions of primary mammary epithelium have been introduced into cleared mammary gland fat pads and shown to repopulate the gland. Long term expression of a retroviral-expression vector transmitted $\beta$ galactosidase gene has been demonstrated in mammary glands derived from infected single cell suspensions of primary mammary epithelium.

Significance to Cancer Research: Provide essential support for the study of mammary tumorigenesis with the specific goal of identifying and characterizing the genes at risk to MMTV activation. Provides essential biological material for other investigators studying the biology of the mouse mammary tumor virus as well as other classes of retroviral genomes.

Project Officer: Dr. Robert Callahan
Program: Immunology Resource
Technical Review Group: DEA; Ad Hoc Intramural Technical Review Group
FY 90 Funds: $ 143,851 B