Division of

CANCER BIOLOGY AND DIAGNOSIS

Volume II
October 1, 1982-September 30, 1983

U.S. DEPARTMENT
OF HEALTH
AND HUMAN SERVICES

National
Institutes of
Health

National
Cancer
Institute

National Cancer Institute

NATIONAL CANCER INSTITUTE

ANNUAL REPORT

October 1, 1982 through September 30, 1983

TABLE OF CONTENTS

DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE

SUMMARY REPORT OF THE DIRECTOR

October 1, 1982 through September 30, 1983

INTRODUCTION

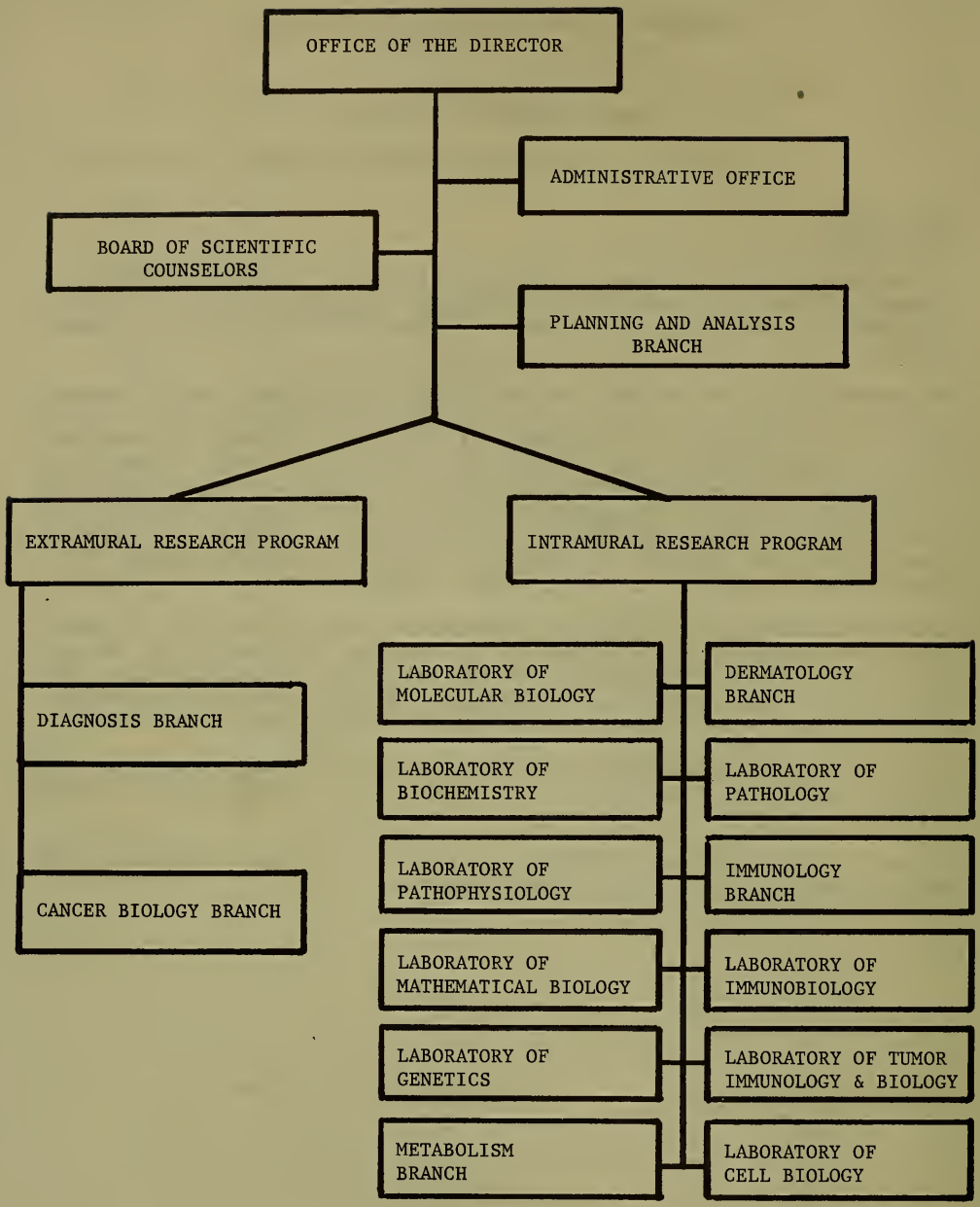
The Division of Cancer Biology and Diagnosis consists of both intramural and extramural research programs in the biology and diagnosis of cancer. A close coordination is maintained with other divisions of NCI in areas of common interest.

The intramural research program consists of three broad areas: cancer biology, immunology and clinical research. Basic research in cancer biology is carried out in the Laboratories of Biochemistry, Molecular Biology, Mathematical Biology, Pathophysiology, Cell Biology and Genetics. Research in immunology is conducted in the Immunology Branch, and the Laboratory of Immunobiology, and the Laboratory of Tumor Immunology and Biology. Three clinical branches work in the fields of Dermatology, Metabolism and Pathology. A fourth organizational element, under the Director for Extramural Research Program, manages three major extramural programs: Tumor Biology, Immunology and Cancer Diagnosis.

During the reporting period two new laboratories joined the Division of Cancer Biology and Diagnosis. The Laboratory of Genetics, directed by Dr. Michael Potter, was formed from a section of the Laboratory of Cell Biology. The Laboratory of Tumor Immunology and Biology, directed by Dr. Jeffrey Schlom, was transferred to DCBD from the Division of Cancer Cause and Prevention.

The Laboratory of Immunobiology moved from the NIH Bethesda campus to the Frederick Cancer Research Center. A current organizational chart is shown on p. XVI.

The report of the Extramural Program is given in the second volume, which includes both contract and grant funded research.


OFFICE OF THE DIRECTOR
ADMINISTRATIVE OFFICE
BOARD OF SCIENTIFIC COUNSELORS
PLANNING AND ANALYSIS BRANCH
EXTRAMURAL RESEARCH PROGRAM
INTRAMURAL RESEARCH PROGRAM
DIAGNOSIS BRANCH
CANCER BIOLOGY BRANCH
LABORATORY OF MOLECULAR BIOLOGY
LABORATORY OF BIOCHEMISTRY
LABORATORY OF PATHOPHYSIOLOGY
LABORATORY OF MATHEMATICAL BIOLOGY
LABORATORY OF GENETICS
METABOLISM BRANCH
DERMATOLOGY BRANCH
LABORATORY OF PATHOLOGY
IMMUNOLOGY BRANCH
LABORATORY OF IMMUNOBIOLOGY
LABORATORY OF TUMOR IMMUNOLOGY & BIOLOGY
LABORATORY OF CELL BIOLOGY

Division of Cancer Biology

The Division of Cancer Biology and Diagnosis conducts laboratory and clinical research in tumor biology and immunology. This report will include descriptions of the research programs of the intramural laboratories in the division. They are: The Laboratory of Molecular Biology (Dr. Ira Pastan, Chief); The Laboratory of Biochemistry (Dr. Maxine Singer, Chief); The Laboratory of Pathophysiology (Dr. Pietro Guillino, Chief); the Laboratory of Mathematical Biology (Dr. Charles DeLisi, Acting Chief), the Laboratory of Genetics (Dr. Michael Potter, Chief), the Metabolism Branch (Dr. Thomas Waldmann, Chief); the Dermatology Branch (Dr. Stephen Katz, Chief); the Laboratory of Pathology (Dr. Lance Liotta, Chief), the Immunology Branch (Dr. David Sachs, Chief), the Laboratory of Immunobiology (Dr. Tibor Borsos, Acting Chief); the Laboratory of Tumor Immunology and Biology (Dr. Jeffrey Schlom, Chief), and the Laboratory of Cell Biology (Dr. Lloyd Law, Chief).

Laboratory of Molecular Biology

The research activities of the Laboratory of Molecular Biology (Dr. Ira Pastan, Chief) are directed towards examining the factors regulating gene expression in animal and human cells, and using this information to define the biochemical basis for abnormal growth and behavior of neoplastic cells. In addition, there has been a continuing research interest in elucidating the role of the plasma membrane in receiving signals from hormones, growth factors, and other cells, and on determining how these signals are transmitted to the genetic apparatus.

Previous work in this laboratory showed that the phosphorylation of vinculin, a protein important for maintenance of cell shape and adhesion, by the tyrosine specific src kinase is stimulated by anionic phospholipids. Vinculin binds to anionic phospholipids and appears to undergo a structural change that makes it a better substrate for the src kinase. Whether these phospholipids also interact with the src kinase itself is currently under study. Evidence suggests that cAMP treatment of Rouse sarcoma virus (RSV) transformed cells stimulate src kinase activity. Studies of the regulation of collagen gene activity by transformation by RSV have continued. Structures of two different collagen genes have been compared. It was found that the size distribution of the exons that encode the helical portion of collagen has remained unchanged in these two genes although the sequences in these exons, as well as the size and sequences of introns, have changed. This suggests that many recombinational rearrangements responsible for the assembly of the ancestral collagen genes were no longer tolerated after the two genes diverged from a common ancestor. Regulation of the expression of the alpha-2(I) collagen gene has been examined. Data suggest that there are three levels of control in the expression of this gene. Mouse cell lines have been established in which the cloned chick alpha-2(I) collagen promoter is stably integrated in the genome of these cells. It was found that this cloned promoter responds to the same regulation by the products of oncogenes as the endogenous alpha-2(I) collagen promoter.

Gene transfer techniques have been refined to enable the identification of genes that control mammalian cell growth. These improved techniques have increased the efficiency with which foreign genes can be introduced and stably expressed in multiple primate cell types. A model system for human mammary neoplasia has been developed using the inbred C3H/Sm mouse. This strain, which has a low occurrence of mammary tumors and is free of fully expressed endogenous mouse mammary tumor virus (MMTV) genomes, may be relevant to human neoplasia. Work is underway to elucidate how virus and chemical or hormonal stimulation act on developmentally controlled murine genes and defective MMTV provirus genes carried on C3H/Sm chromosomal DNA. Increased levels of a new, unique mRNA transcript homologous to the long terminal repeat of MMTV have been demonstrated in both spontaneous and experimentally-induced C3H/Sm mammary tumors.

Recombinant DNA clones spanning the entire fibronectin gene of chick embryo fibroblasts have been isolated and partially characterized. It has been shown

that the rate of fibronectin synthesis is markedly decreased by RSV transformation. The structure and function of the transformation-sensitive fibronectin has been examined. Carbohydrate residues in the collagen-binding domain are necessary for protection of this domain against proteases. Experimental results suggest that fibronectin may interact with gangliosides on the cell surface; it was found to share some of the biological activities with the epithelial adhesive protein laminin.

A lysosomal protein (major excreted protein [MEP]) whose synthesis and secretion are stimulated by malignant transformation, tumor promoters and growth factors was examined in detail. A cDNA clone coding for MEP has been isolated and used as a probe to demonstrate increased MEP mRNA levels in transformed and tumor promoter treated cells.

A new organelle, the receptosome, has been identified that moves by saltatory motion carrying ligands from clathrin-coated pits on surface of fibroblasts to the cell interior. Investigations have demonstrated that purified receptosomes are rich in cholesterol, have a unique polypeptide pattern, are enriched in transferrin receptors and contain phosphomannosyl receptors, but contain no detectable amounts of clathrin. One ligand internalized in receptosomes is alpha-$_2$-macroglobulin. The alpha-$_2$-macroglobulin receptor has been purified. A method has been developed to modify the Pseudomonas exotoxin (PE) so that it no longer binds to cellular receptors but is fully enzymatically active. This modified toxin has been coupled to epidermal growth factor (EGF), and the antibody to the human transferrin receptor. These conjugates enter cells through coated pits and receptosomes. Experiments have shown that adenovirus enhances the toxicity of these conjugates by up to 10,000-fold by disrupting the receptosome that contains the virus and toxin, giving the toxin free access to the cytosol. These studies have important implications for cancer chemotherapy where monoclonal antibody toxin conjugates are being widely investigated.

Affinity labeling techniques were employed to identify and characterize the plasma membrane receptors for 3,3',5-triiodo-thyronine (T_3) in three tissue culture lines. A 55 kilodalton (kd) protein was detected. One-dimensional peptide mapping showed that there are structural similarities in the 55 kd protein from the three different species, indicating that the plasma T_3 receptors are highly conserved. The plasma membrane T_3 and T_4 receptors were shown to have structural similarities. Evidence suggests that one plasma membrane thyroid hormone receptor mediates the uptake of both T_3 and T_4 into cells.

Electron microscopic immunochemistry has been used to localize transforming proteins in cells. A monoclonal antibody against Harvey MSV p21 srx has demonstrated that a cross-reactive p21-like protein is present in normal and malignant human epithelial cultured cells. This suggests that there is a family of normal p21 proteins in most cells, some of which probably induce maliganancy when introduced by certain retroviruses that carry selective mutations of these

genes. Immunochemical methodology has been further developed and used to determine the intracellular location of calmodulin in cultured cells. Contrary to the findings of other investigators, it was shown that little calmodulin is concentrated in macrofilament structures.

The Laboratory of Molecular Biology also conducts basic research aimed towards understanding the control mechanisms of protein synthesis, cell division and host-virus interactions. The gal operon of E. coli is used as a model system. The two gal promoters, which are controlled by cyclic AMP in opposite ways, are negatively regulated by a gal repressor protein. The repressor gene has been cloned into a high expression vector and the protein purified. Identification of the repressor binding sites has revealed a two-element gal operator--one located upstream to the promoters and the other inside the first structural gene. This suggests that repressor bound at two distal sites may inactivate the activities of the promoters located in between by changing DNA conformations. This is the first example of a local alteration in DNA structure caused by a protein bound at a different site. The nature of transcription termination of E. coli has been examined. E. coli phage P1 can stimulate the precise extension of transposons, and the responsible P1 gene has been subcloned. Currently work is underway to clarify the mechanism of the reaction. A vector system is being constructed in which DNA fragments are cloned adjacent to a promoter active in E. coli, to permit rapid assessment of coding gene activity. A shuttle vector system is being developed that is tailored for the cloning of large and/or unstable DNA fragments as well as for the reconstruction of genes from overlapping DNA clones. The control of cell division in E. coli is being studied as a model for understanding cell division in more complex cells. Investigators have demonstrated that the sul A protein is rapidly turned over in normal cells and has a longer half life in cells with defective cell division. This system is important in the timing of cell division in E. coli. Studies are underway to investigate the degradation of sul A in vitro.

Laboratory of Biochemistry

The research program of the Laboratory of Biochemistry (Dr. Maxine Singer, Chief), includes studies of the control of gene expression by chromatin structure and by environmental, hormonal and developmental signals, the organization of genomes, moveable eukaryotic elements, the regulation of physiological processes by calcium ions, the mechanism of muscle contraction, cellular immunology, nutrition, and improved methods for separation of proteins.

The regulation of the expression of rat growth hormone (RGH) has been studied as a model of the biochemistry of gene expression. A variety of vectors have been constructed which contain rat growth hormone (RGH) gene. These vectors were successfully transfected and expression of RGH was observed in a rat pituitary adenoma cell line (GH_3) and hybrids between GH_3 and a mouse line (ML) which contain but do not express the RGH gene.

These transfectants process RGH transcripts to proper mature mRNA size, perhaps by some informational molecules supplied by the GH_3 cell portion of the hynrid. One of these transfected cells is inducible. A vector consisting of the 650 bases comprising the promoter region of the gene linked to the E. coli galactose gene was also constructed. Initial studies on cross-linking glucocorticoid receptors with DNA suggest that DNA methylation has little to do with expression, inducibility or hybrid cell extinction of the RGH gene. Studies have been initiated to determine whether RGH induction in GH_3 cells is a primary consequence of steroid treatment or whether it requires intervening protein induction(s).

Human (and rat) glucocorticoid receptors have been purified and proteolytic analysis of the receptors is underway. It is hoped that enough purified receptor can be obtained to enable amino acid sequencing, for subsequent identification and characterization of human glucocorticoid receptor gene. Studies on the regulation of collagen biosynthesis in animals and in normal and transformed cells have continued. Recent work has shown that the specific decrease in collagen synthesis in the bone of scorbutic guinea pigs is related primarily to the decreased food intake and weight loss associated with scurvy, and occurs independently of the effects of ascorbate deprivation on hydroxylation of proline in collagen.

Another group is concerned with the enzymes and accessory protein factors involved in DNA synthesis. Recent results on the structure of the mammalian alpha-polymerases emphasize a 195 kilodalton (kd) polypeptide that appears to be an alpha-polymerase constituent in calf and monkey cells. Experiments were conducted to validate and improve a new method of gel analysis for DNA polymerase activity in crude cell extracts. This method has been used to examine the levels of beta-polymerase in cultured cells from patients with ataxia telangiectasia (AT), a disease characterized by defects in DNA repair synthesis. This enzyme species, which is thought to function in DNA repair, was found to be equally abundant in AT cells and those of normal individuals.

Previous work in this laboratory had confirmed that intracisternal A-particle (IAP) genes make up a family of extensively reiterated retrovirus-like elements in the genomic DNA of Mus musculus and some other rodent species. Recent data show that one randomly cloned IAP gene has a retroviral form of long terminal repeat units (LTR) and is bracketed by short duplications of cellular sequence. The IAP LTRs can effectively promote gene expression in mammalian cells when introduced in an expression vector carrying the bacterial gene for chloramphenicol acetyl transferase (CAT). Thus, IAP genes have a number of attributes associated with the conventional integrated proviruses, even though the IAPs themselves are not known to have an infectious extracellular phase. It has recently been established that an IAP gene insertion is responsible for the rearrangement and activation of a c-mos oncogene. This is the first demonstration of cellular oncogene activation by insertion of an endogenous retroviral element. The results indicate that IAP genes can function as movable elements in the mouse genome and may occasionally have a role in cell transformation and/or tumor progression.

Analysis of DNA isolated from somatic cell hybrids that segregate human chromosomes has continued. New methodology has permitted the localization of several human cellular oncogenes to specific human chromosomes and subchromosomal regions. Many of these c-onc genes map to the same sites as nonrandom chromosome rearrangements in specific human cancers, supporting the assumption that these genes may be involved in human oncogenesis.

The heavy metal induction of metallothionein (MT) has been used as a model system to study eukaryotic gene regulation. MT genes from mice and humans have been cloned and characterized. An upstream activator region and a more distal control region required for heavy metal inducibility were identified. Although heavy metals and glucocorticoids both induce MT-I expression, gene transfer experiments indicate that they regulate this gene by independent mechanisms. MT induction may require a positive transcriptional activator. A versatile set of mammalian cell expression vectors have been constructed which will enable essentially any coding sequence to be placed under the control of the MT promoter. This will allow an exceptionally high level of production of several appropriately processed and post-translationally modified biologically active polypeptides. At least 12 different fragments with homology to MT probes have been found in the human genome. Analysis of DNA from human/rodent somatic cell hybrids indicates that the MT genes are located on at least four different human autosomes but not on the X-chromosome.

A series of experiments have been designed to investigate the relationship between function and DNA structure in the control of initiation and termination of RNA synthesis, as well as in the translational expression of mRNAs, in both prokaryotic and eukaryotic systems. Recombinant DNA techniques have been used to develop plasmid, phage, and bacterial vector systems which allow the isolation, characterization, and comparison of prokaryotic transcriptional regulatory systems. Previous work demonstrated that under appropriate conditions prokaryotic genes could be expressed efficiently in eukaryotic cell-free systems and in mammalian cells. An SV40-plasmid vector system has been constructed which carries two independent gene transcription units which function in eukaryotic cell systems. Each transcription unit controls the expression of a different selectable gene function which can be easily quantified in cell extracts. The system is being used to evaluate transcriptional initiation and translational regulatory sequences in eukaryotes.

Cloned cDNA probes have been used to isolate the genomic sequences for the following chicken proteins: alpha skeletal muscle actin, alpha cardiac actin, beta cytoplasmic actin, myosin light chains 1 and 3, vimentin (a major intermediate filament protein), pyruvate kinase, and GAPDH (glyceraldehyde phosphate dehydrogenase). The structure and regulation of expression of the genes for each of these proteins is being determined. The various actin genes have been subcloned to enable the study of the developmental regulation of these genes after transfection into the C2 mouse muscle cell line. Probes specific for the various chicken actin genes have been used to monitor expression during differentiation in vitro.

The sequential arrangement of nucleosomes along the chromatin fiber is punctuated by sites that are highly sensitive to nuclease attack. Previous work had mapped such sites to the 5' terminus of several genes in Drosophila. An in vitro transcription system is being developed for investigation of the functional relationship of 5' terminal hypersensitive sites in chromatin to gene activity. Another group has been studying the regulation of expression of the immunoglobulin gene family. They have shown that the complete kappa gene is transcribed after transfection into antibody-producing myeloma cells but not into nonlymphoid 3T3 or L cells. Thus the lymphoid cells appropriately regulate the kappa gene even when it is not in its usual chromosomal environment. Further studies have demonstrated that certain sequence elements downstream of the promoter are necessary for gene transcription in myeloma cells.

A continuing research interest has been the regulation of cellular processes by calcium ions (Ca^{2+}). One problem under study is the role of Ca^{2+} dependent phosphorylation of myosin light chains in the assembly of myosin filaments in smooth muscle and nonmuscle cells. Recent data have shown that the equilibrium between monomeric and filamentous myosin is dependent not only on phosphorylation but also on the nature of the anions used for the experiments. Studies on the roles of fodrin and calmodulin in the regulation of the actin-myosin ATPase system have been initiated. Fodrin is a widely distributed, membrane associated protein that binds actin and calmodulin. Calmodulin and fodrin form a one to one complex. No effects of actin on fodrin-calmodulin interaction or of calmodulin on fodrin modulation of actin-stimulated myosin ATPase have yet been detected.

In most, if not all, eukaryotic cells the action of Ca^{2+} is mediated by the CA^{2+}-receptor protein, calmodulin. Ca^{2+} binding to calmodulin is sequential and accompanied by stepwise conformational transitions. These separate states would allow calmodulin to transfer quantitative changes in Ca^{2+} concentration into qualitatively different cellular responses. Investigators have now identified the high and low affinity Ca^{2+} binding sites. This has enabled studies of the interaction of calmodulin with its target proteins and with anti-calmodulin drugs.

Most calmodulin-regulated enzymes are also regulated by cAMP-dependent phosphorylations which enhance or inhibit their stimulation by calmodulin. Conversely, calmodulin and Ca^{2+} modify cAMP dependent responses. A major calmodulin binding protein, calcineurin, has been identified as a calmodulin regulated phosphoprotein phosphatase. This enzyme contains a Ca^{2+} binding protein as an integral subunit. This subunit has been sequenced and was shown to contain four Ca^{2+} binding loops analogous to the Ca^{2+} loops of calmodulin. The amino terminal blocking group is myristic acid, which may serve to anchor the enzyme reversibly in the membrane. This calmodulin-stimulated phosphoprotein phosphatase may serve as an important link between Ca^{2+} and cAMP mediated regulations of cellular processes.

Simian virus 40 (SV40) has been used as a model to study the use of common regulatory elements in viral-host interactions. Several years ago, segments of

the African green monkey genome that are homologous to the control region of the SV40 genome were cloned from a monkey library. Work this year concentrated on studying whether the monkey segments, which are homologous in structure, are similar in function. One of the monkey segments, designated clone 7, was used. The SV40-ori-like region in clone 7 is embedded in the genomic region that is rich in interspersed repeated sequences, and is flanked by two members of the Alu family, one of which is a member of an Alu subfamily whose members contain a potential Z-DNA forming segment. The SV40-ori-like region in clone 7 serves as an efficient cellular promoter for RNA transcription. It does not, however, include enhancer activity, which must be separately supplied in the vector. The cellular promoter is notable for the absence of the TATAAA box typical of RNA polymerase II transcription units. It is possible that it represents a rare class of polyII promoters that play a special regulatory role in cellular metabolism.

An ongoing research project concentrated on the organization and function of highly repeated sequences in primate genomes. Recent work focused on the deca-satellite and on the KpnI family of long interspersed repeated sequences. The most striking feature of the deca-satellite is its long range organization which is not fixed; no two monkeys thus far investigated show the same set of decasatellite containing restriction fragments. The abundant KpnI family is dispersed in primate genomes. Sequencing studies of several cloned monkey family members have confirmed that family members vary in the total length and internal arrangement of family sequences. These moveable KpnI elements appear to belong to a distinctive class of eukaryote moveable elements characterized by the absence of long terminal repeats and the presence of a 3'-terminal polyA stretch. Such elements may move by the intermediary formation of transcripts and reverse transcripts. Primary sequence data has been compared with recently published sequences for an analogous interspersed rodent DNA family. There was nearly 70 percent homology between most of the base pairs, suggesting a common ancestor which was conserved in evolution.

Immunization of cytotoxic effector cell (CTL) precursors in vivo involves adherent accessory cells (macrophages), T helper cells, Class I MHC antigens (H-2 in mice), Class II MHC antigens (Ia), and at least two different nonspecific helper factors (IL1 and IL2). Researchers are utilizing a variety of in vitro approaches to better understand this system. High performance liquid chromatography which can detect and quantify granulocyte differentiation proteins has been applied to the study of leukocytes obtained from chronic myelogenous leukemia (CML) patients known to have the Philadelphia chromosome defect, in the hope of finding a protein marker that would facilitate early diagnosis and clinical staging of this disease. The HPLC patterns of acetonitrile-trifluoroacetic acid extracts of mature granulocytes from patients in the stable phase of CML revealed marked variations from the highly reproducible normal pattern. These variations apparently reflect elevated levels of some of the normal granule proteins and thus a defect in the regulation of granule protein synthesis rather than the synthesis of abnormal proteins.

The GL-13-BC guinea pig model for human CML developed previously in the Laboratory of Biochemistry has facilitated the study of molecular aspects of this disease, and, in addition, offers a means of rapidly screening new therapeutic regimens. Cyclophosphamide and Alkeran were tested in both early and late stages of the disease. The greatest therapeutic effect was observed when cyclophosphamide was given during the early stage. Significant new simplifications of the procedures for purifying and fractionating the carboxymethyldextrans (CM-Ds) have not only facilitated their preparations but improved resolution of CM-Ds in the low to moderate range. Recent studies have eased the earlier concern that the use of CM-Ds for the displacement chromatography of proteins might result in the removal of required metal ions from enzymes. The complex of alpha-lactoglobulin with Ca^{2+} and that of conalbumin with iron retained their metal ions when chromatographed with CM-D.

Laboratory of Pathophysiology

The research effort of the Laboratory of Pathophysiology (Dr. Pietro Gullino, Chief), is focused on the study of the mammary gland and mammary carcinomas. A continuing research objective is to establish whether neoplastic transformation of mammary epithelium alters the organization of genes involved in the production of secretory milk proteins. Molecules similar to mammary gland alpha-lactalbumin but distinct in their ability to modify the specificity of galactosyl-transferase have been found in the epididymal fluid. Unlike mammary gland alpha-lactalbumin, this activity transfers galactose to glucose and to myo-inositol with equal efficiency. In the male reproductive tract where lactose is absent and free glucose levels are very low, this activity may modulate sperm surface glycoproteins and be an important factor in cell-to-cell interaction.

Alpha-lactalbumin bears a structural homology with lysozyme, an enzyme catalyzing the hydrolysis of a 1-4 glucosilic linkage in polysaccharides. The complete nucleotide sequence of the rat alpha-lactalbumin gene carrying the code and intervening sequences, including its 5'-flanking region has been established. Galactosyl-transferase has been purified from human and rat milk, and whey-acidic proteins have been isolated and sequenced. The isolation and sequence analysis of the cDNA clones corresponding to galactosyl-transferase are being pursued. The goal of these studies is to better understand the role of galactosyl-transferase activity in the generation of specific cell surface antigenic determinants. To date, a library of cDNA clones from rat lactating mammary glands has been generated.

The effects of cyclic nucleotides and hormones on growth and regression of mammary carcinomas have been examined. Past studies showed that protein kinase type II increases sharply during regression of hormone-dependent mammary carcinomas. Monoclonal antibodies generated against the regulatory subunits of type I and II protein kinase cross-reacted with the regulatory subunits of MCF7 human mammary carcinoma line. In regressing tumors, two new receptor proteins

appeared that were specifically precipitated by the anti-receptor II antibody. Indirect immunofluorescence revealed that during regression the nuclei of MCF7 tumors are enriched in type II cAMP-dependent protein kinase.

The role of the ras gene expression in neoplastic transformation is being investigated in growing and regressing mammary tumors, hormone-dependent versus hormone-independent mammary tumors, in the mammary gland with dysplastic-hyperplastic lesions or one undergoing chemical carcinogenesis. Factors that affect phenotypic reversion of transformed cells are also being analyzed in cell line 433 of NIH 3T3 cells containing the transforming ras gene of Harvey sarcoma virus flanked by LTR of MMTV which acts as promoter and is influenced by glucocortocoids. The controlling effects of cyclic AMP on 433 phenotype and p21 production is being investigated. A clinical study has been initiated in which the ratio of steroid receptor to cyclic AMP binding proteins is being used to discriminate between hormone dependent and independent tumors.

A major project in this laboratory has been to evaluate the role of the microenvironment in the growth and neoplastic transformation of the mammary epithelium. Previous work demonstrated that production of faulty basement membrane causes regression of mammary tumors, and may be controlled by modulation of collagen turnover, increased synthesis as a consequence of cell contact with "foreign" substrate, or by stimulation by growth factors and hormones. One growth factor, isolated and partially purified from rat, mouse and human milk, is produced by the mammary cells and stimulates production of more collagen. A similar factor is produced by MCF7, a human mammary carcinoma.

In another set of experiments, substances that block proline incorporation into collagen substantially reduced cell growth both in vivo and in vitro, and proline was shown to stimulate the growth of tumor cells in culture. Studies on breast cancer and dietary lipids revealed that mammary epithelium is dependent on essential fatty acids for proliferation, and that prolactin stimulated epithelium recruits the fatty acids from the adipocytes. Mast cells are the source of histamine that triggers release of fatty acids from adipocytes. Some of these fatty acids are transformed into a potent growth stimulator of mammary epithelia. Alterations of cell membrane-associated receptors modify the response of target tissues to various hormones and thus control cell growth or function.

In previous work it was shown that the number of detectable prolactin receptors is controlled in vivo by the level of circulating prolactin or growth hormone. Prolactin up-regulates its receptor by modifying target membrane fluidity via modification of prostaglandin synthesis. An assay for prostaglandin receptors has been developed which has demonstrated that regressing tumors have an increased capacity to bind prostaglandin and that copper ions increase the binding capacity. Patients afflicted with adrenoleukodystropy or adrenomyeloneuropathy accumulate long chain fatty acids in their cellular membranes, which alters the fluidity of the erythrocyte membrane. The role of prostaglandins in the ovarian function was also studied. Suppression of endogenous prolactin

secretion caused an increase in the number of prostaglandin receptors in ovarian membranes. This suggests that prolactin exerts its effects through the prostaglandin cascade and explains, in part, the mechanism by which infertility occurs in hypoprolactinemic women.

The methodology of cell cultures in artificial capillaries has been further improved to enable the continuous production of hormones for laboratory and clinical use. Modifications permit study of nutrients and product transport through the tissue formed in the intercapillary spaces, the effect of lymphatic-type drainage upon cell function and the use of monoclonal antibodies directed against specific types of neoplastic cells.

Another area of research interest within this laboratory is the study of the role of hormones and various growth factors on mammary gland development, differentiation and neoplastic transformation. Using explants of mid-pregnancy mammary gland in culture it was found that T3 increased alpha-lactalbumin synthesis but not that of casein. The activity of lactose synthetase was enhanced but not that of galactosyl-transferase. Lactose production was enhanced by T3 addition to the media.

Development and function of the mammary gland was studied in vitamin D deficient mice. No major morphological differences were found; however, serum prolactin levels were decreased in vitamin D deficiency. In vitro studies demonstrated that the glands of vitamin D deficient mice synthesized 10 to 20 percent less alpha-lactalbumin and 50 to 60 percent less casein. In another set of experiments, it was shown that aldosterone and cortisone, in the presence of T3, determine milk protein synthesis both in vivo and in vitro. Both epidermal growth factor and mammary tumor derived growth factor appear to play a role in the development of the mammary gland, but the mechanism of this involvement is not clear. The purification and characterization of the human prolactin receptor is underway, as a model for understanding the interaction of lactogenic hormones and their receptors.

A series of studies has focused on the partition of wheat germ agglutinin receptor sites over the protoplasmic and the exoplasmic faces of several cell types in an attempt to correlate the localization of cell membrane components with their physiological functions. In cultured cells from acute lymphoblastic T leukemia and from mycosis fungoides, wheat germ agglutinin receptor sites always localized in the exoplasmic face of the plasma membrane, as did the glycolipid binding Con A in Acanthamoeba castellanii. A new freeze-fracture radioautographic technique is being developed to enable the further characterization of cell components.

A major research thrust within this laboratory has been to determine the relationship between angiogenesis and neoplastic transformation. Acquisition of angiogenesis is associated with an increased risk of neoplastic transformation. Previous studies demonstrated that copper ions are required for angiogenesis to occur. Recent studies have demonstrated the appearance of chemotactic factors

for capillary endothelium in rabbit corneas treated with angiogenesis effectors. Purification and characterization of the chemoattractant is underway. In previous studies it was shown that anti-fibronectin serum can block endothelial cell migration and impair angiogenesis. The significance of this observation regarding tumor growth is being evaluated.

Laboratory of Mathematical Biology

The activities of the Laboratory of Mathematical Biology (Dr. Charles DeLisi, Acting Chief), fall into several broad areas: macromolecular structure and function, membrane structure and function, immunology, pharmacokinetics, and computational and modeling methodology.

One group of investigations deals with the insertion and organization of molecules (proteins, lipids) in membranes and studies changes in membrane organization both in the lateral and perpendicular directions. Spectroscopic techniques are used to study lipid-protein interactions, and measure conductance across planar black lipid membranes to approach questions of membrane protein topology.

Work on liposomes has centered on developing a mechanism to direct liposome-encapsulated agents to tumor targets. Approaches have included antibody-mediated targeting to tumor cells, with immunoglobulin attached to the liposome, temperature-sensitive liposomes, designed to release encapsulated drug selectively in a tumor; and compartmental delivery of liposomes of their contents to lymph node micro-metastases.

A new approach has been developed for the use of monoclonal antibodies for diagnosis and therapy of tumor in lymph nodes: delivery to the nodes via lymphatic vessels after subcutaneous injection. In vitro binding characteristics of the antibody were combined with in vivo pharmacokinetic parameters to develop a computer system for the simulation analysis and modeling of the delivery system. The specific uptake of antibody via the lymphatics was obtained in lymph node micrometastases of guinea pig tumors. Recent studies suggest that the lymphatic route will provide higher sensitivity, higher efficiency, and less cross-reaction than intravenous injection for diagnostic imaging of early lymph node tumors.

Work has continued on the analysis and modelling of experimental data bearing on molecular mechanisms of endocytosis, exocytosis and chemotaxis of various cellular systems. A comprehensive analysis of data related to the early and late stages of cellular responses to epidermal growth factors has been undertaken to gain a better understanding of the effects of phorbyl esters and various other tumor promoters on cellular division. Studies of exocytosis have focused on mechanisms controlling the initial stages of release of histamine from mast cells and basophils, and the antigen specific and nonspecific desensitization of these systems.

A series of experiments has been performed to elucidate the maintenance phase of the immune response and impairments in that phase. A mathematical model has been formulated with a view towards analyzing experiments on complex time dependent behavior characteristic of immune response maintenance.

The development of a comprehensive data base containing primary, secondary and tertiary structural information in all sequenced nucleic acids and proteins has continued. Algorithms are being developed for the retrieval and analysis of large amounts of information related to sequence and structural homologies. Methods are being developed for predictive classification of the location and general function of proteins, such as oncogene products, which should serve as a guide to locating and elucidating their function.

Research within the laboratory encompasses biological macromolecules and their properties. Stabilities of macromolecular conformations are determined by interatomic interactions. The relative importance, for proteins, of short range and long range interactions is being assessed in detail. Calculation of approximate free energies are used together with optimal selection methods to yield secondary structure predictions. Several simple models of long range intramolecular interactions have been formulated to facilitate investigations of protein folding pathways. Folding appears to proceed by growth from only a few centers within the molecule. Structural models of membrane receptor proteins are being developed.

Laboratory of Genetics

The Laboratory of Genetics (Dr. Michael Potter, Chief) was formed in 1982 from a section of the Laboratory of Cell Biology. The primary research interests of this laboratory are in the genetic systems that control neoplasia and antibody structure.

Using the mouse plasmacytoma system as a model it now has been conclusively shown the 95 percent of the pristane induced tumors have nonrandom chromosomal translocations involving immunoglobulin gene bearing chromosomes 6 and 12 with a specific region on chromosome 15. Recent studies have revealed that the break sites occur at the location of the mouse c-myc oncogene. The process of the chromosomal translocation appears to activate c-myc oncogene expression. Work is underway to find the genetic basis for the translocation in the c-myc locus. These findings led to a study of B-cell lymphocytic tumors and plasmacytomas induced in BALB/c mice by Abelson virus. Data suggest that Abelson plasmacytomas are a distinct group of tumors, four of which lack translocations, but which still express large amounts of v-abl. This suggests that their transformed state depends upon the activity of this oncogene.

Abelson lymphosarcomas also express large amounts of v-alb. However, a distinct morphological subset of these tumors has been identified--the Abelson plasmacytoid lymphosarcomas (ABPL), many of which have lost the A-MuLV proviruses. These tumors have the remarkable characteristics of having rearranged

c-myb genes. Thus, three of the major oncogenes associated with hematopoietic neoplasms in the chicken and the mouse have been implicated in B-cell neoplasm in the mouse.

A major effort is being made to identify the genes that determine the resistance of plasmacytoma induction in the DBA/2 mouse. Over 30 marker gene differences are known between the plasmacytoma susceptible BALB/c and DBA/2 strains. Two minor resistance gene loci and possibly one stronger locus have neen found. BALB/c Jax strain is resistant to plasmacytoma development and attempts are underway to determine the genetic loci responsible for this change.

A 75 kd T-cell glycoprotein antigen has been isolated from the RBL-5 lymphoma. This 75 kd protein is probably a degradation product of a 175 kd protein commonly expressed in many normal cells. Work is underway to sequence this protein and recover the gene that controls it. The 175 kd protein system may have very important implications for the role of somatic mutations in neoplasia.

Using the Friend virus system as a model, investigations have shown that two retroviruses that lack cellular oncogene inserts induce erythroleukemia in genetically susceptible strains of mice. Recent data suggest that erythroleukemia may be associated with the expression of the viral env gene glycoprotein products. The env gene from one of the retroviruses has been sequenced, and the primary structure of the gp52 protein product has been deduced. Further studies have revealed a unique mechanism for resistance to erythroleukemia development. The resistant DBA/2 strain produces an MCF/xenotropic related env gene product and renders DBA/2 mice resistant. The localization of this gene, and the mechanism of resistance are under investigation.

The properties of single ion channels in the thyroid cell membrane which control the movement of the Na+, K+, Ca++, Cl-, and I- are being studied. Innovative new techniques have been developed which allow investigators to measure ion flow directly. Studies are underway to determine how intracellular Ca++ acts on the internal surface of the cell membrane to control Ca+ and I- channels following stimulation of the cells with noradrenaline. The complete variable region sequences for 12 monoclonal antibodies that specifically bind beta 1, 6-D-galactan structures have been determined. These proteins appear to be products of the same V_L and V_H genes; however, all are different. The major structural differences are determined by the D region and antigenicity (idiotype) but not function. Evidence of somatic mutation of IgM beta 1, 6-D-galactan binding antibodies implies that a mechanism other than the error proneness associated with DNA rearrangement is involved.

Research continues on the evolution of immunoglobulin V-gene families and the underlying mechanisms for this evolution. Genomic and rearranged V-genes in specific antibodies are being studied to assess the importance of gene conversion in antibody diversity. The region 3' of the mouse phi and alpha-C_H genes has been sequenced to demonstrate how RNA splicing mechanisms generate

multiple RNAs for membrane IgD and IgA. V_H regions from two antilysozyme monoclonal antibodies, HyHEL 8 and HyHEL 10, have been sequenced. These two proteins appear to have V_L and V_H structures from closely related genes.

Laboratory of Dermatology

The Dermatology Branch (Dr. Stephen Katz, Chief), conducts both clinical and laboratory investigations of the etiology, diagnosis and treatment of inflammatory and malignant diseases involving the skin and the host's response to these diseases. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center.

The Dermatology Branch has continued its study of the immunopathology of skin diseases. Investigations are directed towards elucidating the role of epidermal Langerhans cells in the induction and expression of cell mediated immunity. In vivo studies indicate that Langerhans cells function in the induction of contact hypersensitivity. In vitro studies showed that Langerhans cells have strong stimulatory activity in TNP specific and allostimulatory proliferation and cytotoxic T lymphocyte systems, but do not appear to perform accessory cell functions. Recent studies have demonstrated that in graft versus host disease keratinocytes can be induced to synthesize Ia antigens which do not induce allogeneic T-cell proliferation. The function(s) of these Ia positive keratinocytes is currently under study.

Work continues on the identification and characterization of antigens and antibodies involved in the pathophysiology of the auto-immune blistering skin diseases. Current research activities focus on the basement membrane zone constituents involved in normal physiology and in pemphigoid. Previous experiments had shown that pemphigoid antigen was a disulfide-linked glycoprotein of molecular weight of approximately 220K. Monoclonal antibodies are being used to identify and characterize skin specific antigens, some of which may be important in the pathogenesis of certain diseases.

Circulating antigen-antibody complexes have been implicated in the pathogenesis of a variety of dermatologic, rheumatologic, neoplastic and infectious disease states. In previous studies, the immune complexes which exist in several diseases were identified and characterized. Patients with dermatitis herpetiformis, gluten-sensitive enteropathy and IgA nephropathy were shown to have circulating IgA containing immune complexes. In dermatitis herpetiformis these soluble immune complexes contain both IgA_1 and IgA_2. Recent studies concentrate on examining the influence of certain MHC genes on immune function in vivo and in vitro in humans. An Fc receptor reticuloendothelial system clearance defect occurs in 50 percent of patients with dermatitis herpetiformis. A high percentage of HLA-B8/DRw3 positive normal individuals have abnormal Fc IgG receptor function on the splenic macrophages. Additional studies have demonstrated that normal HLA-B8/DRw3 positive individuals and HLA-B8/DRw3 positive dermatitis herpetiformis patients also have decreased numbers of peripheral

blood lymphocytes bearing receptors for the Fc portion of IgG, and a significant decrease in the number of T-suppressor cells. These HLA-B8/DRw3 patients and controls also have increased numbers of spontaneous immunoglobulin secreting cells in their peripheral blood.

Clinical investigations continue to evaluate the safety and effectiveness of new oral and topical synthetic retinoids in the treatment of skin cancer, disorders of keratinization and cystic acne. Studies are designed to establish an optimal dosage and treatment schedule to permit maximum therapeutic effectiveness with minimal toxicity. In conjunction with these clinical studies investigators are evaluating the morphologic and biochemical effects of Vitamin A and its analogs on skin. Freeze fracture studies suggest that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms.

UV-radiation is believed to be the major cause of skin cancer. Patients with Xeroderma pigmentosum (XP) are particularly susceptible to the carcinogenic action of UV radiation. Studies indicate that XP patients have a marked impairment in the rate and/or efficiency of repair of DNA damage induced by chemical carcinogens or UV irradiation. Understanding the relationship between DNA repair deficiency and skin tumor development in XP patients may elucidate the role of DNA repair in preventing those cancers induced by chemical and physical carcinogens. Some XP patients develop neurological abnormalities due to the early death of neurons. Other neurodegenerative disorders have been studied with a view towards understanding their pathogenesis as well as developing presymptomatic diagnostic tests. A hypersensitivity to X-rays has been demonstrated in lymphocyte lines from patients with a variety of degenerative diseases. It is possible that the degeneration of excitable tissue in these hypersensitive disorders may be caused by the accumulation of unrepaired DNA damage as the result of faulty DNA repair mechanisms.

The biochemical characterization of normal and malignant mammalian melanosomes has been an area of continued research interest. These studies are aimed at elucidating the mechanism of formation of melanoma proteins, their importance in the immunology of melanoma and their possible immunotherapeutic potential. Melanosomal proteins from melanoma tissues vary in structure from those of normal tissues. Previous work had shown that both proteins have amino acid contents which are identical with respect to 13 amino acids, but differ significantly with regard to four amino acids. Tumor-specific proteins can be found in the serologic fluids of melanoma patients and mice and large quantities of these proteins are shed from melanoma cells in vitro. It has been recently shown that one of these shed proteins has structural homology to the albumins, and may represent a normally occurring gene which is abnormally expressed in neoplastic tissues. This implies that these tumor-specific proteins may have a critical immunologic importance to the survival of the tumor in the host.

The role of tyrosinase in melanogenesis has been studied as a model for understanding enzymatic control mechanisms in normal and maligant tissues. Recent

investigations have revealed that tyrosinase is responsible for melanin synthesis of many different pigmentary systems, and that it is specifically activated by L-DOPA. It has been further shown that tyrosinase is under allosteric control and that phospholipids may play a part in the expression of the enzyme's activity. Recent evidence supports the theory that the enzyme is additionally controlled by enzyme-associated factors which can further modify the production of pigment; these may prove to be critical to the control of pigment formation in mammals.

The expression of tumor viruses in vivo and in vitro has been studied in the bovine papilloma virus (BPV) system. These viruses are capable of causing malignant transformation in mouse cells. Previous work demonstrated that a 2.3 kb segment of the viral DNA which cannot by itself induce transformation can be activated by a retroviral LTR to be transforming. Cells transformed by such a construction contain integrated copies of the viral DNA. BPV transformed cells can be cured of their viral DNA by long term treatment with interferon. A clinical trial of human leukocyte interferon in patients with chronic widespread wart virus infection has been initiated. Preliminary results suggest that intralesional or systemic treatment with human leukocyte interferon results in a marked diminution in the size of warts and a decrease in virus-positive cells in lesional skin.

The studies of viral and cellular oncogenes have continued. Four different human p21 ras genes have been cloned which are homologous to the p21 transforming genes of Harvey and Kirsten murine sarcoma viruses, and these genes have been localized on four different chromosomes. An activated form of one of these genes was shown to be a tumor oncogene which differs from the normal homologue by one base pair. Results indicate that p21 ras genes can be oncogenic by either increased levels of the normal gene product or normal levels of a structurally altered gene product.

The polypeptide chains which comprise the subunits of the keratin filaments of normal bovine and murine epidermis have been isolated and individually characterized. Results from a variety of experimental studies have revealed that the polypeptides are present in the filaments in the precise molar ratios of 1:2 and that the filaments contain regions of three-chain coiled-coil alpha-helix. cDNA cloned probes that encode human and mouse epidermal keratins have been isolated and are being used to determine the amino acid sequences of the proteins, and to study the structure and expression of keratin genes.

Metabolism Branch

The Metabolism Branch (Dr Thomas Waldmann, Chief), conducts basic laboratory and clinical research to define the host factors that result in a high incidence of neoplasia, with special emphasis on understanding those factors involved in the control of the human immune response. A broad range of immunological investigations are carried out in patients with immunodeficiency diseases and a high incidence of malignancy, as well as in cancer patients,

especially those with T- and B-cell leukemias. A second research focus is to determine the physiological and biochemical effects produced by a tumor on host metabolism. In this regard, the regulatory role of cell membranes, homeostatic mechanisms, and the metabolic derangements of biochemical control mechanisms are being investigated.

Studies of the arrangement of immunoglobulin genes, their rearrangement and the deletions that control immunoglobulin synthesis have continued. In their embryonic state, human immunoglobulin light and heavy chain genes are organized into a discontinuous system of multiple germline regions. During the differentiation of a stem cell into a mature B cell, there is a rearrangement of the cellular genome to combine a single variable (V_H), diversity (D_H), and joining (J_H) region to form a heavy chain gene. Similarly, a single light chain variable region must combine with an appropriate J_K or J_L region to activate a light chain gene. Recombinant DNA techniques have been used to study the gene arrangements in B cell, T cell and non-T, non-B forms of lymphocytic leukemias. All B cell leukemias have rearranged the immunoglobulin genes of at least one set of heavy chain genes and one set of light chain genes. In contrast, human hematopoietic cells, T cells and non-B cells usually retain the germline configuration of their immunoglobulin genes. A series of leukemias of controversial origin have been analyzed with immunoglobulin gene probes to define their origin in order to accurately categorize these malignancies. Immunoglobulin genes were examined in the cells of patients with chronic myelogenous leukemia. In the chronic granulocytic phase or in myloid blast crisis the immunoglobulin genes remained in the germline configuration. In virtually all cases of lymphoid blast crisis there was a rearrangement of heavy chain genes and in three of eight, cases of the light chain genes as well, suggesting that the disease in these patients reflects a B cell precursor form of leukemia. Serial examination of a single patient revealed lymphoid blast crises with different clonal patterns of immunoglobulin gene rearrangement, showing that the stem cell which represents the site of the malignant transformation in chronic myelogenous leukemia has the capacity to mature along different pathways including different lineages of B cell development. Studies of the cells of patients with acute lymphocytic leukemia suggest that immunoglobulin gene rearrangements proceed in hierarchical order, with heavy chain gene rearrangements preceeding light chain, and with kappa gene rearrangements or deletions preceeding those of lambda light chain genes. Those cells that have had an aberrant V, D, J region on both of the 14th chromosomes would have deleted the residual D regions as well as the signal flanking nucleotides required for immunoglobulin gene reorganization. Thus, these cells may be frozen in the B cell precursor stage of maturation as a result of these aberrant rearrangements. This would explain the failure of maturation of B-cell precursors in certain patients with acute B-cell precursor lymphocytic leukemia.

Clonal populations of B cells, B-cell precursors and plasma cells were shown to have specific individual immunoglobulin gene rearrangements. The detection of such rearrangements by the identification of a new band on Southern hybridization blots with immunoglobulin gene probes provides a sensitive marker for clonality and B cell lineage within lymphoid tissue lacking expression of

definitive surface phenotypes. These genetic markers enable the early detection of neoplasia and diagnosis of a lymphoma in a malignancy of uncertain type.

The critical importance of genetic factors in regulating the immune response, and especially of the immune response of Ir genes, has become increasingly apparent. A primary research objective has been to define the mechanism by which Ir genes linked to the major transplantation antigens (HLA in man and H-2 in mice) function, with the hope of understanding the modes of recognition involved at this regulatory level, the cells which participate, and the cell membrane receptors which mediate the regulation. These studies may provide us with a better understanding of the mechanisms of genetic control of immune responses involved in human disease: normal defense mechanisms, excessive responses (as in autoimmune disease) or deficient responses (as in malignancy or immunodeficiency diseases).

Myoglobin has been used as a well-defined model protein antigen. Two Ir genes mapping in different subregions (I-A and I-C) of the H-2 complex control the murine immune response to different determinants on the same myoglobin molecule. These genes operate in the antibody responses both in vivo and in vitro, and control T-cell interactions with both macrophages and B lymphocytes. In the current studies, an immunodominant determinant, i.e., a site for which the majority of T cells are specific, has been localized. Importantly, the site recognized by T cells is different from the sites recognized by antibodies previously described. This will enable the construction of synthetic peptides, which will elicit antibodies but not immunize helper T cells which could promote an anamnestic response upon later challenge.

To further explore T-cell recognition with homogenous populations of cells, long term cultured T-cell lines were prepared and cloned. One group of clones was specific for the immunodominant site and recognized only the IA^d site on macrophages. A second group of clones specific for Lys 140 required myoglobin presentation by cells bearing IE^d. The Lys 140-specific T-cell clones enabled the specific blocking of T-cell proliferation with a slight molar excess of antibody over antigen, suggesting that most T cells are specific for sites distinct from those recognized by most serum antibodies. These Lys 140-specific T-cell clones also allowed investigators to test the necessity for antigen cleavage during "processing" for antigen presentation. These clones respond equally well to the fragment which contains Lys 140 as to the native myoglobin. However, inhibitors of lysozomal proteases or of intracellular transport inhibited presentation only of native myoglobin, not of the fragment. The requirements for "processing" a large protein differ from those for a small peptide, supporting the hypothesis that large proteins require fragmentation for T-cell recognition. The mechanism of antigen handling by macrophages in presentation of the antigen to T cells is an important site of immune regulation.

Additional studies with this system support the hypothesis that helper T cells specific for one site on an antigen molecule will selectively help B cells specific for particular sites on that antigen, thus selectively inducing antibodies to only a subset of determinants on the molecule. This may explain how Ir genes which regulate T cell responses also can regulate antibody specificity.

A major accomplishment of the Metabolism Branch over the past year has been the definition of T-cell growth factor receptors TCGF or interleukin-II on the cells of the immunoregulatory T-cell circuit. This 14,000 dalton glycoprotein hormone is essential for the expansion and continued proliferation of cytotoxic, suppressor and some helper T cells. TCGF has been characterized, its gene has been cloned, and a monoclonal antibody, termed anti-Tac, that appears to identify the TCGF has been generated. This antibody enables the further study of the role of the T-cell growth factor receptor in the development of various lymphocyte functions. The antibody selectively binds activated T cells, but not resting T cells or various B-cell and pre-B cell lines.

Utilizing anti-Tac monoclonal antibody, a series of reactions has been defined which require an interaction of T-cell growth factor with its inducible receptor on activated T cells. The inhibition of immunoglobulin synthesis by B cells stimulated by T helper cell dependent activators may either reflect an inhibition of the secretion of a B cell growth and differentiation factor by the helper T cells or a direct action on the B cells themselves. The issue of whether B cells can be controlled directly by T cell growth factor is a controversial one. Evidence in this laboratory suggests that under certain circumstances B cells may manifest an antigen recognized by the anti-Tac monoclonal antibody. All Hairy Cell Leukemic cells (which are B cells as defined by their immunoglobulin gene reorganization) are Tac positive. Similarly, certain B-cell lines from patients with Burkitt's lymphoma or with HTLV induced T-cell leukemia, or B-cell lines from normal individuals induced with Epstein-Barr virus, can be induced to manifest the Tac antigen by exposure to phorbol esters. Preliminary studies imply that binding of the anti-Tac monoclonal to Tac positive B cells is inhibited by purified TCGF, providing further support for the hypothesis that anti-Tac recognizes the human TCGF receptor, and illustrates ways in which this antibody can be used to modulate the human immune response.

A major effort of this Branch has been to define the events of cellular differentiation and cellular interaction involved in the specific circulating immune response. Special emphasis has been placed on defining the defects of B-cell maturation and of regulatory T-cell and macrophage interaction with B lymphocytes and plasma cells that occur in patients with immunodeficiency diseases associated with a high incidence of neoplasia, in patients with autoimmune disorders, and in cancer patients. These studies are directed at defining the factors in normal and abnormal states controlling the production of antibodies and the synthesis of immunoglobulin molecules.

The regulatory T-cell network represents a complex array of different cells with different, and at times, opposing functions. Clonal T-cell leukemias with retained immunoregulatory function have been studied as a model for understanding this regulatory T-cell network. The Sezary syndrome is characterized by circulating pleomorphic malignant mature T lymphocytes. The adult T-cell leukemia (ATL) has a more aggressive course and appears to be clustered geographically in the Southwest of Japan, the Carribbean basin and in certain areas of the Southeastern United States. Recently, a unique human type C retrovirus, human T-cell leukemia/lymphoma virus (HTLV) has been isolated from the neoplastic T cells of patients with ATL. The immunoregulatory function and cell surface phenotype of leukemic cells has been compared in patients with the clinical diagnosis of ATL and from patients with the Sezary syndrome with circulating antibodies to HTLV, but with leukemic cells which are functionally and phenotypically like those Sezary patients who lack serum antibodies to HTLV. Leukemic cells of both groups were of the T3, T4+, T8- phenotype. However, HTLV negative Sezary leukemic cells frequently functioned as helper T cells whereas most HTLV+ ATL cells functioned as suppressors of immunoglobulin biosynthesis. These and other studies suggest that the cells defined by the T4 and T8 antibodies are complex populations that are not solely helper/inducer and suppressor/cytoxic populations respectively. Cell populations that react with T4 and T8 monoclonal antibodies differ not in terms of their function, but in terms of the class of the antigen with which the T cell interacts.

The monoclonal anti-Tac was able to differentiate cells from the patients with ATL and the HTLV+ Sezary patients from cells of the HTLV-Sezary patients. The Sezary T cell leukemic populations that were HTLV negative were Tac antigen negative, whereas all of the leukemic T-cell populations with antibodies to HTLV were Tac antigen positive. Thus the HTLV associated mature T-cell leukemia cells were Tac antigen positive, thus manifesting the receptor for T-cell growth factor. The infection of mature T cells with HTLV leads to the production of T-cell growth factor by some of these cell populations in culture and to the expression of TCGF receptors in all cases. This finding may be important in understanding the pathogenesis of the uncontrolled growth of these neoplastic cells. For those cells producing TCGF there may be a self stimulatory circuit in which the same cell produces and responds to this growth factor for T cells. HTLV infection may lead to a bypassing of the TCGF-TCGF receptor system causing altered growth by mechanisms that have not been defined. Alternatively, HTLV may directly alter the receptor so that it behaves as if TCGF were bound to it and thus directly stimulates the growth of the malignant T cell.

The response of human peripheral blood lymphocytes to either the Epstein-Barr virus or influenza viruses was studied to define the normal factors regulating immunoglobulin and antibody synthesis as well as cytotoxic systems in normal individuals, and to define the disorders that occur in patients with immunodeficiency disease that are associated with neoplasia.

EBV is a virus of the herpes group which infects B lymphocytes specifically and has been implicated in Burkitt's lymphoma, nasopharyngeal carcinoma and the polyclonal B-cell malignancies occuring in patients receiving immunosuppressive

drug therapy. Patients with a form of acquired immunodeficiency develop hypogammaglobulinemia following EBV infection. This propensity to develop immunodeficiency following EBV infection is inherited in an x-linked fashion. New sensitive techniques have been developed to determine the rate of spontaneous B-cell transformation in the human peripheral blood lymphocyte population as a mechanism to further characterize these cells. It was suggested that suppressor T cells activated in acute EBV infection, while capable of preventing previously uninfected B cells from being activated by EBV, are unable to control cell growth by B cells which acquired EBV infection before this suppressor mechanism became activated.

EBV is also associated with the common form of adult rheumatoid arthritis. When cultures of peripheral blood lymphocytes from normal EBV sero-positive donors are infected with EBV, initially the virus activates B cells to produce immunoglobulin which peaks at 8 to 10 days of culture and then rapidly falls as a consequence of the EBV immune suppressor T cells present in these patients. However, when lymphocytes from EBV sero-positive rheumatoid arthritis were stimulated with EBV in vitro, immunoglobulin production progressively increased, indicating a defect in EBV specific suppressor T cell activity. In other experiments, the antigen-specific human immune responses to hemophilus influenza antigens have been used to study the generation of immune cytotoxic T cells and the production of specific antibody production by B cells. Previous studies have demonstrated profound defects in the generation of "self"-MHC restricted cytotoxic T cells from chemical and viral antigens in patients with ataxia telangiectasia and the Wiskott-Aldrich syndrome. Both these immunodeficiency diseases are associated with recurrent infections and a high incidence of cancer. Recent studies examining alloimmune phenomenon and cytotoxic responses in these diseases confirm the existence of defects in immune cytotoxic T-cell production which are not limited to a few antigenic specifications. These defects may contribute to the recurrent infections and high incidence of cancer in these patients.

Peripheral blood mononuclear cells from normal individuals and patients with immunodeficiency disease were compared for their capacity to produce antigen specific antibody responses in vitro when stimulated with influenza virus antigens in the absence of any polyclonal activator. The data show that this antigen-induced antibody production by human B cells was highly antigen-specific, required de novo protein synthesis, and depended on the cooperative interaction of macrophages and a specific subset of T cells. B cells stimulated with whole virus produced antibody to viral hemagglutinin, the virion surface glycoprotein responsible for viral infectivity. These in vitro responses recapitulate the in vivo response to the same antigens, and implies that the B-cell repertoire for influenza viral antigens differs from the T-cell repetoire. The ability to study antigen-induced specific antibody production in vitro has furthered our understanding of the specific immunoregulatory defects involved in immunodeficiency diseases.

Studies on the production and characterization of SISS-B, an inhibitor of immunoglobulin synthesis, have continued. This lymphokine was produced by

mitogenic stimulation of polyclonal mononuclear cells or by continuous cultures of human T cells grown in T-cell growth factor. Recent work demonstrated that the SISS-B is produced by human T-T cell hybridomas, by cultured T-cell lines from patients with common variable hypogammaglobulinemia and excessive suppressor T-cell numbers, as well as by lines derived from the malignant T cells of patients with the HTLV associated adult T-cell leukemia.

Additional studies have provided evidence that cellular cooperation, recognition, and regulation may in part be mediated by endogenous lectins and complex carbohydrate receptors. By blocking various in vitro immunological reactions with simple sugars, the role of carbohydrate recognition has been studied. The purification of complex carbohydrates from pregnancy urine has been initiated in an effort to characterize more complex sugars with immunoregulatory capacity. Some of these purified complex sugars may represent specific receptors for endogenous lectins. Pregnancy urine was chosen since it contained immunoregulatory glycoproteins which might play an important role in maintenance of the fetal allograft. Utilizing a multistep purification procedure, two immunosuppressive sugar-containing materials have been found.

The mechanism of action of insulin-like growth factors, and the regulatory role of amino acids in controlling normal growth have been studied as they relate to our understanding of malignant growth. Past studies showed that MSA or rat insulin-like growth factor-II (IGF-II) may be a fetal growth factor. This was confirmed in recent studies which showed that rat embryo fibroblasts synthesize IGF-II, have cell surface receptors for IGF-II, and respond to IGF-II with increased DNA synthesis. Placental lactogen, which stimulates the production of IGF-II by rat embryo fibroblasts has extensive amino acid sequence homology with growth hormone and may regulate the producton of a fetal insulin-like growth factor. These studies on the rat model have been extended to human fetal fibroblasts. These investigations have revealed that there are two different size classes of IGFs as well as IGF binding protein produced by the human fetal fibroblasts.

The regulatory role of proline and its metabolite pyrroline-5-carboxylate (P5C) are being investigated further. The interconversions of proline and P5C constitute a metabolic cycle in which oxidizing potential in the form of P5C can be generated and transferred between cellular compartments as well as between different cells with asymmetric enzyme capacities. This implies that proline-P5C functions as a system for transferring metabolic information within cells and as a simple, but generalized "hormone system" between cells. This new hypothesis for a mechanism of cellular regulation may be important in defining pathogenetic mechanisms in a variety of human diseases including oncogenesis.

Laboratory of Pathology

The Laboratory of Pathology (Dr. Lance Liotta, Chief) is responsible for all the diagnostic services in Pathological Anatomy for the Clinical Center of the NIH. Approximately 5,666 surgical specimens or biopsies were assessioned in

the past year, and nearly 1,000 specimens of fresh human tissues were furnished to NIH scientists. Diagnostic services in cytology (both exfoliative and fine needle aspiration), medical genetics and in diagnostic electron microscopy are provided to the Clinical Center. In addition, the Laboratory provides all types of histological services and staining procedures for NCI scientists.

Experimental work in ultrastructural pathology has concentrated on investigating the disposition of the IgE Fc receptor in the basophil membrane, and the biological fate of cross-linked IgE (as in allergen binding). These studies have demonstrated that only cross-linked IgE with its associated receptors is internalized, followed by rapid lysosomal degradation and exocytosis. Unlike most other systems, no surface re-expression of either IgE or its receptor was found. Correlative morphological and biochemical studies on homo- and heterozygous cystinotics and normal controls have demonstrated that heterozygotes, though clinically normal, have a diminished capacity to lysosomally process cystine. Such impaired lysosomes are detectable in peripheral blood neutrophils. This may allow detection of suspected or unknown carriers. A long term study of matrix synthesis of Ewing's tumor has clearly established that this tumor is a sarcoma, since it synthesizes types I and III (stromal) collagens, but that it is also very primitive, since it also synthesizes type IV collagen, an "epithelial" collagen.

Structural studies on the carbohydrate chains of human haptoglobin 1-1 and hCG have been completed, and studies on oligosaccharides released from glycolipids of human and mouse cells are underway. The structure of a ganglioside that is the antigen recognized by a mouse antihuman colorectal carcinoma hybridoma has been determined. A hybridoma antibody useful as a reagent for radioimmunoassay of a urinary tetrasaccharide $(G)_4$ has been prepared and studies on urinary excretion of $(Glc)_4$ in patients with soft tissue sarcomas are in progress. Preliminary data suggests that elevated excretion of $(Glc)_4$ in patients with rhabdomyosarcoma, Ewing's sarcoma, ALL, and some other tumors might be useful indicators of tumor regression or recurrence during therapy.

A primary research interest within the Laboratory of Pathology is the study of the biochemical mechanisms which play a role in tumor cell invasion and formation of metastases. Two biochemical factors have been identified that are quantitatively enhanced in actively invading tumor cells: specific new types of proteases and a new type of matrix receptor. Antibodies against the protease which react with actively invading breast carcinoma cells may prove diagnostically useful. The matrix receptor can be readily measured in human breast carcinoma tissue. Attempts to correlate receptor content with clinical state of disease are in progress. Histologic studies have shown that basement membranes are disrupted at the point of tumor cell invasion.

Recent studies have shown that metastatic tumor cells of different histologic origins consistently elaborate a family of metalloproteinases which can degrade components of the basement membranes. One of the enzymes selectively degrades type IV collagen, which is found only in the basement membrane. A separate metalloprotease degrades type V collagen, which is a collagen found at the

basement membrane-stromal interface. Both of these enzymes have been purified and characterized. Classic mammalian collagenase failed to degrade collagens IV and V, under conditions where collagens I, II and III were totally degraded. This suggests that different classes of proteases degrade different types of collagen. A quantitative in vitro assay for studying the biochemical mechanism of tumor cell invasion has been developed which utilizes the human amnion membrane. Cells placed on one side invade through the full thickness of the amnion and are collected and counted on the other side. Findings to date suggest that tumor cell invasion does not absolutely require living host cells or tumor cell proliferation. Purified inhibitors of metalloproteinases inhibit tumor cell invasion of amnion in vitro, are not cytotoxic, and do not inhibit cell motility or chemotaxis. Chemotactic agents stimulate tumor cell invasion in this system.

Using purified monospecific antibodies to basement membrane components and antibodies to different collagen types, immunohistology studies of human breast cancer have been performed. All types of noninvasive breast neoplasms studied exhibit an intact extracellular basement membrane. However, all invasive and metastatic tumors studied exhibit markedly defective or absent extracellular membrane.

Cytoplasmic staining can be used to aid identification of micro-metastases of breast cancer in lymph nodes. The production of collagen in human breast carcinoma desmoplasia was also studied. It was shown that the source of the collagen in desmoplasia is the host, not the carcinoma cell. The host cells responsible for collagen and elastin synthesis are probably myofibroblasts. Human breast cancer desmoplasia may be a stromal host response to invading tumor cells.

In order to further study the interactions of tumor cells with laminin, a glycoprotein of basement membranes, the domain of the laminin molecule which attaches to the tumor cells has been identified and the specific cell membrane receptor for laminin has been characterized for the first time. Rotary shadowing electron microscopy has revealed the configuration of laminin and its molecular binding site. A fragment of the laminin molecule which binds to the receptor and blocks attachment will inhibit metastases formation in animal models.

The research program of the Laboratory of Pathology includes investigative studies aimed at understanding the mechanisms by which animal viruses interact with cells susceptible to lytic productive infection as well as the nonproductive malignant transformation of cells. Recent data on the Fc binding receptor induced by herpes simplex virus strongly suggests that the receptor for protein A IgG complexes can be discriminated from a receptor for aggregated IgG. Biochemical studies indicate that the receptor for IgG-protein A complexes present in the HSV-infected cell is a glycoprotein.

Studies have continued on human and bovine papillomavirus transformation. Investigators have shown that the BPV-1 genome remains exclusively extra-chromosomal in transformed mouse cells. The viral transcripts present in BPV-transformed cells as well as those present in productive fibropapillomas of cattle have been mapped. The sequence of the bovine papillomavirus has been completed and only one strand has open-reading frames. Two additional transcripts are present in productively-infected cells which are not present in the transformed cells. One of these messages appears to encode the major capsid protein, VP1. Additional studies have examined the effect of mouse cell interferon on BPV transformation of mouse cells and on the stability of the BPV plasmid in transformed mouse cells. It was found that mouse cell interferon markedly reduces the level of transformation by BPV on mouse cells. Continued treatment of established transformed lines with mouse L-cell interferon lead to an overall reduction in the number of plasmid BPV copy numbers in the cell lines under study.

A major diagnostic and experimental study of neoplastic and non-neoplastic lesions of the lymphoreticular and hematopoietic system was carried out. It was shown that immunologic phenotype cannot be predicted by morphologic criteria alone. The clinical importance of immunological phenotype has been evaluated in patients with diffuse, aggressive non-Hodgkin's lymphomas. This study demonstrated that regardless of immunotype, patients have a similar complete remission rate and survival with aggressive chemotherapy. Additional studies in progress include an analysis of the morphologic, immunologic, and clinical aspects of a subset of peripheral T-cell lymphomas. Most of these cases display a helper/inducer phenotype, but other T-cell associated antigens are inconsistently demonstrated. The transferrin receptor, OKT9, is variably expressed and appears to correlate with rapidity of growth of the malignant lymphoma. Previous studies demonstrated that hypercalcemia occurs in certain patients with peripheral T-cell lymphomas and that this hypercalcemia may be due to the production of osteoblast-activating factor (OAF) by the neoplastic cells. Additional studies have shown that both normal and neoplastic T cells can elaborate factors which induce phagocytic activity in a cell line of histiocytic origin. The factor produced by these cells can act independently of an effect of Fc receptors. A similar factor was identified in supernatants from T cells stimulated by concanavalin A, in which an increase in phagocytosis was seen independent of an effect on Fc receptors.

The expression of various antigens has been investigated in non-Hodgkin's lymphomas. It has been demonstrated that lymphoblastic lymphomas are heterogeneous and include cases of T-, pre-B, and pre-pre-B origin. The lymphoblastic lymphomas with a T-cell phenotype usually reflect different stages in late thymic differentiation in contrast with T-ALL, which normally corresponds to an earlier stage of T-cell differentiation. Unlike T-ALL, the helper and suppressor phenotypes are mutually exclusive, and the thymocyte antigen, OKT6, is present in less than half of the cases.

Additional studies have shown for the first time that immunoglobulin synthesis can be induced by TPA in acute lymphocytic leukemia with a common ALL phenotype. Although the cells have the immunoglobulin gene rearrangement of normal B cells, immunoglobulin synthesis either in the cytoplasm or on the surface normally is not identified. While TPA induced immunoglobulin secretion, TDT, a marker of lymphoblasts, decreased in these cells, further indicating induction of differentiation.

Other investigators have used the small family of genes which code for the three polypeptide chains of fibrinogen as a model for the study of developmentally and hormonally controlled gene expression. These studies focused on understanding the regulation of fibrinogen mRNA levels, as well as the molecular cloning and structure of the rat and human fibrinogen genes. cDNA genes have been obtained for each of the three chains of fibrinogen, and the amino acid sequence of each of these clones has been determined.

Computer aided 2-dimensional (2D) electrophoretic gel analysis (GELLAB), developed by Image Processing Section of the Laboratory of Pathology, continues to be a major tool in a large series of substantive biomedical research problems involving 2D gels. The two drawing algorithms for nucleic acid molecules developed in this Section have been enhanced. The polygonal algorithm now allows the searching for specific base patterns and the superposition of these base patterns onto the secondary structure to determine in what types of structures these patterns reside. Over 150 molecules have been drawn, and the algorithm can accommodate molecule sizes up to 1500 bases.

In order to analyze secondary structures, the predicted structure(s) of natural sequences have been compared with the same sequence which has been randomly shuffled. In sequences known to have high secondary structure it was found that the natural sequence has a more stable predicted structure and agrees better with the chemical data. This approach is being pursued on selected adenovirus sequences.

Immunology Branch

The research program of the Immunology Branch, (Dr. David Sachs, Chief), conducts studies of the regulation and control of immune responses, structure and function of cell surface molecules, transplantation biology, molecular biology, and tumor immunology. In addition, the Immunology Branch maintains a fluorescense activated cell sorter facility which is involved integrally in many of the studies carried out in the Branch and in a large number of collaborative investigations.

A major research effort has been directed toward studies of the generation and regulation of T-cell dependent responses to both conventional and alloantigens, and at the mechanisms of interaction among T cells, B cells and accessory cell populations. The regulation of cytotoxic T-lymphocyte responses to alloantigens and modified self antigens has been studied as a model of allograft tolerance and regulation of T-cell mediated immunity. Recent work has shown that

T-cell dependent antibody responses are regulated by two independent T suppressor (T_S)cell pathways. Antigen-specific and major histocompatibility (MHC) restricted T_S cells suppress only those responses generated by T helper (T_H) cells expressing the same antigen specificity and MHC restriction specificity. These cloned T_S cells are now being characterized.

In another series of experiments, the effect of immune response (Ir) genes on the interaction of T cells with B cells and/or accessory cells was shown to depend upon the pathway of B cell activation functioning under given response conditions. This suggests that ability to circumvent Ir gene restrictions may depend upon the ability to select specific pathways for immune response activation.

Idiotypic determinants on anti-Staphylococcal nuclease antibodies have been used as markers for investigations of genetic control of the immune response to nuclease. Several hybridomas reactive with nuclease and/or anti-idiotype were produced. Recent studies using this in vitro system suggest that T_H cells bear idiotype on their surface.

Further investigations have concentrated on identifying the cell surface elements which regulate cellular collaborations. Recent data suggest that the activation of B cells can involve a genetically regulated interaction with macrophages. Elucidation of the roles performed by macrophages in the activation of allospecific cytotoxic T lymphocytes (CTL) has demonstrated that there are two distinct T-cell activation pathways mediated by macrophages: an Ia dependent activation pathway which involves self-recognition of macrophage Ia determinants, and an Ia independent activation pathway which involves recognition by macrophage K/D determinants.

Of increasing research interest has been the immunosuppressive potential of chronic graft-versus-host (GVH) reactions in mice. These studies indicate that allogeneic class II major histocompatibility antigens alone can initiate immunosuppression in vivo; and that such suppression can be prevented by treating donor or recipient mice with anti-H-2 monoclonal antibodies. These observations may be important in the etiology of acquired immune deficiency syndrome (AIDS). In a prospective study, a number of immune response profiles of healthy homosexual men, at high risk for AIDS, are being followed. Approximately 20 percent of donors repeatedly exhibit reduced cytotoxic T cell and interferon responses to influenza virus in vitro. These findings may have important prognostic and/or diagnostic clinical value.

The work of other investigators has focused on regulation of the immune response by the idiotype network. Recent findings indicate that anti-idiotype antibodies will trigger T-helper lymphocytes to secrete helper lymphokines and B cells to secrete antibody. These studies provide a mechanism for perpetuation of humoral immunity and a theoretical model for obtaining immunity in the absence of antigen. Investigations of the role of cell surface molecules in the triggering and regulation of immunocompetent cells have been pursued. Current studies suggest that monoclonal anti-Fc gamma receptor antibodies trigger B cells to secrete antibody.

A major research thrust has been the study of the structure and function of products of the major histocompatibility complex (MHC). A large number of hybridoma cell lines producing antibodies to H-2 and Ia antigens have been characterized. Anti-idiotypic antibodies against these hybridomas have been produced and the effects of such anti-idiotypic reagents on in vitro and in vivo parameters of histocompatibility have been examined. Mechanisms of tolerance in bone marrow chimeric animals have also been investigated. Evidence suggests suppression is due to clonal deletion rather than active suppression. A series of investigations directed toward dissecting the recognition structures of alloreactive T cells suggests that either these recognition structures are substantially different or the allodeterminants recognized by these cells are not the same.

Transplantation biology studies using the miniature swine model have continued. Milligram quantities of histocompatibility antigens have been prepared, and N-terminal amino acid sequences have been obtained for the SLA antigens of all three partially inbred strains. Two new recombinants within the MHC were detected, both of which separate the MLC stimulatory locus (SLA-D) from the serologic loci (SLA-ABC). Transplantation studies using these recombinants indicate that selective matching for class II antigens frequently permits long term kidney graft survival across class I differences.

The alloreactive repertoire has been examined for responses to cell surface antigens, including $\underline{K}^b$ mutant-encoded class I antigens and the non-MHC-linked mls antigens. Recent findings indicate that the T-cell repertoire for recognizing $\underline{K}^b$ mutant products depends upon the interaction of responder genotype and T-cell maturation environment. These results may have practical implications for immune competence in clinical bone marrow transplantation.

Work continues on the induction of T-cell tolerance to self components. Experiments have demonstrated that immature T-cell precursors are tolerized to MHC antigens pre-thymically, but are first tolerized to non-H-2 encoded mls antigen intra-thymically. Other studies have shown that the induction of T-cell tolerance to self antigens involves recognition of self H-2 determinants. These findings are among the first demonstration that MHC genes not only regulate T-cell activation but also regulate the induction of self-tolerance. Rapid progress has been made on defining the genetics, structure and function of the new HLA gene, HLA-SB_1 and its products. This locus is proving to be important in regulation of human immune responses and association with disease states.

The Immunology Branch also conducts molecular biology studies of the structure, genomic organization and regulation of expression of porcine MHC genes. Previous work established that the structure of a porcine class I gene is homologous to those of mouse and human, although sequences flanking the gene are not. These flanking sequences appear to be MHC-specific, which may have implications concerning the evolution of this multigene family. The role of MHC-linked sequences in regulating gene expression is being studied and the mechanisms leading to differential expression of MHC genes in vivo are also under investigation.

Studies in tumor immunology have focused on understanding the mechanisms in mice which generate cell-mediated cytotoxic reactions against chemically-induced, syngeneic tumor cells. One T-cell mediated mechanism is generated by a response which differs from other cytotoxic T-cell responses, presenting opportunities for new possibilities for manipulating host anti-tumor activity.

Ultrastructural studies of lytic conjugates between human NK cells and their targets have indicated that the contents of the characteristic cytoplasmic granules of these lymphocytes undergo morphologic changes and are secreted during the cytotoxic activity of these effectors. This suggests that the granule contents might contain the product(s) that mediate cytotoxic function. The isolated granules have been found to be highly cytotoxic by a strictly calcium dependent mechanism.

Laboratory of Tumor Immunology and Biology

The Laboratory of Tumor Immunology and Biology (Dr. Jeffrey Schlom, Chief), conducts research to identify immunologic markers specifically associated with various carcinomas, with the ultimate aim of employing these in the diagnosis, prognosis and treatment of human cancer. The Laboratory is also developing immunoassays to aid in the characterization of human carcinoma cell populations, and in the diagnosis or prognosis of human carcinomas. A major focus of the Laboratory involves investigation of the association between specific genetic elements and tumorigenesis. Research is also conducted on those factors involved in the differentiation and antigenic modulation of human tumor populations.

A primary research interest is the generation and characterization of monoclonal antibodies reactive with mammalian carcinoma cells. Thirteen monoclonal antibodies reactive with human mammary carcinoma cells, two of which react with carcinoembryonic antigen have been developed. Some of the monoclonals reacted with the surface of some nonbreast carcinomas, but none reacted with the cell surface of non-epithelial cancers or normal cell lines tested.

Monoclonal antibodies generated to human mammary tumor metastases were tested for reactivity to novel and known tumor associated antigens. Studies are in progress to develop radioimmunoassays with several of the monoclonals.

Antigenic variation was observed in the expression of specific tumor associated antigens within individual human mammary tumor masses using monoclonal antibodies. This diversity was observed in human mammary tumor cell lines grown in vivo and in vitro. The expression of some tumor associated antigens is related to S-phase of the cell cycle. Membrane expression of the reactive antigens appeared to be stable, although antigenic drift was observed with continued passage of mammary tumor cell lines. The relationship of antigenic phenotype to specific biologic parameters is currently being investigated. Studies are in progress involving the localization of human mammary tumors using radiolabeled monoclonal antibodies. Several monoclonal antibodies are now being tested for possible clinical application.

Lymphocytes from lymph nodes obtained at mastectomy from breast cancer patients have been fused with murine and human myeloma cells to obtain human-mouse and human-human hybridoma cultures that synthesize human monoclonal antibodies. The immunologic reactivities of the human Ig's were assayed. One human IgM monoclonal antibody from a human-mouse fusion was used to discriminate between mammary carcinoma cells, and normal mammary epithelial cells, stroma, or lymphocytes of the same breast. This same antibody reacted with selected non-breast carcinomas and metastatic mammary carcinoma cells in lymph nodes and at distal sites. Several human-human hybridoma cultures are currently being assayed for immunoreactivity to human breast carcinomas.

The role and mechanisms by which hormones, growth factors (particularly epidermal growth factor (EGF)), EGF related transforming growth factors (TGF's), and tumor-promoting phorbol esters regulate cellular proliferation, the production of various extracellular matrix proteins and the expression of specific tumor-associated cell surface antigens have also been studied. It has been demonstrated that EGF and/or tumor promoters (TPA) stimulate an increase in the production of basement membrane collagen and laminin. This response is preceded by an increase in the activity of a phospholipid-dependent protein kinase and by the appearance of newly phosphorylated, soluble proteins. Experiments are in progress to identify these phosphoproteins and to ascertain their role in modulating the production of these matrix proteins. TGF's are peptides produced by a variety of rodent and human neoplasms which reversibly confer upon normal cells several properties associated with the transformed phenotype. TGF's have been isolated from several human breast tumor samples. Experiments are in progress to purify this activity and to generate monoclonals against it.

A continuing research interest is the role of infectious mouse mammary tumor virus (MMTV) and genetically transmitted MMTV proviral DNA in the etiology of mammary neoplasia. Several recombinant clones of MMTV related human cellular DNA have been isolated. The major region of homology was found at the 3' end of the MMTV pol gene. Previous studies suggested that the MMTV and mammalian type C proviral genomes do not hybridize, however, low stringency hybridization conditions permit stable hybrids to form between the pol gene sequences of infectious type A, Type B (MMTV), and type D (squirrel monkey retrovirus, SMRV). Similarly, the type A and D pol gene sequences also hybridize with the MMTV pol related human DNA sequences, and the SMRV LTR and gag gene hybridize to the human recombinant clones. The organization of the retroviral related sequences in five independent human recombinant clones has been determined. Variations in the restriction sites present in each of the human recombinant clones suggest that they represent a family of related but nonidentical retroviral genomes which are located on four chromosomes. In earlier studies, pedigreed breeding colonies of feral mice were identified which lack MMTV proviral DNA in their germ line. Experiments are in progress to assess the role of genetically transmitted MMTV proviral genomes in mammary neoplasia.

The ability of oncogenes from various sources to retransform revertant cell lines has been studied. The revertants were almost completely resistant to

retransformation by the Ki-MuSV oncogene, and to some, but not all other oncogenes. These studies imply that the revertant cells have undergone genetic alterations which render them resistant to transformation by certain oncogenes and not others. The basis for this type of resistance may involve functional similarities and differences among various transforming agents. Initial findings have indicated that changes in ion transport might be associated with cell transformation. Transfection studies designed to show whether or not ouabain resistance is causally related to the suppression of transformation in the revertants are in progress. The role of hormones and cyclic nucleotides in mediating cell growth and differentiation is being investigated.

Hypusine formation is virtually absent in quiescent lymphocytes and other growth-arrested cells, but rises in proportion to the rate of protein synthesis when these cells are activated. Recent studies suggest that hypusine formation modulates the activity of eIF-4D as an initiation factor, thereby contributing to the control of protein synthesis and cell growth. Hypusine formation is the only specific biochemical reaction yet described for polyamines in intact cells which can be related to a particular biochemical function.

The effects of the tumor promoting phorbol ester (PMA) on HL-60 promyelocytic leukemia cells, human lymphocytes, and several other cell lines has been studied. In HL-60 cells, PMA causes a cessation of cell growth and terminal differentiation into monocytes, accompanied by the synthesis of a characteristic set of proteins which provide a marker for monocytic differentiation. During the initial response of HL-60 to PMA, there is a rapid increase in phosphorylation of a 17 kd cytosolic protein (pp17), which does not occur with phorbol ester analogues lacking tumor promoting activity. Data indicate that induction of phosphorylation of pp17 is a general initial consequence of the action of PMA on cells, independent of the final effects on growth and differentiation. Studies are in progress to characterize the protein kinase involved in this phosphorylation.

The synthesis and turnover of HLA-A,B,C molecules in human peripheral lymphocytes has been studied. These proteins are among the most rapidly turned over of all cell proteins. This is undoubtedly related to the function of the HLA proteins in the immunological activity of the peripheral T lymphocyte. Since immunological surveillance for alteration of cell surface configurations due to viral infection or, possibly, neoplastic changes, is a major activity of T lymphocytes, rapid turnover of HLA molecules may be a biochemical concomitant of this process.

Laboratory of Immunobiology

The research program of the Laboratory of Immunobiology (Dr. Tibor Borsos, Acting Chief) is directed towards understanding the mechanisms of the effector arm of the immune system. The primary focus has been the study of cytotoxic mechanisms of humoral immune factors and the analysis of the behavior of cells under immune attack.

Past studies demonstrated that the acquisition of the complement fixing property by immunoglobulin molecules (Igs) at cell surfaces is a function of density and distribution of antigen epitopes on the cell. A minimal number of epitopes must be present to bind sufficient numbers of Igs for C fixation to occur and recent results have demonstrated that C_1 activation is directly dependent on density. These results clearly differentiated between binding (fixation) and activation of C1 by Igs. It was hypothesized that activating properties of Igs were governed by the angles of the Fab arms as determined by the average distance between epitope molecules. Activation of C1, however, was not always followed by cytotoxic activity. Analysis of the blockage in the sequence leading to cytolysis suggests that the gamma chain is important in the generation C3 convertase, the key enzyme in the pathway of C activation. Studies on the structural, biochemical and immunological properties of C4 and C2 are in progress to eludicate the molecular basis of generating C3 convertase.

The antibody dependent C-mediated killing of in vivo grown guinea pig hepatoma cells has been studied to better understand the mechanism of sensitivity of nucleated cells to immune attack. It was demonstrated that cellular lipid and/or fatty acid composition play a major role in influencing the sensitivity of the cells to C killing. When cultured human (Raji) and mouse (P815) cells were studied it was found that cells removed at different stages of asynchronous cell growth in vitro varied in their sensitivity to C killing. However, unlike the hepatoma cells no correlation between sensitivity to C attack and the synthesis and composition of cellular lipids was observed. These studies have been expanded to include sensitivity to cellular mechanisms of immune attack. Preliminary experiments with P815 cells have indicated these cells vary in their sensitivity to cytotoxic T lymphocyte (CTL) attack but are resistant to natural killer cells (NK). The P815 cells were resistant to CTL in their stationary phase of growth. This resistance of tumor cells to immune attack is not restricted to humoral immune mechanisms.

The Laboratory of Immunobiology has continued to examine the factors that activate mouse peritoneal macrophages for tumor cytotoxicity. The prototype material is found in antigen or mitogen stimulated murine spleen cell culture supernatants, and also in the culture fluids from a phorbol myristate acetate stimulted El-4 thymoma cell line. By four functional criteria, activity from the two sources was found to be comparable. Physicochemical studies are underway to further characterize this spleen cell activity.

It was shown previously that human blood monocytes comprise two subpopulations, only one of which migrates to the chemoattractant, fMet-Leu-Phe (FMLP) and has saturable binding sites for this peptide. Studies were initiated to determine if expression of binding sites was a function of monocyte maturation. The results suggest that there are two distinct lineages of monocytes with respect to expression of receptors for FMLP.

Most naturally arising rodent tumors are nonimmunogenic. Investigators in the Laboratory of Immunobiology have evaluated three methods for ability to alter

host response to nonimmunogenic tumors: selection of nontumorigenic variants after chemical mutagen treatment; alteration of tumor cell membrane fluidity with cholesterol esters; and super-infection of tumor cells with murine retroviruses. Immunogenic tumors, syngeneic to strain 2 guinea pigs, were selected for study. These tumors might provide a rigorous test of the concept that introduction of new antigenic determinants into tumor cell membranes would produce immunological recognition of occult tumor antigens.

Investigations were initiated to determine whether it was possible to produce, in vitro, tumor cell variants with potent transplantation protection antigens and whether immunization with such variants provides protection against challenge with the parent tumor. Tumor cell populations were grown in vitro, treated with chemical mutagen and then variants were cloned and evaluated for growth. One cloned line (M 48) grew and regressed in conventional animals but grew progressively and metastasized in immunosuppressed "B" guinea pigs, suggesting that this clone had acquired a potent transplantation protection antigen(s). Alteration in tumor cell membrane fluidity by treatment with cholesterol esters was shown to be associated with increased immunogenicity.

The basis of rejection of MuLV-infected guinea pig fibrosarcoma cells has been analyzed. Evidence indicates that the host response was directed to viral antigens and required an intact T-cell system. MuLV-infected tumor cells grew and regresssed in conventional animals but grew progressively in "B" guinea pigs. Occasionally, tumors recurred in conventional animals at injection sites. Tumor recurrences invariably were composed of cell populations which neither released infectious virus nor expressed virus antigen. Clonal analysis of the parent cell population indicated marked heterogeneity of virus experession with rare clones expressing neither infectious virus nor viral antigen. The results suggest that virus antigen positive cells are selected and rejected by a T-cell dependent mechanism, which, in a hyperimmunized animal, spares virus negative cells.

Human mammary carcinomas have been reported to regress after infusion of autologous plasma adsorbed with protein A. A small animal model has been developed to facilitate evaluation of this antitumor therapy. Buffalo/N rats were given a single injection of NMU. When animals developed a single 5 to 10 mm mammary they were entered into the treatment protocol. Animals received four intravenous injections of plasma. Antitumor effects occurred with transfer of plasmas from tumor bearing rats after adsorption with Sepharose, CNBr Sepharose, or Sepharose Protein A.

Laboratory of Cell Biology

The research program of the Laboratory of Cell Biology (Dr. Lloyd Law, Chief) is focused on the biological and immunogenic characterization of tumor specific rejection antigens (TATA). Antigens from chemically induced sarcomas such as Meth A and CI-4 have been purified and characterized. These two antigens differ in their chromatographic behavior, isoelectric points, and amino acid

compositions. Recently, two additional 75 kd proteins have been isolated and purified. Like Meth A and CI-4, these proteins are immunogenic in the 5-10 μg range. Sarcoma mKSA has its own group specific nuclear antigen (T antigen) but the 75 kd protein purified from mKSA cells is a unique TATA and is not related to T antigen. Data suggest that the 75 kd protein is a normal cell constituent which has been activated in mKSA by malignant transformation by SV40. Further studies suggest that the 75 kd proteins represent a family of closely related proteins that are present in all neoplasms. Through analysis of somatic cell hybrids of Meth A microcells and Chinese hamster E36 cells, it has been shown that the gene for Meth A TATA is located on the distal region of chromosome 12, the region coding for immunoglobulin (IG) heavy chains. No evidence for linkage of the Meth A coding gene and the H-2 complex was observed. Unlike the TATAs of chemically induced and spontaneous neoplasms, the TATA of RBL-5, a T-cell leukemia, is group specific, crossreacting in all FMB leukemias but not with other leukemias. This TATA was purified. It has a MW of 75 kd, but is a glycoprotein. As with Meth A, CI-4 and CII-7 proteins, this glycoprotein is being sequenced with a view towards preparing synthetic probes that will allow isolation of the determinant encoding this protein.

Other investigations are directed towards understanding the regulation of T-cell mediated immunity. The mechanisms for tumor cell induced suppressions, the development of tumor immunity in tumor bearing hosts, and prostaglandin regulation of immune responses have been studied. The immune responses to a series of 15 and 16 residue cytochrome C synthetic peptides have been studied. T-cell memory was found to be controlled by residue 11 (Lys) and by at least one residue between position 7 and 10. The specificity of antigen-Ia interaction was controlled by insertion or deletion of the penultimate residue (Ala). These results suggest that the presenting cell and the T cell independently recognize their respective sites, and that interactions between antigen and Ia control responses of T-cell clones.

Investigators in this Laboratory have isolated a rat hepatoma leukemia-helper virus (RHHV) and a wild rat leukemia-helper virus (WR-RaLV). Analyses of the genomic complexity of these endogenous viruses have continued. The aim of this research is to understand the significance and function of "helper sequences," especially when recombined with C-src sequences in the genome of a transforming rat virus. These viral DNAs have been successfully cloned and cultured in E. coli.

Mouse trophoblast cell lines established in this laboratory have been shown to have the characteristics of trophectoderm. Unlike more differentiated cells, these trophoblast cells are resistant to the growth of wild type polyoma virus. However, several mutant viruses which can overcome this restriction have been isolated and found to have genetic changes within a regulatory region of the polyoma virus genome. Mutants growing in embryonal sarcoma cells (EC) differ in both phenotype and genome from mutants grown in trophoblast cells. Nucleotide sequences are being determined upon a series of virus mutants. Preliminary data suggest that the deletions in the regulatory region of the viral genome may be important in determining the phenotype of these mutants.

Macromolecular Biology Section

The goal of research of the Macromolecular Biology Section (Dr. Peter Mora, Chief) is to better understand the role of cell surface changes in mammalian cells in the regulation of cell growth, by studying the molecules which are expressed on the cell surface and also interact directly with cellular DNA. The SV40 T antigen and the p53 cellular protein are the only two known macromolecules in mammalian systems which fulfill these requirements. The SV40 early gene coded T antigen is expressed on the surface of SV40 transformed mouse cells and can cause immune recognition and rejection in the mouse. The p53 is a phosphoprotein of approximately 53,000 MW which forms a complex with the T antigen in most SV40 transformed cells. It has been isolated from numerous cells not transformed by SV40. The SV40 p53, and the various embryogenesis related stage dependent p53 molecules are the same protein within the same mammalian species. The complexing of p53 with the SV40 T antigen in SV40 transformed cells, and also the stability of the non-complexed p53 in embryonal carcinoma cells relate to differences in phosphorylation of the p53. Various p53 molecules have associated phosphokinase activity. Studies are underway to fully characterize p53 and to define how changes in this molecule may relate to its role in embryogenesis and cell division in both normal and malignant cells.

Summary Statement
Laboratory of Molecular Biology
DCBD, NCI
October 1, 1982 through September 30, 1983

Research in the Laboratory of Molecular Biology focuses on understanding the factors that control gene expression in animal cells and bacterial cells. This information is used to define the biochemical basis for the abnormal growth and behavior of cancer cells. In addition, there is a strong emphasis on investigating the role of the plasma membrane in receiving signals from hormones, growth factors, the extracellular matrix, and other cells and on determining how these signals are transmitted to the genetic apparatus.

Gene Activity and Malignant Transformation

Pursuing last year's observation that the phosphorylation of vinculin, a protein important for maintenance of cell shape and adhesion, by the tyrosine specific src kinase is stimulated by anionic phospholipids, S. Ito has directly studied the interaction of vinculin with phospholipids. He has shown that vinculin binds to anionic phospholipids and appears to undergo a structural change that makes it a better substrate for the src kinase. Thus one way anionic phospholipids stimulate vinculin phosphorylation is by interacting with vinculin itself. Whether these phospholipids also interact with the src kinase itself is currently under study. Evidence has also been obtained by C. Roth that cAMP treatment of RSV-transformed cells stimulates src kinase activity.

B. de Crombrugghe and coworkers have continued their studies on the regulation of collagen gene activity by transformation by Rous sarcoma virus. Y. Yamada and M. Mudryj have extended their previous analysis on the structure of the collagen genes by comparing the structure of two different collagen genes. They found that the size distribution of the exons that encode the helical portion of collagen has remained unchanged in these two genes although the sequences in these exons as well as the size and sequences of introns have changed. Their data show that many recombinational rearrangements that were responsible for the assembly of the ancestral collagen genes were no longer tolerated after the two genes diverged from a common ancestor.

Regulation of the expression of the $\alpha 2(I)$ collagen gene was examined by several different approaches. C. McKeon's studies on the methylation of this gene and on its nuclease sensitivity in the chromatin of different tissues suggest that there are three levels of control in the expression program of this gene. A. Schmidt has established mouse cell lines in which the cloned chick $\alpha 2(I)$ collagen promoter is stably integrated in the genome of these cells and has found that this cloned promoter now responds to the same regulation by the products of oncogenes as the endogenous $\alpha 2(I)$ collagen promoter.

Because defined segments of several viral promoters, called enhancing sequences, are essential elements for the activity of these promoters and might be present in every promoter, they have begun to study their function and have obtained evidence that suggests that enhancing sequences are entry sites for RNA polymerase.

B. Howard and his coworkers have concentrated on the development and application of gene transfer techniques to identify genes that control mammalian cell growth. By constructing improved mammalian vectors and optimizing conditions for DNA-mediated gene transfer, they have dramatically increased the efficiency with which foreign genes can be introduced and stably expressed in multiple primate cell types, including human embryo fibroblasts, HeLa cells and monkey kidney CV-1 cells. They have further applied these techniques to obtain preliminary evidence for DNA sequences from human embryo fibroblasts that inhibit HeLa cell replication.

G. Smith has developed a model system for human mammary neoplasia based on the inbred C3H/Sm mouse. This strain, because it has a low occurrence of mammary tumors and is free of fully expressed endogenous mouse mammry tumor virus (MMTV) genomes, may have greater relevance to human neoplasia than other mouse mammary tumor models. G. Smith and his coworkers hope to elucidate how virus, chemical and hormonal stimulation act on developmentally controlled murine genes and defective MMTV provirus genes carried on C3H/Sm chromosomal DNA. They have demonstrated increased levels of a unique new mRNA transcript homologous to the long terminal repeat of MMTV in both spontaneous and experimentally-induced C3H/Sm mammary tumors.

Recombinant DNA clones spanning the entire fibronectin gene of chick embryo fibroblasts were isolated and partially characterized by H. Hirano. Using clones of these genes, J. Tyagi has investigated fibronectin mRNA synthesis in nuclei isolated from normal and RSV-transformed cells and found that the rate of fibronectin synthesis is markedly decreased by RSV transformation.

K. Yamada and coworkers have examined the structure and function of the transformation-sensitive, adhesive glycoprotein fibronectin. Its functional organization was determined by mapping domains for binding to the plasma membrane, collagen, heparin, fibrin, actin and DNA. Carbohydrate residues in the collagen-binding domain were found to be necessary for protection of this domain against proteases, which attack a disulfide-linked loop in non-glycosylated fibronectin. Fibronectin may interact with gangliosides on the cell surface, since its interactions with a ganglioside-deficient mutant were defective and were restored by the addition of exogenous gangliosides. Fibronectin was compared to the epithelial adhesive protein laminin, and it was found to share some, but not most, of a series of biological activities. In addition, new transformation-sensitive proteins besides fibronectin were identified using temperature-sensitive tumor viruses.

M.M. Gottesman and coworkers have continued studies on a lysosomal protein (major excreted protein [MEP]) whose synthesis and secretion are stimulated by malignant transformation, tumor promoters and growth factors. A cDNA clone coding for MEP has been isolated and used as a probe to demonstrate increased MEP mRNA levels in transformed and tumor promoter treated cells.

G. Johnson has developed evidence that ADP-ribosylation of non-histone chromosomal proteins might negatively regulated MMTV expression and that glucocorticoids decrease such levels of ADP-ribosylation.

Membrane Molecular Biology

Many growth factors, hormones and viruses enter cells. M. Willingham and I. Pastan have identified a new organelle, the receptosome, that carries ligands from clathrin-coatd pits on the surface fibroblasts to the cell interior. This organelle moves by saltatory motion carrying ligands such as epidermal growth factor (EGF), α_2-macroglobulin, insulin, low density lipoprotein, β-galactosidase and many viruses. Using a careful homogenization method, R. Dickson, L. Beguinot and J. Hanover have developed a method to purify receptosomes which involves four steps. They have characterized the purified receptosomes and shown they are rich in cholesterol, have a unique polypeptide pattern when analyzed by SDS gel electrophoresis, are enriched in transferrin receptors and also contain phosphomannosyl receptors. They do not contain detectable amounts of clathrin. One ligand internalized in receptosomes is α_2-macroglobulin. J. Hanover has now developed a purification method for the α_2-macroglobulin receptor and has identified it as a dimer with each subunit having a molecular weight of approximately 85,000. N. Richert has prepared a monoclonal antibody to the epidermal growth factor receptor of A431 cells and shown in collaboration with V. Ginsberg, NIAMDD, that the antibody recognizes a carbohydrate determinant on the EGF receptor which is the human H Type I determinant also found in glycolipids.

Using Pseudomonas exotoxin (PE) prepared by S. Leppla, a method has been developed to modify the toxin so that it no longer binds to cellular receptors but is fully enzymatically active. This modified toxin has been coupled to hormones such as EGF and antibodies such as that to the human transferrin receptor. Both of these conjugates are specifically cytotoxic for cells with the appropriate recognition markers. The conjugates are about equally potent to conjugates with ricin A chain. When these toxins enter cells they do so through coated pits and receptosomes, a pathway used by many ligands including adenovirus. D. FitzGerald has found that adenovirus enhances the toxicity of these conjugates by up to 10,000-fold. It does this by disrupting the receptosome that contains the virus and toxin giving the toxin free access to the cytosol. Enhancement by adenovirus has been observed with PE conjugates of EGF and PE conjugates with monoclonal antibodies for the human transferrin receptor. These studies have important implications for cancer chemotherapy where monoclonal antibody toxin conjugates are being widely investigated.

S.-y. Cheng has been investigating the receptors for thyroid hormones on cells. Affinity labeling techniques were employed to identify and characterize the plasma membrane receptors for 3,3',5-triiodo-L-thyronine (T_3) in cultured Swiss 3T3-4 mouse fibroblasts, GH_3 rat pituitary tumor cells and human epithelioid carcinoma A431 cells. A major specifically labeled protein with an apparent molecular mass of 55 kDalton (kDal) was detected in three cell lines. One-dimensional peptide mapping showed there are structural similarities in the 55-kDal protein from three different species. Thus, the plasma membrane T_3 receptors are highly conserved. Using ^{125}I-labeled L-thyroxine([^{125}I]T_4), the binding and uptake of T_4 in cultured GH_3 cells and Swiss 3T3-4 cells were shown to be saturable and specific. These results showed that the uptake of T_4 is receptor-mediated. Using affinity labeling and peptide mapping techniques, plasma membrane T_3 and T_4 receptors were shown to have structural similarities. The results from equilibrium binding studies indicate that one plasma membrane thyroid hormone receptor mediates the uptake of both T_3 and T_4 into cells. Using electron spin resonance (ESR), the dynamic interactions of thyroid hor-

mones with liposomes derived from L-α-dimyristoyl-phosphatidylcholine (DMPC) and plasma membranes of GH_3 cells were studied. The results suggest that $SL-T_3$ binds to a protein component.

M. Willingham has continued his study on the localization of transforming proteins in cells using electron microscopic immunocytochemistry. He has extended his previous studies on Harvey MSV p21^{src} to human epithelial cultured cell lines, both normal and transformed, and has shown that a cross-reactive p21-like protein is present in normal and malignant human cells using a monoclonal antibody against Harvey MSV p21^{src}. These studies together with those in the literature suggest the existence of a family of normal p21 proteins in most cells; some of these probably induce malignancy when introduced by certain retroviruses that carry selective mutations of these genes. M. Willingham has further developed immunocytochemical methodology and described a new method referred to as the "GBS" (glutaraldehyde-borohydride-saponin) method which is useful for determining the intracellular location of some proteins. Using such methods he has identified the intracellular location of calmodulin in cultured cells and has shown, contrary to previous studies by others, that there is little calmodulin concentrated in microfilament structures.

With J. Wehland he has performed microinjection studies of a monoclonal antibody to α-tubulin in living cells and shown the antibody reacts with the surface of microtubules, interferes with microtubule functions such as the formation of mitotic spindles and the saltatory motion of lysosomes. I. Abraham and M.M. Gottesman have demonstrated that CHO cells with mutant α- and β-tubulins also have abnormal mitotic spindles.

Using morphologic methods, M. Willingham has demonstrated that colloid gold coupled EGF enter cells through coated pits and receptosomes, but when adenovirus is also present, the colloid gold EGF conjugate is released into the cytosol. Thus adenovirus can be used to selectively introduce molecules into the cytosol that enter the cell through the normal internalization pathway.

S. Akiyama and M.M. Gottesman have begun to develop human cell lines that are simultaneously resistant to adriamycin, vincristine, vinblastine, puromycin and actinomycin-D. The mechanism of drug resistance is probably due to changes in the cell membrane. The biochemical defects in these human cell lines are now under analysis.

S. Wollman has continued his studies on thyroid growth and differentiation. He has found that when thyroid follicles are embedded in collagen gels, epithelial cells migrate out from the follicles. Ultimately the separate follicles approach each other and form aggregates. C. Garbi has continued his studies on the inversion process of thyroid follicles in suspension culture.

Gene Regulation in Prokaryotes

Because cancer cells are characterized by deranged control of synthesis of some proteins and of cell division, frequently a result of viral take-over of specific host machinery, the Laboratory of Molecular Biology also conducts basic research to understand the control mechanisms of protein synthesis, cell division and host-virus interactions using prokaryotic cells as model systems.

S. Adhya and coworkers conduct a program to study the genetic regulatory mechanisms in prokaryotic cells. This is followed at two levels. The first involves identification of the DNA elements and regulatory proteins that modulate the expression of a gene at the level of transcription and translation. In the other, the nature of the protein-protein and DNA-protein interactions between these regulatory elements that bring about the individual regulatory steps are being studied with purified proteins. The gal operon of E. coli is used as one of the model systems. The two gal promoters, which are controlled by cyclic AMP in opposite ways, are negatively regulated by a gal repressor protein. The repressor gene has been cloned into a high expression vector and the protein purified. Identification of the repressor binding sites has revealed a two-element gal operator--one located upstream to the promoters and the other inside the first structural gene. This discovery immediately suggested a new mechanism of gene regulation. Repressor bound at two distal sites may inactivate the activities of the promoters located in between by changing DNA conformations. This would be the first example of a local alteration in DNA structure caused by a protein bound at a different site.

Besides the gal operon, the genes for the following proteins have also been cloned and their regulation studied: adenylate cyclase (cya), cyclic AMP receptor protein (crp), NusA (nusA) and Rho (rho). S. Garges has found that the cya, crp and rho genes are autogeneously regulated at the level of transcription. There is also evidence to suggest that cyclic AMP may modulate the translation of the rho gene. These are now being studied in vitro.

M.E. Gottesman and coworkers have continued their study on the nature of transcription termination in E. coli. Lu Deru has examined the ability of E. coli phage P1 to stimulate the precise excision of transposons. The responsible P1 gene has been subcloned. Currently Shen Dong Lu is attempting to clarify the mechanism of the reaction. M.E. Gottesman is collaborating with D. Court in the construction of a vector system in which DNA fragments are cloned adjacent to a promoter active in E. coli. This will permit rapid assessment of coding gene activity. M.E. Gottesman in collaboration with B. Howard is developing a shuttle vector system that is tailored for the cloning of large and/or unstable DNA fragments as well as for the reconstruction of genes from overlapping DNA clones.

S. Gottesman and coworkers are examining the control of cell division in E. coli and have demonstrated that the sul A protein is rapidly turned over in normal cells and has a longer half life in cells defective in cell division. This system is important in the timing of cell divison in E. coli and serves as a model for understanding cell division in more complex cells. They are beginning to investigate the degradation of sul A in vitro as well as unstable proteins such as the lambda xis N and CII proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08000-13 LMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

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

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Ira Pastan Chief, Laboratory of Molecular Biology LMB NCI

COOPERATING UNITS *(if any)*
Genet Corporation

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.9	0.6

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

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

Using clones of fibronectin and collagen the expression of these genes has been measured in cell-free extracts and in RSV transformed cells. We find that transcription rates of these genes are diminished by RSV transformation. A low molecular factor has been identified which inhibits collagen gene expression in cell-free extracts.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER PRINCIPAL INVESTIGATORS:

Benoit de Crombrugghe	Chief, GRS	LMB	NCI
Kenneth M. Yamada	Chief, MBS	LMB	NCI

OTHER INVESTIGATORS:

Genn Merlino	Guest Worker	LMB	NCI
Jaya Sivaswami Tyagi	Research Fellow	LMB	NCI
Hideyasu Hirano	Visiting Fellow	LMB	NCI
Young-Hua Xu	Visiting Fellow	LMB	NCI
Margery Sullivan	Genet Corporation		

PROJECT DISCRIPTION:

Objectives: To understand how malignant transformation controls gene activity and behavior of cultured cells.

Methods Employed: Prepare cloned cDNAs and isolate genes to use to study messenger RNA synthesis in intact cells or cell-free systems. Isolate animal cells that have mutations in the control of RSV expression and determine the biochemical basis of these alterations.

Major Findings: H. Hirano in collaboration with K. Yamada has isolated overlapping clones containing the entire fibronectin genome. Partial sequencing of the clones indicated that they coded for the expected sequence as found in bovine fibronectin. The gene is unusual because it has more than 50 introns and exons and probably is made up of repeated pieces of DNA. Using these clone J. Tyagi has measured the rate of fibronectin transcription in nuclei from normal chick embryo fibroblasts and those transformed by Rous sarcoma virus and has found that the rate of fibronectin transcription is diminished in RSV transformed cells.

J. Tyagi has also isolated a factor from chick embryo extracts that preferentially blocks transcription by RNA polymerase 2 in a cell-free system. This is a low molecular rate factor whose properties are now being studied.

G. Merlino has studied the transcription of the chick $\alpha 2(I)$ collagen in cell-free extracts and showed it is faithfully transcribed. He has also looked at the expression of various collagen genes encoding Types I, II and III collagen during chick embryo development and showed the pattern of collagen RNA expression varies at different times of development

In collaborative studies with C. Gorman and B. Howard, it has been shown that the Rous sarcoma virus LTR is a strong promoter when introduced into a variety of eucaryotic cells. Howard and co-workers showed that promoters such as RSV can be attached to the chloramphenicol acetylase gene (CAT) and the activity of this enzyme measured in extracts of transfected cells. Using antibodies to CAT, the cells which are expressing the enzyme can be identified by immunofluorescence.

Significance for Cancer Research and the Program of the Institute: The invasive properties of tumor cells may be related to alterations in collagen, fibronectin and cyclic AMP. Understanding how the synthesis of these molecules is altered in transformation may suggest new methods of cancer treatment.

Proposed Course: The structure of the promoter region of fibronectin will be determined to compare it with that of collagen and to determine if a structural similarity exists that might explain why both of these genes are down regulated in RSV transformation. Fibronectin synthesis will be examined in mutant cells infected with a temperature mutant of Rous sarcoma virus to determine the rate with which fibronectin expression is altered upon transformation.

Publications:

McKeon, C., Ohkubo, H., Pastan, I., and de Crombrugghe, B.: Unusual methylation pattern of the α 2(I) collagen gene. Cell 29: 203-210, 1982.

Merlino, G.T., Tyagi, J.S., de Crombrugghe, B., and Pastan, I.: Transcription of the chicken α2 (I) collagen gene by homologous cell-free extracts. J. Biol. Chem. 257: 7254-7261, 1982.

Pastan, I., Willingham, M., de Crombrugghe, B., and Gottesman, M.M.: Aging and cancer: Cyclic 3',5'-adenosine monophosphate and altered gene activity. National Cancer Institute Monograph 60: 7-15, 1982.

Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I., and Howard, B.H.: The Rous sarcoma virus 3' long terminal repeat is a strong promoter when introduced into a variety of eucaryotic cells by DNA mediated transfection. Proc. Natl. Acad. Sci. U.S.A. 70, 6777-6781, 1982.

Tyagi, J.S., Merlino, G.T., de Crombrugghe, B., and Pastan, I.: Chicken embryo extracts contain a factor that preferentially blocks the accumulation of RNA polymerase II transcripts in a cell-free system. J. Biol. Chem. 257: 13001-13008, 1982.

Hirano, H., Yamada, Y., Sullivan, M., de Crombrugghe, B., Pastan, I., and Yamada, K.: Isolation of genomic DNA clones spanning the entire fibronectin gene. Proc. Natl. Acad. Sci. U.S.A. 80: 46-50, 1983.

Tyagi, J.S., Hirano, H., Merlino, G.T., and Pastan, I.: Transcriptional control of the fibronectin gene in chick embryo fibroblasts transformed by Rous sarcoma virus. J. Biol. Chem. 258: 5787-5793, 1983.

Merlino, G.T., McKeon, C., de Crombrugghe, B., and Pastan, I.: Regulation of the expression of genes encoding types I, II, and III collagen during chick embryonic development. J. Biol. Chem. 1983, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08001-13 LMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of Cyclic AMP and Transforming Viruses in the Regulation of Cell Behavior

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Ira Pastan Chief, Laboratory of Molecular Biology LMB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
National Cancer Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.2	4.2	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.)*

Using highly purified src kinase from RSV induced rat tumors, S. Ito and co-workers have shown that vinculin, an in vivo substrate for the kinase, is also phosphorylated by the kinase in cell-free extracts. This phosphorylation is stimulated by anionic phospholipids. To determine the mechanism for this stimulation, S. Ito has measured the interaction of anionic phospholipids with vinculin and shown that vinculin binds to acidic phospholipids but not neutral phospholipids. Therefore one mechanism by which phospholipids promote vinculin phosphorylation is by binding to the substrate.

C. Roth has found that RSV transformed Chinese hamster ovary (CHO) cells are resistant to the growth inhibitory action of cyclic AMP. In these resistant cells cyclic AMP stimulates the phosphorylation of pp60src and increases src kinase activity. These findings raise the intriguing possibility that RSV transformation changes the response of the cell to cyclic AMP so that instead of being an inhibitory factor it is converted to a stimulatory factor.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER PRINCIPAL INVESTIGATORS:

Mark C. Willingham	Chief, UCS	LMB	NCI
Michael M. Gottesman	Chief, MCGS	LMB	NCI

OTHER INVESTIGATORS:

Nancy Richert	Senior Staff Fellow	LMB	NCI
Diane Werth	Staff Fellow	LMB	NCI
Seiji Ito	Visiting Fellow	LMB	NCI
Charles Roth	IPA	LMB	NCI

PROJECT DISCRIPTION:

Objectives: To understand the role of cyclic AMP and oncogenic viral gene products in malignant transformation and particularly how these factors regulate cell growth, cell morphology and cell adhesion.

Methods Employed: Cell culture, viral transformation and standard biochemical analyses of the enzymes involved in cyclic AMP metabolism and the synthesis of macromolecules. Preparation of antibodies against tumors induced by Rous sarcoma virus. Purification of proteins.

Major Findings; Using highly purified src kinase from RSV induced rat tumors, S. Ito and co-workers have shown that vinculin, an in vivo substrate for the kinase, is also phosphorylated by the kinase in cell-free extracts. This phosphorylation is stimulated by anionic phospholipids. To determine the mechanism of this stimulation, S. Ito has measured the interaction of anionic phospholipids with vinculin and shown that vinculin binds to acidic phospholipids but not neutral phospholipids. Therefore one mechanism by which phospholipids promote vinculin phosphorylation is by binding to the substrate.

C. Roth has found that RSV transformed CHO cells are resistant to the growth inhibitory action of cyclic AMP. In these resistant cells cyclic AMP stimulates the phosphorylation of pp60src and increases src kinase activity. These findings raise the intriguing possibility that RSV transformation changes the response of the cell to cyclic AMP so that instead of being an inhibitory factor it is converted to a stimulatory factor.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approaches 1, 2 and 5; Objective 4, Approach 2, and Objective 6, Approach 3.

Various aspects of this work will lead to a better understanding of how cells become cancer cells and how the growth of cancer cells is controlled. It also has therapeutic implications.

Proposed Course: To understand the mechanism by which cyclic AMP dependent phosphorylation of src kinase increases its activity. To search for kinases that phosphorylate the src kinase and thereby regulate its activity. To determine natural substances for the src kinase.

Publications:

Blithe, D.L., Richert, N.D., and Pastan, I.H.: Purification of a tyrosine-specific protein kinase from Rous sarcoma virus induced rat tumors. J. Biol. Chem. 257: 7135-7142, 1982.

Richert, N.D., Blithe, D.L., and Pastan, I.: Properties of the src kinase purified from Rous sarcoma virus induced rat tumors. J. Biol. Chem. 257: 7143-7150, 1982.

Ito, S., Richert, N., and Pastan, I.: Phospholipids stimulate the phosphorylation of vinculin by the tyrosine-specific protein kinase of Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 79: 4628-4631, 1982.

Gottesman, M.M., Roth, C., Leitschuh, M, Richert, N., and Pastan, I.: Genetic and biochemical analysis of cyclic AMP effects in transformed cells. Proc. ICN-UCLA Symposia "Tumor Viruses and Differentiation," 1983, in press.

Ito, S., Richert, N., and Pastan, I.: Vinculin phosphorylation by the src kinase: Inhibition by chlorpromazine, imipramine and local anesthetics. Biochem. Biophys. Res. Commun. 107: 670-675, 1982.

Roth, C.W., Richert, N.D., Pastan, I., and Gottesman, M.M.: Cyclic AMP treatment of Rous sarcoma virus transformed Chinese hamster ovary cells increases phosphorylation of pp60src and increases pp60src kinase activity. J. Biol. Chem. 1983, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08006-12 LMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Control of Gene Expression in Bacteriophage Lambda

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Max Gottesman, Chief, Biochemical Genetics Section, LMB, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

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

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

We are continuing our study on the nature of transcription termination in E. coli and of the mechanism of action of the bacteriophage lambda N-protein, which suppresses transcription termination.

The synthesis and function of the lambda cII-protein is under investigation. Synthesis of pcII is regulated at a post-transcriptional level by the E. coli himA-hip gene products.

We are constructing two new vector systems. The first of these is designed to clone DNA fragments bearing promoters active in E. coli. The second, a shuttle vector, is tailored for the cloning of large, and/or unstable DNA fragments, as well as for the reconstitution of genes from overlapping DNA clones.

We are examining the ability of coliphage P1 to stimulate the precise excision of transposons. The responsible gene has been subcloned, and we are attempting to clarify the molecular mechanism of this reaction.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Douglas Ward	Visiting Fellow	LMB	NCI
Susan Gottesman	Research Chemist	LMB	NCI
Sheng Dong Lu	Visiting Fellow	LMB	NCI
George Gaitanaris	Visiting Fellow	LMB	NCI

PROJECT DESCRIPTION:

Objectives: Our objective is to understand how DNA recombination and gene regulation is changed when E. coli carries an active λ or P phage. One goal of this work is to design vector systems, based on E. coli and phage λ, useful for the cloning and expression of a variety of prokaryotic and eukaryotic genes.

Methods Employed: Standard microbial genetic and biochemical techniques required for plasmid, phage and bacterial constructions, and for enzyme assays.

Major Findings: (1) The capacity of phage lambda N-protein to suppress transcription termination is reduced in certain E. coli nusA or rho mutants. In the former, failure to suppress the tR2 terminator results in the inability of lambda to propagate. With Donald Court, we find that introduction of promoter-containing DNA fragments into the phage chromosome immediately distal to tR2 restores phage growth. In this way, a suitably modified lambda was constructed which serves as a vector for the cloning of promoters active in E. coli.

In the rho026 mutant strain Susan Gottesman, Patsy Trisler, and I, in collaboration with A. Das, have demonstrated that transcription termination at some, but not all, terminators, is affected. N-function, for example, can no longer suppress termination at the Rho-dependent tL1 terminator, but can do so at tL2, which is Rho-independent.

By selecting for N-activity in nusA1 and rho026 mutants,we have isolated outside suppressors of these mutations. Suppressors of nusA1 for lambda growth reside in the E. coli nusB gene or in the L11-"protein U" region. Mutations in or near gene N that restore function in rho026 have been found. Analysis of these various suppressors is expected to clarify the process of transcription termination and of the regulation of lambda gene expression.

(2) The E. coli himA-hip genes encode a low-molecular weight protein, IHF, which binds to specific DNA sequences near the lambda attachment site, and thus promotes phage integration and excision. IHF is also required for the translation of the cII transcript; an IHF binding sequence

is observed just upstream of the cII ribosome binding site. In collaboration with Amos Oppenheim, a number of cII-lacZ gene fusions have been constructed; their dependence on IHF for expression is under investigation.

(3) In collaboration with Bruce Howard and Mary McCormick, we have constructed a shuttle vector system for the cloning of large and/or unstable DNA fragments, and for the reconstitution of genes from overlapping gene segments. The system is based on a plasmid capable of replicating autonomously or of integrating into the E. coli chromosome. In the integrated, single-copy form, we expect to alleviate certain problems encountered in maintaing cloned DNA. Results obtained with Sandra Weller indicate that a DNA fragment carrying a Herpes Simplex origin of replication is more stable in our vector than in other cloning systems.

(4) The excision of transposons in E coli is a recombination event between the terminal repeats created by the insertion of the transposon. Excision restores the integrity of involved host gene. The bacterial functions responsible for the excision reaction are unknown. We have detected a function encoded by bacteriophage P1 that stimulates excision, and have subcloned the responsible gene, ref, into lambda and pBR322. Ref activity requires the product of the host recA gene, but is independent of the recB and himA gene products.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 1.

In cancer cells, the expression of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using λ as a model system. This understanding might help to prevent the conversion of normal cells to those capable of forming cancers.

Proposed Course: (1) We plan to use the promoter cloning vector to study the regulation of promoters and to obtain mutations that alter this regulation. Our single-copy vector system resembles the natural situation more closely than the currently utilized multi-copy plasmid systems, and it is hoped that the cloned promoters will be subject to their normal control mechanisms.

We plan to study the host and phage mutations that affect transcription termination and to determine the sequence and protein changes involved.

(2) To study how IHF promotes translation, we will test, in vitro, the effect of this protein on cII synthesis in a cII-lacZ fusion. We will attempt to demonstrate specific binding of IHF to the cII transcript, and to determine the role of IHF in the translation of the transcript.

(3) We are attempting the cloning of: a. very large (>50kb) DNA fragments, and b. unstable DNA fragments, e.g. head to head dimers, in our shuttle vector system. In addition, we are reconstituting the his operon

from clones carrying incomplete, overlapping segments of the operon. We will then proceed to attempt a reconstitution of a globin gene from clones of incomplete, overlapping genomic globin DNA.

(4) We will study the mechanism of transposon excision by isolating the P1 ref gene product.

Publications

Ward D. F., and Gottesman, M. E.: Suppression of transcription termination by phage lambda. Science 216: 946-951, 1982.

Oppenheim, A. B., Gottesman, S., and Gottesman, M.: Regulation of bacteriophage λ int gene expression. J. Mol. Biol. 158: 327-346, 1982.

Adhya, S., and Gottesman, M.: Promoter occlusion: Transcription through a promoter may inhibit its activity. Cell 29: 939-944, 1982.

Ward, D. F., and Gottesman, M. E.: E. coli nusB mutation that suppress nusA1 exhibit λ N specificity. J. Mol. Biol., 1983, in press.

Das, A., Gottesman, M. E., Wardwell, J., Trisler, P., and Gottesman, S.: A mutation in the E. coli rho gene that inhibits the N gene activity of phage lambda. Proc. Natl. Acad. Sci. U.S., 1983, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08010-09 LMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Morphological Mechanisms of Organelle Function and Transformation in Culture

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Mark C. Willingham Chief, Ultrastructural Cytochemistry Section LMB NCI

COOPERATING UNITS *(if any)*
Laboratory of Human Carcinogenesis, CIP, DCCP, NCI
Macromolecular Interactions Section, LB, DCBD, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Ultrastructural Cytochemistry Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	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.)*

Neoplastic transformation produces many changes in cell physiology. We have utilized morphologic techniques to study these changes and to understand basic cellular mechanisms of organelle movement, function and compartmentalization of intracellular materials. We have employed ultrastructural immunocytochemistry, single cell microinjection, image intensification microscopy, and biochemical preparative techniques to follow substances from the outside of the cell to the interior. The study of the pathway of receptor-mediated endocytosis and Golgi traffic by these methods has revealed: (1) Epidermal growth factor (EGF) clusters in coated pits of the Golgi prior to lysosomal delivery. (2) Adenovirus enters cells through the same pathway, and coincubation of toxic conjugates of EGF and Pseudomonas toxin with adenovirus leads to a 10,000-fold enhancement of toxicity, apparently through an adenovirus-mediated lysis of receptosomes. (3) Microinjection of a monoclonal antibody to tubulin decorates microtubules in living cells and blocks saltatory movement of organelles such as receptosomes and lysosomes. In other studies, it was shown that an antibody to Harvey MSV p21^{src} reacts with a normal cell protein in human epithelial cell lines in culture.

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Ira H. Pastan	Chief, LMB	LMB	NCI
David J.P. FitzGerald	Staff Fellow	LMB	NCI
Nancy D. Richert	Staff Fellow	LMB	NCI
Jürgen Wehland	Guest Worker	LMB	NCI
Susan Banks-Schlegel	Guest Worker	LHC	NCI
Claude B. Klee	Chief, MIS	LB	NCI

PROJECT DESCRIPTION:

Objectives: To investigate the mechanisms that control cell motility, endocytosis, exocytosis, intracellular protein traffic, viral infection and the morphologic and growth manifestations of malignant transformation.

Methods Employed: Cell culture, specialized light and electron microscopic morphologic and immunocytochemical methods, including the EGS and GBS ultrastructural localization procedures, single cell microinjection techniques and specialized biochemical purification and analytical methods.

Major Findings: The movement of cells and subcellular organelles has been directly studied using electron microscopic immunocytochemistry, single cell microinjection and image intensification techniques. We have previously established the location and quantitative levels of a number of proteins within the cell to understand their relationships and effects on morphologic alterations following transformation. These studies included locating the position of virally-coded src proteins on the inner aspect of the plasma membrane (ASV p60^{src} and Ha-MSV p21^{src}) in transformed animal cells. Recently, we have extended those studies to human epithelial cultured cell lines, both normal and transformed, and have demonstrated a cross-reactive p21-like protein in both normal and malignant human cells using a monoclonal antibody against Harvey murine sarcoma virus p21 ^{src}. These studies demonstrate the presence of a normal human cell protein in the same location as the virally-induced p21^{src} in rodent cells, and taken together with genetic and biochemical data from other laboratories suggest the existence of a family of normal p21 proteins in most cells, some of which induce malignancy when introduced by certain retroviruses that carry selective mutations of these genes.

Development of immunocytochemical methodology that lead to the EGS method has continued in this laboratory, and recently a new method referred to as the "GBS" (glutaraldehyde-borohydride-saponin) method has been developed for use in certain intracellular locations. This new method allows greater latitude in processing and handling of tissue than the EGS method, but it is not uniformly applicable to all antigen locations. Further studies using the EGS method have allowed precise localization of calmodulin in cultured cells, revealing that there is little if any concentration of calmodulin in microfilament structures,

as had previously been reported by others. A monoclonal antibody to a unique intracellular filamentous structure has been isolated, which demonstrates these structures, previously unrecognized, in a wide variety of cells. The subunit protein of these unique intermediate filament-like structures has been named "nematin." This antigen has been localized by both light and electron microscopic immunocytochemistry and by microinjection of monoclonal antibody into living cells.

Microinjection of a monoclonal antibody to alpha-tubulin in living cells has demonstrated that the antibody reacts with the surface of microtubules and interferes with microtubule functions such as formation of the mitotic spindle and saltatory motion of lysosomes and other organelles in living cells. This same monoclonal antibody was also successfully coupled to 5 nm colloidal gold particles and still showed selective localization to microtubules when injected as a gold-antibody conjugate into living cells.

Studies of receptor-mediated endocytosis have revealed details of the processing of epidermal growth factor (EGF) in the trans elements of the Golgi system. EGF conjugated covalently to horseradish peroxidase was found to concentrate in the clathrin-coated pits of the Golgi system prior to delivery to lysosomes. This finding firmly establishes the role of the Golgi system in the processing of ligands that enter through plasma membrane coated pits and receptosomes (the free endocytic vesicles derived from coated pits). Receptosomes appear to deliver their contents selectively to the trans-reticular elements of the Golgi and deliver ligands and, in some cases, receptors to lysosomes from the Golgi, or recycle these elements back to the cell surface. In studies of the internalization of EGF and adenovirus, we have found that adenovirus appears to lyse receptosomes during their infection cycle, releasing the intact virion into the cytosol. When colloidal gold-labeled EGF was coincubated with adenovirus, both virus and gold particles were released into the cytosol, a result never seen in the absence of virus. A cytotoxic conjugate of EGF and Pseudomonas exotoxin, which requires entry into the cytosol for its action, was shown to be enhanced in its toxicity by 10,000-fold in the presence of adenovirus, presumably by adenovirus-induced release of the toxin conjugate from lysed receptosomes. These studies suggest a mechanism for the selective delivery of cytotoxic conjugates and their enhanced toxicity that may be of therapeutic value for the treatment of tumors.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 6, Approach 3.

Transformation of cultured cells appears closely linked with the ability to form malignant tumors in vivo. The understanding of the basic mechanisms that control cell movement, organelle function, response to growth-promoting factors, virus infectivity, and the morphologic changes that occur following transformation by certain viruses are likely to be of great value in understanding the basic mechanisms that are altered in most cancer cells. This understanding is likely to have significant impact on the ability to design successful therapeutic procedures for malignancy.

Proposed Course: We will continue to study the basic cellular mechanisms that regulate movement of organelles and cells, the entry and exit of cellular proteins, viruses, and hormones, and the interactions of specific transformation-linked molecules with these systems. Through the study of the alterations in cell function related to transformation, we will try to gain a more precise understanding of the mechanisms by which cancer cells are able to grow uncontrollably and metastasize.

Publications:

Willingham, M.C., Pastan, I.H., and Sahagian, G.G.: Ultrastructural immunocytochemical localization of the phosphomannosyl receptor in Chinese hamster ovary (CHO) cells. J. Histochem. Cytochem. 31: 1-11, 1983.

Willingham, M.C., and Pastan, I.H.: Transit of epidermal growth factor through coated pits of the Golgi system. J. Cell Biol. 94: 207-212, 1982.

Via, D.P., Willingham, M.C., Pastan, I., Gotto, A.M., and Smith, L.C.: Co-clustering and internalization of low density lipoproteins and alpha$_2$-macroglobulin in human skin fibroblasts. Exp. Cell Res. 141: 15-22, 1982.

Dickson, R.B., Schlegel, R., Willingham, M.C., and Pastan, I.: Binding and internalization of alpha$_2$-macroglobulin by cultured fibroblasts: effect of monovalent ionophores. Exp. Cell Res. 142: 127-140, 1982.

Schlegel, C.R., Dickson, R.B., Willingham, M.C., and Pastan, I.: Amantadine and dansylcadaverine inhibit Vesicular Stomatitis virus uptake and receptor-mediated endocytosis of alpha$_2$-macroglobulin. Proc. Natl. Acad. Sci. U.S.A. 79: 2291-2295, 1982.

Dickson, R.B., Schlegel, C.R., Willingham, M.C., and Pastan, I.H.: Reversible and irreversible inhibitors of clustering of alpha$_2$-macroglobulin in clathrin-coated pits on the surface of fibroblasts. Exp. Cell Res. 140: 215-225, 1982.

Willingham, M.C., Wehland, J., Klee, C.B., Richert, N., Rutherford, A.V., and Pastan, I.: Ultrastructural immunocytochemical localization of calmodulin in cultured cells. J. Histochem. Cytochem. 31: 445-461, 1983.

Willingham, M.C., and Pastan, I.H.: Receptor-mediated endocytosis: General considerations and morphological approaches. In Cuatrecasas, P., and Roth, T.F. (Eds.): Receptor Mediated Endocytosis, Receptors and Recognition, Series B. London, Chapman and Hall, 1983, pp. 4-17.

Willingham, M.C., and Pastan, I.H.: Receptor-mediated endocytosis of peptide hormones and macromolecules in cultured cells. In Weinstein, I.B., and Vogel, H.J. (Eds.): Genes and Proteins in Oncogenesis. New York, Academic Press, 1983, pp. 183-195.

FitzGerald, D.J.P., Padmanabhan, R., Pastan, I., and Willingham, M.C.: Adenovirus-induced release of epidermal growth factor and Pseudomonas toxin into the cytosol of KB cells during receptor-mediated endocytosis. Cell 32: 607-617, 1983.

Willingham, M.C., and Pastan, I.H.: Image intensification techniques for detection of proteins in cultured cells by fluorescence microscopy. Methods Enzymol. 1983, in press.

Willingham, M.C., Haigler, H.T., FitzGerald, D.J.P., Gallo, M.G., Rutherford, A.V., and Pastan, I.H.: The morphologic pathway of binding and internalization of epidermal growth factor in cultured cells: studies on A431, KB, and 3T3 cells using multiple methods of labeling. Exp. Cell Res. 1983, in press.

Willingham, M.C.: An alternative fixation-processing method for pre-embedding ultrastructural immunocytochemistry of cytoplasmic antigens: the GBS procedure. J. Histochem. Cytochem. 1983, in press.

Pastan, I.H., and Willingham, M.C.: Receptor-mediated endocytosis: coated pits, receptosomes, and the Golgi. Trends Biochem. Sci. 1983, in press.

FitzGerald, D.J.P., Trowbridge, I.S., Pastan, I., and Willingham, M.C.: Enhancement of toxicity of an anti-transferrin receptor antibody- Pseudomonas exotoxin conjugates by adenovirus. Proc. Natl. Acad. Sci. U.S.A. 1983, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1CB08011-09 LMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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 on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Kenneth M. Yamada, Chief, Membrane Biochemistry Section, LMB, NCI

COOPERATING UNITS *(if any)*

Diabetes Branch, NIAMDD

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Membrane Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.7	2.7	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 major cell surface glycoprotein fibronectin is usually decreased on tumor cells and is involved in cell adhesion. We have determined the functional organization of human plasma fibronectin. Protease-resistant domains for the binding of fibronectin to the plasma membrane, collagen, heparin, fibrin, actin, and DNA were mapped. The A and B subunits of human fibronectin were found to be similar but not identical in structural organization.

Fibronectin was compared to the epithelial adhesive protein laminin. Laminin had equal hemagglutination activity, but much less activity in other assays for adhesion and effects on transformed cells.

A cell mutant lacking gangliosides was defective in fibronectin distribution. Reconstitution of gangliosides restored a normal distribution, suggesting some role in fibronectin-membrane association.

Recombinant DNA clones spanning the entire fibronectin gene were isolated and partially characterized. The gene was found to be unusually large and complex, with at least 48 exons of similar size. It may have evolved by gene duplication of primordial gene(s) 150 base pairs in size.

New transformation-sensitive proteins of plasma membranes were identified by two-dimensional gel electrophoresis of membranes from cells transformed by wild-type and temperature-sensitive viruses. In collaborative studies, both fibronectin and high cell density were found to be necessary for rapid migration of embryonic neural crest cells, and the insulin receptor was further characterized.

Our future objectives will be to evaluate the function of fibronectin using synthetic hybrids between different isolated domains, and by continuing structural and functional comparisons of the cellular and plasma forms of fibronectin. Immunological approaches will be extended to characterize further the membrane components interacting with fibronectin and with insulin. The fibronectin gene will be characterized further in terms of structure.

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Masao Hayashi	Visiting Associate	LMB	NCI
Hideyasu Hirano	Visiting Fellow	LMB	NCI
Takayuki Hasegawa	Visiting Fellow	LMB	NCI
Steven K. Akiyama	Guest Researcher	LMB	NCI
Etsuko Hasegawa	Guest Researcher	LMB	NCI

PROJECT DESCRIPTION:

Objectives: Fibronectin, a major cell surface and extracellular matrix glycoprotein, is depleted after neoplastic transformation. Our objectives are:

(1) To determine the structural organization of fibronectin and its gene.

(2) To evaluate fibronectin's role in cell behavior and to determine its mechanism of action.

(3) To identify and characterize other transformation-sensitive cell surface proteins.

Methods Employed: Fibronectins were isolated from monolayer cultures of normal fibroblasts or from plasma by urea-extraction or gelatin- and heparin-affinity chromatography. Human plasma fibronectin was cleaved into protease-resistant domains by a graded series of concentrations of trypsin. Structural and functional domains were purified by affinity chromatography on columns containing covalently immobilized gelatin or heparin, as well as by DEAE column chromatography. Fragments were characterized by one-dimensional and two-dimensional peptide mapping, measurement of binding to a standard series of ligand-affinity columns including heparin, actin, and DNA, analysis for free sulfhydryl groups by DTNB after denaturation in 6 M guanidinium chloride, and analysis for carbohydrate by the phenol-sulfuric acid method. Biological activities of proteins or fragments were measured in standardized assays for hemagglutination, attachment of cells to type I collagen, spreading of cells on cell culture substrates, and restoration of normal morphology to transformed fibroblasts.

Plasma membranes were isolated from chick embryo fibroblasts or from parallel cultures of these cells transformed by the Schmidt-Ruppin strain of Rous sarcoma virus, or by the tsNY68 temperature-sensitive mutant of this virus. After isolation in a two-phase flotation system, membranes were homogenized in boiling SDS, then analyzed by two-dimensional gel electrophoresis. Analyses compared cells labeled by ^{35}S-methionine or ^{3}H-mannose for 2 or 24

hours as well as by lactoperoxidase-mediated iodination and concanavalin A binding using various cell culture temperatures to compare normal and transformed phenotypes.

Genomic DNA encoding fibronectin was first identified using a cDNA probe corresponding to the 3' untranslated region of fibronectin mRNA. Overlapping recombinant DNA clones were isolated by stepwise screening of a library of chicken genomic DNA fragments cloned in the bacteriophage λ Charon 4A vector. Successive clones were obtained by using defined restriction endonuclease fragments from the 5' end of each clone to identify other clones extending further in the 5' direction. The relationships and overlaps between clones were determined by electron microscope R-loop analysis, restriction mapping, and heteroduplex analysis. Nucleotide sequencing of an exon in the clone most 5'-distal to the 3' site first identified by the cDNA probe was performed by standard procedures to confirm the identification of the clones as corresponding to the fibronectin gene.

Major Findings: The glycoprotein fibronectin provides a well-characterized model system for beginning to understand the mechanisms of cell-to-substrate and cell-to-cell adhesion. A series of protein domains on fibronectin for mediating interactions with fibrin, heparin, collagen, actin, DNA, and the plasma membrane were isolated by affinity and ion exchange chromatography from human plasma fibronectin. They were then mapped on the A and B subunits of the intact, dimeric molecule. Although the overall domain organization was similar on A and B chains, there was a site of apparent difference located between the heparin- and fibrin-binding domains near the carboxyl terminus of the molecule.

Fibronectin was compared to laminin, a putative epithelial cell adhesion protein, in a series of biological assays. Laminin, like fibronectin, was found to be a potent agglutinin. In addition, laminin was shown to mediate the spreading of fibroblasts, but with only 5% of the specific activity of fibronectin; it was unable to mediate adhesion of fibroblasts to type I collagen or to restore normal morphology to transformed cells. Laminin and fibronectin therefore share certain biological activities, yet differ markedly in others.

Interactions of fibronectin with the cell surface were found to be defective in a mouse L-cell mutant lacking higher-order gangliosides. Reconstitution of purified gangliosides such as G_{T1b} into these cells resulted in a restoration of fibronectin binding in characteristic fibrillar patterns on the cell surface, suggesting that gangliosides or related molecules are involved in the interactions of fibronectin with cells.

The gene for chicken fibronectin was isolated as a set of five overlapping genomic clones. It is an unusually large and complex gene, with at least 48 exons and a length of 48 kilobases. The relatively uniform size of most exons suggests a possible evolution of the fibronectin gene by multiple gene duplications of a primordial gene(s) 150 base pairs in size.

Plasma membranes were examined systematically by several methods to identify new transformation-specific alterations besides the well-documented decrease in fibronectin. Chick fibroblasts transformed by wild-type or temperature-sensitive mutant Rous sarcoma viruses were analyzed by various protein labeling methods and the sensitive two-dimensional gel electrophoresis procedure. Polypeptides that were found to be consistently transformation-sensitive included a group of five polypeptides that were detected only by short-term labeling with methionine, fibronectin, a mannose-containing glycoprotein of 48,000 daltons with an unusually high isoelectric point of 8.4, and two other polypeptides of 19,000 and 180,000 daltons. Several of these polypeptides should be of interest for further characterization in understanding cell surface changes in transformation.

In collaborative experiments using antibodies against the cell-binding site of chicken cellular fibronectin and purified fibronectins, this glycoprotein and high cell density were shown to be necessary for rapid migration of neural crest cells, which is an important and well-regulated invasive event in embryonic development. In addition, the insulin receptor is being further characterized in terms of its overall structure and the existence of a possible single pro-receptor for the different subunits.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 5.

Decreases in fibronectin and alterations in other cell surface glycoproteins are thought to contribute to the abnormal behavior of transformed and malignant cells. Our studies are providing a characterization of human fibronectin and of other transformation-sensitive molecules. Since fibronectin and laminin provide models for understanding cell adhesion, obtaining molecular insights into their structure and function may help to explain the aberrant adhesive behavior of malignant cells. The identification and characterization of other cell surface molecules may provide further insights into the role of the cell surface in regulating the cell-cell interactions and metabolic regulation of normal and tumor cells.

Proposed Course: We propose to define further the functional relationships between the domains of fibronectin by (a) testing the ligand-binding and biological activities of crosslinked, synthetic hybrids between our isolated domains, and (b) continuing structural and functional comparisons between the cellular and plasma forms of fibronectin using extensive two-dimensional tryptic and chymotryptic peptide mapping and immunological analyses. We will also use existing polyclonal antibodies and monoclonal antibodies being developed to attempt a further characterization of the specialized plasma membrane structures, receptors, and pro-receptors involved in cell interactions with fibronectin and insulin. We will also attempt to continue nucleic sequence analyses of the fibronectin gene.

Publications:

Yamada, K. M.: Isolation of fibronectin from plasma and cells. In Furthmayr, H. (Ed.): Immunochemistry of the Extracellular Matrix. CRC Press, Boca Raton, FL., 1982, pp. 111-123.

Yamada, K. M.: Biochemistry of fibronectin. In The Glycoconjugates. Academic Press, New York, 1982, Vol. III, pp. 331-362.

Hayashi, M., and Yamada, K. M.: Divalent cation modulation of fibronectin binding to heparin and to DNA. J. Biol. Chem. 257: 5263-5267, 1982.

Katow, H., Yamada, K. M., and Solursh, J.: Occurrence of fibronectin on the primary mesenchyme cell surface during migration in the sea urchin embryo. Differentiation 22: 120-124, 1982.

Kasuga, M., Hedo, J. A., Yamada, K. M., and Kahn, C. R.: The structure of insulin receptor and its subunits: Evidence for multiple non-reduced forms and a 210K component. J. Biol. Chem. 257: 10392-10399, 1982.

Hynes, R. O., and Yamada, K. M.: Fibronectins: Multifunctional modular glycoproteins. J. Cell Biol. 95: 369-377, 1982.

Yamada, K. M., Hayashi, M., and Akiyama, S. K.: Structure and function of fibronectin. In Hawkes, S., and Wang, J. L. (Eds): Extracellular Matrix. Academic Press, New York, 1982, pp. 25-34.

Yamada, K. M., Critchley, D. R., Fishman, P. H., and Moss, J.: Exogenous gangliosides enhance the interaction of fibronectin with ganglioside-deficient cells. Exp. Cell Res. 143: 295-302, 1983.

Dubbelman, T. M. A. R., and Yamada, K. M.: A survey of differences between membrane polypeptides of transformed and nontransformed chick embryo fibroblasts. Biochim. Biophys. Acta 693: 177-187, 1982.

Rovasio, R. A., DeLouvee, A., Yamada, K. M., Timpl, R., and Thiery, J. P.: Neural crest cell migration: Requirements for exogenous fibronectin and high cell density. J. Cell Biol. 96: 462-473, 1983.

Yamada, K. M. (Ed.): Cell Interactions and Development: Molecular Mechanisms. (Other contributors: Barondes, S. H., Dazzo, F. B., Goodenough, U. W., Thorner, J., Harris, A. K., Kratochwil, K., Roth, S., Toole, B. P., Underhill, C. B., and Wassarman, P. M.) John Wiley & Sons, New York, 1983, 287pp.

Akiyama, S. K., and Yamada, K. M.: Fibronectin and disease. In Wagner, B., and Fleischmajer, R. (Eds.): Connective Tissue and Diseases of Connective Tissue. Williams and Wilkins, Baltimore, 1983, in press.

Kennedy, D. W., Rohrbach, D. H., Martin, G. R., Momoi, T., and Yamada, K. M.: The adhesive glycoprotein laminin is an agglutinin. J. Cell. Physiol. 114: 257-262, 1983.

Hirano, H., Yamada, Y., Sullivan, M., de Crombrugghe, B., Pastan, I., and Yamada, K. M.: Isolation of genomic DNA clones spanning the entire fibronectin gene. Proc. Natl. Acad. Sci. U.S.A. 80: 46-50, 1983.

Hayashi, M., and Yamada, K. M.: Domain structure of the carboxyl-terminal half of human plasma fibronectin. J. Biol. Chem. 258: 3332-3340, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08700-11 LMB

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Structure and Expression of Collagen Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Benoit de Crombrugghe, M.D. Chief, Gene Regulation Section LMB, NCI

COOPERATING UNITS *(if any)*

Genex Corp.; University of Maryland, Catonsville, MD; University of Chicago; Pasteur Institute, Paris, France

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Gene Regulation Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

1. The comparison of the structure of two collagen genes suggests that very little or no recombinational events took place in these genes after they diverged from a common ancestor about 500×10^6 years ago. This situation contrasts strongly with the intense recombinational rearrangements which the ancestral gene underwent throughout the period of its assembly. We believe the functions of the collagen proteins were the principal selective force against any recombinational rearrangements of the genes once an optimal size for the polypeptide chain was acquired.

2. Studies on methylation of the chick α2(I) collagen gene and its DNase I sensitivity in chromatin from several types of tissues with different levels of collagen synthesis suggest at least three levels of regulation for the expression of this gene.

3. The cloned α2(I) collagen promoter after it has been stably integrated in the genome of mouse cells responds to the same type of down-regulation by oncogenic products as the endogenous α2(I) collagen promoter.

4. Studies with deletions in the LTR promoter of Rous sarcoma virus favor the view that "enhancing sequences" are entry sites for RNA polymerase.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Bruce Howard	Chief, Molecular Genetics Section	LMB	NCI
Cornelia Gorman	Staff Fellow	LMB	NCI
Margery Sullivan	Genex Corp.		
Michael O'Neill	Univ. of Md. Catonsville, MD.		
Bill Uphold	Univ. of Chicago		
Moshe Yaniv	Pasteur Institute, Paris, France		
Yoshihiko Yamada	Visiting Associate	LMB	NCI
Maria Mudryj	Chemist	LMB	NCI
Rodolfo Frunzio	Visiting Fellow	LMB	NCI
Catherine McKeon	Postdoctoral Fellow	LMB	NCI
Azriel Schmidt	Visiting Fellow	LMB	NCI
Gene Liau	Postdoctoral Fellow	LMB	NCI
Kay LeVan	Biologist	LMB	NCI

PROJECT DESCRIPTION:

Objective: To understand the differentiation programs which determine the level of expression of specific genes in animal cells.

Methods Employed: (1) Introduction of segments of animal and bacterial genes in the DNA of bacterial plasmids and bacteriophage; construction of recombinant DNAs; construction and screening of cDNA and gene libraries; (2) DNA transfection of animal cells, selection of transfected cells; (3) purification of specific DNA fragments and RNA species; nucleotide sequence analysis of DNA and RNA; (4) measurement of RNA and protein synthesis in vivo and in cell-free systems; (5) purification of factors active in regulation of gene expression.

Major Findings:

1. Structure of the collagen genes. We have isolated two complete chick collagen genes, the genes for α2 type I collagen and type III collagen and compared their structure. These two genes probably diverged about 500 × 10^6 years ago. The major conclusion of this study is that the size distribution of exons coding for the helical portion of these collagens are conserved in both genes although the exon sequences and the sizes and sequences of the introns have not been conserved. This finding implies that the recombinational rearrangements that occured during the assembly of the ancestor for these genes (which include the amplification of a genetic unit containing a 54 bp exon, unequal crossings over between two pairs of 54 bp exons to generate 99 bp, 45 bp etc ... exons, precise loss of an intron between 54 bp exons to

generate 108 bp exons) were no longer tolerated after these genes diverged from a common ancestor. Presumably, once the polypeptide had reached an optimal length no changes took place which would increase or decrease the size of the polypeptide.

We have also examined the location of repetitive elements in these two collagen genes and found that these genes contain several species of repetitive elements within their DNA but that the location of these elements is different in the two genes. This suggests that these repetitive sequences moved in these genes either from a location outside these genes or from another location within this gene after the two genes were duplicated from a common ancestor.

2. Structure of the promoter of the α2(I) collagen gene. A comparison of the promoter sequences of the α2(I) collagen gene in chicken and in mouse shows a number of homologous segments interrupted by very dissimilar sequences. The homologies are not only found in the TATA and CAT box sequences which are conserved in many RNA Polymerase II dependent promoters, but in several other segments as far as 350 bp upstream of the start of transcription. These conserved homologous sequences might be important for the expression and the regulation of this gene.

This promoter contains a small segment which in chromatin is hypersensitive to DNAse I in tissues that express the gene but not in tissues which do not. This hypersensitive site is found, however, in chromatin of Rous sarcoma virus (RSV) transformed fibroblasts, although the rate of α2(I) collagen RNA transcription is selectively reduced in these cells after RSV transformation. Therefore, the mechanism which is responsible for the DNase I sensitivity is different from the mechanism that causes a decrease in type I collagen RNA transcription in RSV transformed fibroblasts. The same DNase I sensitive segment is also sensitive to the single stranded nuclease S1 in chromatin of fibroblasts. Additionally, supercoiled DNA plasmids that harbor the chick or mouse α2 collagen promoter contain a discrete S1 sensitive site that has been mapped to a site of the promoter that is very rich in pyrimidines on one strand of the DNA. We conclude that some specific sequence in the promoter have the capacity to become single-stranded when the DNA becomes supercoiled. In chromatin the DNA will become supercoiled if one (or a few) nucleosomes disassociate from the DNA. Such dissociation of nucleosomes could explain the DNase I hypersensitivity.

Another sequence that is highly conserved among three different collagen genes is located around the start of translation, whereas sequences which precede and follow this conserved sequence markedly diverge in each gene. This homologous sequence can be drawn in an identical stem-loop structure in each mRNA with two AUG's separated from each other by the same distance in all three mRNAs. The very high degree of conservation of both the sequence and the deduced secondary structure suggests they may play a role in determining the level of expression of these genes by modulating translational efficiency.

3. Functional analysis of the α2(I) collagen promoter. We have constructed a number of recombinant plasmids to study the activity of this promoter

after DNA mediated transfection of chick and mouse fibroblasts. In these plasmids the bacterial gene for chloramphenicol acetyl transferase (CAT) was placed next to the collagen promoter in a construction which also contains the poly(A) addition site of the early region of SV40, the β-lactamase gene and the origin of replication of ColE1. Two different approaches were taken in these studies. The first approach was to establish mouse cell lines (derived from NIH 3T3 cells), where the cloned collagen promoter plasmid is stably integrated in the mouse cell genome and drives the expression of the CAT gene. These cells were then infected with the complex of mouse leukemia and sarcoma virus (Moloney strain) which causes these cells to acquire a transformed phenotype.

We found that infection by this virus completely inhibits, as expected, transcription of the endogenous α2(I) collagen promoter but also expression from the cloned promoter. We conclude, therefore, that the cloned α2(I) collagen promoter responds to the same type of regulation as the endogenous promoter. We will now be able to determine which part of the collagen promoter is responsible for this regulation.

Another approach was to perform bulk transfection experiments and to examine the level of expression of the collagen promoter within 48 hours after transfection. Using this method we detect expression of the CAT gene driven by the α2(I) collagen promoter. The activity of the collagen promoter is increased, however, 30-to 100-fold by introducing "enhancing" DNA elements in our recombinant plasmids. These enhancing elements are found within a number of viral promoters such as in SV40, polyoma and papilloma viruses and also in the long terminal repeats (LTR) of retroviruses. They are able to activate in "cis" other promoters located on the same plasmid. In contrast to the strong stimulating activity of the SV40 and polyoma enhancers, the LTR of Rous sarcoma virus is unable to stimuate transcription of the collagen promoter in chick cells. One explanation for this observation could be the following - If enhancing sequences constitute entry sites for RNA polymerase, the sequences in the rest of the RSV-LTR promoter could very efficiently trap the RNA polymerase molecules that have entered the promoter through the enhancer because the RSV-LT is a very strong promoter. The LTR sequences would, therefore, prevent these RNA polymerase molecules from reaching the collagen promoter. We decided, therefore, to alter the LTR promoter by removing its TATA box and transcription start. This new construction acts as an excellent enhancer for the collagen promoter. Our results are thus clearly consistent with the notion that enhancer sequences are binding sites either for RNA polymerase itself or for factor(s) that determine(s) the binding of RNA polymerase to a promoter.

Proposed Course:

1. Isolate and characterize additional promoters for other collagen genes.

2. Pursue the functional analysis of the α2(I) collagen promoter. Determine the sequences that are needed to observe the inhibition of promoter activity by oncogenic virus. Search for enhancing sequences in the α2(I)

collagen promoter. Determine whether the sequences that are S_1 sensitive, are necessary for expression or regulation.

3. Determine the role of the conserved element found around the start of translation in three different collagen genes by constructing plasmids in which this segment is interposed between a strong promoter and the chloramphenicol acetyl transferase gene or the β-galactosidase gene. Such plasmids will be used in DNA transfection experiments and compared with recombinants where the conserved element is altered by mutation.

4. Preliminary experiments indicate that hybrid plasmids containing both the α2(I) collagen promoter and the DNA of a bovine papilloma virus are maintained as multicopy episomes in mouse cells. We will examine whether factors that may bind to the collagen promoter in such multicopy plasmids can be titrated and eventually purified.

5. Use the complex of the α2(I) collagen promoter driving a dominant selectable marker gene (like the gene for neomycin resistance) as a genetic system in order to isolate mutants in the collagen promoter as well as mutants that map outside the collagen gene.

6. Identify and clone genes which control the collagen genes.

7. Establish an in vitro transcription system, based on the knowledge we will gain from our ongoing in vivo work with this promoter, to study the regulation of the α2(I) collagen gene.

8. The growth of several viruses is inhibited in embryonal carcinoma cells although their DNAs integrate in the genome of these cells. These DNAs are, however, methylated. We will examine whether the DNA of the α2(I) collagen promoter which is unmethylated in sperm cells also escapes methylation after DNA mediated transfection of embryonal carcinoma cells.

Publications

Pastan, I., Willingham, M., de Crombrugghe, B., and Gottesman, M.M.: Aging and Cancer: Cyclic AMP and Altered Gene Activity. Bethesda, Maryland, National Cancer Institute Monograph 60: 7-15, 1982.

Merlino, G.T., Tyagi, J.S., de Crombrugghe, B., and Pastan, I.: Transcription of the chicken α2(I) collagen gene by homologous cell-free extracts. J. Mol. Chem. 257: 7254-7261, 1982.

Hirano, H., Yamada, Y., Sullivan, M., de Crombrugghe, B., Pastan, I., and Yamada, K.: Isolation of genomic CNA clones spanning the entire fibronectin gene. Proc. Natl. Acad. Sci. USA., 80: 46-50, 1983.

de Crombrugghe, B., Yamada, Y., McKeon, C., Ohkubo, H., Pastan, I.: Structure and Expression of Chick Collagen Genes. Perspectives on Genes and the Molecular Biology of Cancer, 1983.

Yamada, Y., Mudryj, M., Sullivan, M., de Crombrugghe, B.: Isolation and Characterization of a Genomic Clone Encoding Chick α1 Type III Collagen. J. Biol. Chem., 258: 2758-2751, 1983.

Yamada, Y., Kuhn, K., and de Crombrugghe, B.: A conserved nucleotide sequence, coding for a segment of the C-propeptide, is found at the same location in different collagen genes. Nucleic Acids Research, in press.

de Crombrugghe, B., Busby, S., and Buc, H.: Activation of Transcription by the Cyclic AMP Receptor Protein. In Goldberger, R.F., and Yamamoto, K.R. (Eds.): Biological Regulation and Development. New York, Plenium Press, vol. 3B, in press.

Tyagi, J.S., Merlino, G.T., de Crombrugghe, B., and Pastan, I.: Chicken embryo extracts contain a factor that perferentially blocks the accumulation of RNA polymerase II transcripts in a cell-free system. J. Biol. Chem. 257: 13001-13008, 1982.

Cerreghini, S., Herbomel, P., Jouanneau, J., Saragosti, S., Katinka, M., Bourrachot, B., de Crombrugghe, B., and Yaniv, M.: Structure and Function of the Promoter Enhancer Region of Polyoma and SV40. Cold Spring Harbor Symposium on Quantitative Biology, Vol. 47, in press.

Herbomel, P., de Crombrugghe, B., and Yaniv, M.: Relative Efficiencies of Eucaryotic Promoters in F9 Embryonal Carcinoma vs. differentiated cells as assayed by transient expression of chloramphenical acetyltransferase. Cold Spring Harbor Conferences on Cell Proliferation, Vol. 10, Teratocarcinoma Stem Cells, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08702-22 LMB

PERIOD COVERED
October 1, 1982 through September 30, 1983

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

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Seymour H. Wollman Chief, Cell Organization Section LMB NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Cell Organization Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	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.)*

The typical thyroid epithelial cell can take in colloid from the follicular lumen by macropinocytosis. It can also phagocytose red blood cells. We propose to study the mechanism of these processes by electron microscopy, histochemistry and related techniques.

(This project has been suspended during the year, but will be resumed at a later date).

PHS 6040 (Rev. 1/83) GPO 895-100

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08703-31 LMB

PERIOD COVERED
October 1, 1982 through September 30, 1983

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

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Seymour H. Wollman Chief, Cell Organization Section LMB NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Cell Organization Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	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.)*

This project has as its objectives to learn about: (1) The mechanism by which the thyroid gland maintains a concentration of iodide elevated above that of the blood and (2) the mechanism by which the thyroid gland forms and accumulates protein-bound iodine.

The work is largely based upon in vitro studies and makes use of autoradiography, cytochemistry at ultrastructural level, kinetic studies and inhibitors of steps in the synthesis of thyroid hormones.

(This project has been suspended during the year, but will be resumed at a later date).

PHS 6040 (Rev. 1/83) GPO 895-100

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08704-31 LMB

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Thyroid Growth and Involution

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Seymour H. Wollman Chief, Cell Organization Section LMB NCI

COOPERATING UNITS *(if any)*

Lucio Nitsch, Istituto di Patologia Generale, Universita di Napoli, Naples, Italy

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Cell Organization Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

To study the thyroid growth and differentiation: changes occurring during growth, hyperplasia and involution, properties of thyroid cells in separated follicles in suspension culture and influence of extracellular matrix on these properties.

PHS 6040 (Rev. 1/83)

GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Corrado Garbi	Visiting Fellow	LMB	NCI
Asima Majumder	Visiting Fellow	LMB	NCI
Carlo Tacchetti	Visiting Fellow	LMB	NCI

PROJECT DESCRIPTION:

Objectives: To study thyroid growth and differentiation and the properties of thyroid cells in separated follicles in suspension culture, including the influence of extracellular matrix on these properties.

Methods Employed: Thyroid gland was dissociated by collagenase treatment. Small clusters of epithelial cells were isolated from the dissociated tissue by centrifugation followed by filtration through fine nylon mesh. Clusters were cultured in suspension. They round up around a lumen within one day. Properties were examined by phase microscopy, autoradiography and electron microscopy.

Major Findings:

Effects of embedding separated thyroid follicles within a dense gel (with C. Garbi and A.L. Majumder): When separated thyroid follicles are embedded in a relatively dense but transparent collagen gel (1 mg/ml), cells are observed to migrate out of the follicles starting at about 12 hours after embedding in the gel. The cells are clearly the epithelial cells of the follicle since, if follicles are widely spaced, entire follicles disappear as all the cells migrate away. Also the cells have been observed to form long cytoplasmic projections from their base while still in contact with the lumen and while they maintain junctions with their neighbors. Initially, the cells activated to migrate may emerge in all directions, but after a day or so they tend to migrate toward other follicles that are nearby. Ultimately, the separate follicles approach each other by an unknown mechanism and form aggregates of follicles and unorganized cells.

Reversion of inverted thyroid follicles (with C. Garbi): Thyroid follicles in suspension culture invert when placed in 5% calf serum: the inversion process (turning inside out) occurs when the epithelial cells bounding the lumen reverse their polarity. The lumen of the inverted follicle is electron lucent and is bounded by a single layer of stretched cells. If these inverted follicles are embedded in a collagen gel in a medium containing 5% serum, the inverted follicles change within 2 days: the lucent lumens disappear, becoming filled with cells and multiple small electron dense lumens. The phenomenon is not a simple reinversion, and cell migration within the cell cluster appears to be a prominent feature following the embedding in the gel.

Significance for Cancer Research and the Program of the Institute: These studies are part of a program to determine the effects of extracellular matrix on normal thyroid cells. The observation that contact with collagen activates the cells to migrate suggests that we may be touching on aspects of the phenomenon of metastasis.

Proposed Course: We plan to try to ascertain what factors in the extracellular matrix may be involved in stabilization of thyroid epithelial cells against migration in a collagen gel.

Honors: Honorary M.D. degree awarded by the University of Goteborg Medical School (Goteborg, Sweden), October 23, 1982.

Publications:

Garbi, C., and Wollman, S.H.: Ultrastructure and some other properties of inverted thyroid follicles in suspension culture. Exp. Cell Res., 138: 343-353, 1982.

Tachiwaki, O., and Wollman, S.H.: Shedding of dense cell fragments into the follicular lumen early in involution of the hyperplastic thyroid gland. Lab. Invest., 47: 91-98, 1982.

Garbi, C., and Wollman, S.H.: Basal lamina formation on thyroid epithelia in separated follicles in suspension culture. J. Cell Biol. 94: 489-492, 1982.

Wollman, S.H., Herveg, J.P., and Smeds, S.: Lypolysis and blood capillary enlargement in adipose tissue pads on thyroids of rats fed thiouracil. Endocrinology 111: 1867-1873, 1982.

Smeds, S., and Wollman, S.H.: ^{3}H-Thymidine labeling of endothelial cells in thyroid arteries, veins and lymphatics during thyroid stimulation. Lab. Invest. 48: 285-291, 1983.

Smeds, S., and Wollman, S.H.: Capillary endothelial cell multiplication in adipose tissue pads on the thyroid during the feeding of thiouracil. Endocrinology 112: 1718-1722, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08705-07 LMB

PERIOD COVERED

October 1, 1982 through September 30, 1983

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 on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Michael M. Gottesman Chief, Molecular Cell Genetics Section LMB NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We are utilizing the Chinese hamster ovary (CHO) fibroblast and the cultured human KB carcinoma cell line to study the genetics and biochemistry of some aspects of the behavior of cultured cells. Our work has emphasized morphology and its relationship to growth control, response to cyclic AMP and transforming viruses and the role of receptors and receptor-mediated endocytosis of ligands in cell behavior. We have isolated a variety of different mutants with altered microtubules which express mutated α- or β-tubulin subunits. These mutants are defective in spindle formation. Mutants resistant to cyclic AMP have alterations in their cAMP dependent protein kinases. These mutants have been used to demonstrate that agents such as tumor promoters and interferon which raise intracellular cAMP levels do not work through a mechanism involving cAMP dependent protein kinases. Rous sarcoma virus transformed CHO cells are also resistant to growth inhibition by cAMP, but this resistance appears to be due to stimulation of viral src kinase by cyclic AMP stimulated phosphorylation. We are establishing general procedures for the isolation of mutants unable to internalize specific ligands and have isolated human KB cell mutants resistant to conjugates of Pseudomonas exotoxin and epidermal growth factor. These mutants are analyzed genetically using the techniques of somatic cell hybridization, gene cloning and gene transfer, and biochemically by classical enzymology, immunology, affinity labelling techniques and two-dimensional electrophoresis.

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Charles Roth	Guest Researcher	LMB	NCI
Irene Abraham	Expert	LMB	NCI
Carolyn Whitfield	Expert	LMB	NCI
Shin-ichi Akiyama	Visiting Associate	LMB	NCI
Paul Doherty	Visiting Fellow	LMB	NCI
George Vlahakis	Research Biologist	LMB	NCI
Margaret Chapman	Research Biologist	LMB	NCI

PROJECT DESCRIPTION:

Objectives: To determine by genetic manipulation of CHO and human KB cells the cell proteins and processes needed to maintain the cytoskeleton, response to cAMP, transformation by Rous sarcoma virus and receptor-mediated endocytosis of ligands.

Methods Employed: Cell culture, virus infections, isolation of cell behavior mutants, karyotyping and G-binding, somatic cell hybridization, gene transfer using DNA and chromosomes, recombinant DNA techniques, immunologic techniques including immunoprecipitation, immunolocalization and use of monoclonal antibodies; one and two-dimensional electrophoretic and chromatographic analysis of DNA, RNA and proteins; enzymologic analyses.

Major Findings:

1. We have continued to characterize the physiological defect in several CHO mutants we have isolated which express mutated α- and β-tubulins. These mutants are all temperature sensitive for growth. At the non-permissive temperature they make abnormal mitotic spindles, and cytokinesis is ineffective but they continue to synthesize DNA in a cyclic fashion, with the resultant accumulation of multinucleated cells which eventually die. Revertant cell lines selected for temperature resistance have either lost the altered tubulins or carry outside suppressors of the mitotic defect. These results indicated that both functional α- and β-tubulins are needed to form normal spindles, that normal spindle formation and cytokinesis is not essential for continued cell cycling and that it is possible to use a genetic approach (reversion analysis) to isolate mutants with altered non-tubulin proteins which interact with tubulin in the mitotic spindle.

2. As part of the molecular characterization of the defects in the tubulin mutants, we have isolated cDNA clones coding for both α- and β-tubulin from a CHO cDNA library cloned in the Berg-Okayama (full-length cDNA preferred) vector system. These clones will be used as probes to analyze the number of genes and the nature of the mRNAs expressed in our wild-type, tubulin

mutant and revertant cell lines.

3. Cyclic AMP resistant CHO mutants with altered cAMP dependent protein kinases isolated in the laboratory have been used to analyze the involvement of this enzyme in the mechanism of action of the tumor promoter TPA, which induces ornithine decarboxylase (ODC) in CHO cells. ODC induction by cyclic AMP is blocked in our protein kinase mutants, but TPA induction of ODC occurs in these mutants. These results indicate that TPA and cAMP work by independent mechanisms to stimulate ODC activity.

4. The cAMP dependent protein kinase mutants have also been used to determine whether the antiviral and antiproliferative effects of interferon treatment, which raises cAMP levels in many cells, are mediated through cAMP dependent protein kinase. Using encephalomyocarditis virus (EMCV) and human β-interferon we have found that the antiviral and antiproliferative effects of interferon occur in the protein kinase mutants. However, EMCV does not grow as well in these mutants as in the wild-type CHO cells, suggesting that viral maturation involves a step or steps dependent on the host cAMP dependent protein kinase.

5. cAMP treatment of Rous sarcoma virus (RSV) transformed CHO cells does not inhibit their growth. We have found that cAMP treatment of RSV-CHO stimulates the phosphorylation of the transforming protein of RSV, termed pp60^{src}, and that this phosphorylation correlates with an increase in the tyrosine phosphorylation activity of this viral protein. This increase in tyrosine kinase activity in transformed cells may counteract the usual growth inhibitory and morphological effects of cAMP on CHO cells, accounting for their continued growth in the presence of cAMP. We have also found that RSV-CHO cells treated with cholera toxin (which raises intracellular cAMP levels) are considerably more tumorigenic in nude mice than untreated RSV-CHO cells.

6. We have begun to isolate mutants in the human KB cell line which are defective in receptor mediated endocytosis. This cell line was chosen for study because of its rapid doubling time, high cloning efficiency, flat morphology and the presence on its surface of receptors to EGF and transferrin. Conjugates of Pseudomonas toxin with EGF efficiently kill KB cells in the presence of verapamil, and resistant mutants can be readily isolated. These resistant mutants fall into several classes, some of which are cross-resistant to other ligand-toxin conjugates (i.e. ricin-anti-transferrin receptor antibody), suggesting that they may have a general defect in receptor-mediated endocytosis.

Significance of Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 3.5

CHO and KB cells will cause tumors in appropriate hosts. The identification of mutant CHO and KB cells with specific defects in cell surface functions, ligand internalization, and in regulation of cellular growth and morphology will enable us to determine whether any of these functions are needed for tumor formation. Once this information is obtained, specific therapy aimed at

neutralizing those cell surface or cytoskeletal functions required for tumor formation can be devised.

Proposed Course: We plan to continue to isolate many classes of mutants with abnormal cell behavior and determine specific protein alterations in these mutants. We will continue to study mutants we have already isolated: spindles from tubulin mutant and revertant clones will be analyzed biochemically, the mechanism of these tubulin alterations at the DNA level will be determined, altered genes involved in the cAMP resistance phenotype will be cloned by gene transfer techniques and endocytosis mutants will be characterized genetically by dominance and complementation analysis and biochemically, by studies of ligand binding and uptake.

Publications:

Cabral, F., Abraham, I., and Gottesman, M.M.: Revertants of a CHO cell mutant with an altered β-tubulin: Evidence that the altered tubulin confers both colcemid resistance and temperature sensitivity on the cell. Mol. Cell Biol. 2: 720-729, 1982.

Pastan, I.H., Willingham, M., de Crombrugghe, B., and Gottesman, M.M.: Aging and cancer: Cyclic 3',5'-adenosine monophosphate and altered gene activity. Natl. Cancer Inst. Monogr. 60: 7-15, 1982.

Lichti, U., and Gottesman, M.M.: Genetic evidence that a phorbol ester tumor promoter stimulates ornithine decarboxylase activity by a pathway that is independent of cyclic AMP-dependent protein kinases in CHO cells. J. Cell Physiol. 113: 433-439, 1982.

Gottesman, M.M.: Using mutants to study cyclic AMP dependent protein kinase. Methods Enzymol. 1983, in press.

Abraham, I., and Gottesman, M.M.: Gene transfer in CHO cells. New York, N.Y., Raven Press, 1983, in press.

Gottesman, M.M., Roth, C., Leitschuh, M., Richert, N.D., and Pastan, I.H.: Genetic and biochemical analysis of cyclic AMP effects in transformed cells. Proc. ICN-UCLA Symp. 1983, in press.

Roth, C., Richert, N.D., Pastan, I.H., and Gottesman, M.M.: Cyclic AMP treatment of Rous sarcoma virus transformed CHO cells increases phosphorylation of pp60^{src} and increases pp60^{src} kinase activity. J. Biol. Chem. 1983, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08706-12 LMB

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Alteration in Gene Expression During Mammary Gland Tumorigenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Gilbert H. Smith Research Biologist LMB NCI

COOPERATING UNITS *(if any)*

LCMB, DCCP, NCI
Department of Cell Biology, Baylor University

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The inbred C3H/Sm mouse is unusual because, unlike most C3H strains, it has a low occurrence of mammary tumors and does not have a fully expressed MMTV proviral gene; that is, no MMTV antigens or virions are detectable in either normal or neoplastic mammary C3H/Sm tissues. Total RNAs from normal (C3Hf/He or C3H/Sm) and neoplastic (C3H/Sm) tissues were analyzed for amounts of MMTV specific sequences (dot blots or Northerns) and their relative sizes (Northerns). Increased amounts of MMTV transcripts were observed in the RNAs of spontaneous and experimentally-induced C3H/Sm mammary tumors compared to the RNAs from C3H/Sm lactating mammary gland. This suggests that regulatory processes influencing tumor maintenance may involve MMTV endogenous transcription. The RNAs from all C3H/Sm mammary neoplasias studied had increased amounts of a unique new LTR-containing sequence. This anomalous 2.2 Kb sequence may represent either an abnormal splice into an inappropriate MMTV sequence, an LTR-mouse transcript or a normally spliced MMTV transcript containing divergent sequences encoded within the 3' half of one or more of the 4 C3H/Sm proviral genes.

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATOR:

Dale E. Graham Expert LMB NCI

PROJECT DESCRIPTION:

Objectives: The aim of the project is to elucidate the genetic events in the mouse mammary gland leading to epithelial cell hyperplasia and eventually to neoplasia. Multiple factors, including virus, chemicals, hormonal stimulation of the gland and genetic susceptibility, have been identified as playing important roles in the development of mammary cancer. Therefore, our approach includes a multidisciplinary analysis of the changes in gene expression associated with mammary gland differentiation and development. Our objective is to identify and isolate genes or gene activities which may be important in the proliferation and maintenance of the neoplastic phenotype in mammary epithelium. We are presently examining mammary alveolar hyperplasias arising under the influence of a variety of oncagens (virus, chemical, hormone) and tumors which develop from these lesions. We are taking special interest in genes which become activated during growth and differentiation of normal mammary epithelium which are also expressed in transformed epithelium. To this end we have established a new mouse model for experimental breast cancer which gives promise of greater relevancy to the human condition. Special emphasis will be given to the physiological state of the epithelial tissue in relation to its response to a given carcinogenic agent.

Major Findings: The hormonal regulation of casein and α-lactalbumin genes was studied in stable serially-transplanted preneoplastic mammary tissues. All eight preneoplastic alveolar lines established from C3H/Sm mice were found to constitutively express these differentiation-specific genes. All preneoplasias were originally isolated from virgin females and were transplanted into gland-free fat pads of virgin mice. Three preneoplastic lines were developed from hyperplastic alveolar nodules (HANs) induced by MMTV, 3 were from HANs arising in C3H/Sm females carrying pituitary isografts (no MMTV) and 2 were induced by treatment of C3H/Sm virgins with DMBA. Although there was a wide disparity in the tumor incidence among these preneoplastic mammary populations, there was no correlation between tumor incidence and the extent of casein or α-lactalbumin gene expression. Increased synthesis rates of casein and α-lactalbumin were inducible in all the preneoplastic lines upon explant culture in the presence of hormones. Unlike normal virgin mammary epithelium, DNA synthesis was not required for hormonal stimulation of milk protein synthesis. Mammary tumors arising from these HAN populations did not show significant expression of casein or α-lactalbumin genes. Preliminary observations have indicated that the expression of casein and α-lactalbumin genes is stimulated in the normal mammary glands of virgin females carrying preneoplastic mammary transplants. The mechanism(s) which may underlie this

unusual activity is presently under study. Preliminary experiments with keratinizing and ductal mammary preneoplasias indicate that neither casein nor α-lactalbumin genes are activated during these transformations. Unlike HAN-transplant bearing females, normal mammary tissues in mice carrying keratinizing mammary preneoplasias are not stimulated to express their milk protein genes. Further examination of the interrelationship of specific gene activities and preneoplasia are planned.

Endogenous retroviral genes appear to play an important role in mammary tumorigenesis. None of the four MMTV proviral genes in C3H/Sm mice are fully expressed in normal or neoplastic mammary tissues. Nevertheless, transcription of one or more of these genes occurs during mammary gland differentiation (pregnancy and lactation) and MMTV-specific RNA is detectable in normal or neoplastic glands at a relatively high concentration considering the fact that MMTV virions and virus-specific structural proteins are invariably absent. We have demonstrated that the equilibrium levels of MMTV RNA increases during preneoplastic transformation of C3H/Sm mammary epithelium and becomes even higher in mammary tumors. This increase was not accompanied by the occurrence of MMTV antigens or virions. Examination of the RNA transcripts from C3H/Sm mammary tumors has revealed an increase in the MMTV transcripts found in normal C3H/Sm mammary tissue and in addition an apparently new transcript which contained the MMTV LTR (promoter) sequences but not sequences homologous with other MMTV genes (env, gag, pol). This new transcript was found in spontaneous, chemically and hormonally induced C3H/Sm mouse mammary carcinomas. This anomalous transcript may represent an abnormal splice into an inappropriate MMTV sequence, an LTR-mouse gene transcript or a MMTV transcript containing divergent sequences encoded within the 3' half of one of the 4 C3H/Sm MMTV proviral genes. In order to analyze these possibilities, we have made a cDNA recombinant library which we are screening for LTR-containing plasmids that are negative for MMTV env, gag and pol sequences. Isolation and characterization of these LTR-containing recombinant DNAs will lead to the identification of this mammary gland transformation-specific sequence.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 3.

Mouse mammary gland tumorigenesis is a relevant experimental model for understanding human mammary neoplasia. The project is designed to provide a scientific basis to further our understanding of cellular and genetic events controlling normal development and differentiation and their relationship to malignant transformation of the mammary gland.

Proposed Course: We propose to define the tumorigenic influence of endogenous retroviral (MMTV) genes and their possible relationship to normal cellular differentiation. This will be accomplished by studying the regulation of expression of differentiation-specific genes in mammary epithelium in relationship to the expression of MMTV proviral sequences. These events will be examined over the course of malignant transformation by comparing the regulation of these in normal, preneoplastic and malignant mammary tissues.

Publications:

Medina, D., Socher, S.H., Smith, G.H., Dusing-Swartz, S., Arthur, L.O., and Butel, J.S.: Separate pathways for viral and chemical carcinogenesis in the mouse mammary gland. In Rich, M., and Furmanski, P. (Eds.): Biological Carcinogenesis. Marcel Dekker, Inc., New York, 1981, pp 169-182.

Smith, G.H., and Vlahakis, G.: Separation of high mammary tumor incidence from high hepatoma incidence in backcross mice during segregation of the viable yellow gene. Int. J. Cancer 29: 587-590, 1982.

Smith, G.H., Henry, T.J., Vlahakis, G., and Arthur, L.O.: Suppression of spontaneous mammary tumorigenesis despite Mtv-1 gene expression in hybrid and backcross C3H-A^{vy}fb x C3H/Sm mice. Int. J. Cancer 29: 591-598, 1982.

Drohan W.W., Benade, L.E., Graham, D.E., and Smith, G.H.: MMTV proviral sequences congenital to C3H/Sm mice are differentially hypomethylated in chemically-induced, virus-induced and spontaneous mammary tumors. J. Virol. 43: 876-884, 1982.

Hogan, D., and Smith, G.H.: Unconventional application of standard light and electron immunocytochemical analysis to aldehyde-fixed, araldite-embedded tissues. J. Histochem. Cytochem. 30: 1301-1306, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08707-10 LMB

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Relationship of Genetic and Non-Genetic Factors in Mouse Tumorigenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

George Vlahakis	Research Biologist	LMB	NCI
Bernard Sass	Research Pathologist	CGT	NCI

COOPERATING UNITS *(if any)*
Carcinogenesis Testing Program, DCCP, NCI

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Cell Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	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.)*

Collaborative work continues on the relationships of genetic and non-genetic factors in spontaneous mouse tumorigenesis. A histogenesis study will be started this year on the spontaneous occurring ovarian tumors observed in our strains of female mice. A variety of other tumors will also be of interest.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

PROJECT DESCRIPTION:

Objectives: To study the relationship of genetic and non-genetic factors in mouse tumorigenesis.

Findings and Results: A manuscript with Bernard Sass of the Registry of Experimental Cancer, Division of Cancer Cause and Prevention, has now been published in Toxicological Pathology. It deals with the histogenesis and biology of spontaneous mammary gland lesions occurring in female mice. One of the precursor lesions we studied was the hyperplastic alveolar nodule from which mammary tumors arise in females carrying mammary tumor virus (MTV). Another precursor lesion of interest to us was the plaque which occurs in several of the high spontaneous mammary tumor mouse strains not native to the United States. We were also interested in the hormone dependent mammary gland tumors observed during pregnancy in the European strain GR. These hormone dependent tumors normally regress at parturition but reappear in subsequent pregnancies and eventually become malignant. One morphologically distinct mammary tumor occurring in GR and classified as pale cell carcinoma was of particular interest because it apparently is associated with the line of MTV present in strain GR.

This year we will prepare for publication a paper on the spontaneous occurring ovarian tumors observed in our laboratory strains of female mice. We also plan to provide slides of the ovarian tumors as a study set for the Registry of Experimental Cancer here at NIH.

Two papers with Gilbert Smith of our laboratory have now been published in the International Journal of Cancer. They deal with the biology, virology and biochemistry of spontaneous mammary gland tumors in hybrid and backcross female mice made with the high mammary tumor and hepatoma strain C3H-A^{vy}fB and the low tumor strain C3H/Sm.

Significance to Cancer Research and the Program of the Institute: National Cancer Plan Objective 1, Approach 4.

The occurrence of spontaneous tumors in laboratory mouse strains depends on the interplay of several factors: host genotype, viruses, hormones and environment. Mouse mammary tumorigenesis, for example, is an area where further study of oncogenic relationships considering all of the factors might provide insights into the disease process in humans.

Publications:

Sass, B., Vlahakis, G., and Heston, W.E.: Precursor lesions and pathogenesis of spontaneous mammary tumors in mice. Toxicol. Pathol. 10: 12-21, 1982.

Smith, G.H., and Vlahakis, G.: Separation of high mammary tumor incidence from high hepatoma incidence in backcross mice during segregation of the viable yellow gene. Int. J. Cancer 29: 587-590, 1982.

Smith, G.H., Henry, T.J., Vlahakis, G., and Arthur, L.O.: Suppression of spontaneous mammary tumorigenesis despite Mtv-1 gene expression in hybrid and backcross C3H-A^{vy}fB x C3H/Sm mice. Int. J. Cancer 29: 591-598, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08709-08 LMB

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
NAD^+ Metabolism and ADP-ribosylation of Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

George S. Johnson Research Chemist LMB NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Molecular Cell Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

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

B

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

A possible involvement of ADP-ribosylation of chromosomal proteins in the regulation of specific gene expression has been explored. Inhibitors of ADP-ribosylation increased the levels of mRNA for growth hormone and prolactin in cultured GH_3 cells and mouse mammary tumor virus (MMTV) in cultured mammary tumor cells. In the latter system endogenous ADP-ribosylation of chromosomal proteins was analyzed. As expected, the inhibitors decreased ADP-ribosylation, but interestingly, glucocorticoids, known regulators of MMTV synthesis, also decreased ADP-ribosylation. This action of the steroids was very rapid; the fall in ADP-ribose was noted within 30 min, the time span in which mRNA synthesis increases. The results show that a decrease in ADP-ribosylation is associated with an increase in mRNA synthesis and that a decrease in endogenous ADP-ribosylation may be an essential step in glucocorticoid activation of specific genes.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATOR:

Sei-ichi Tanuma Visiting Fellow LMB NCI

PROJECT DESCRIPTION:

Objectives: Nicotinamide adenine dinucleotide (NAD^+) is known to undergo two major types of reactions in mammalian cells, reduction to NADH and cleavage at the nicotinamide-ribose linkage to form nicotinamide and adenosine diphosphate ribose. ADP-ribose can be covalently attached to proteins (mono ADP-ribosylation) or it can be polymerized to poly (ADP-ribose). NAD^+ is present in the cell at a concentration of about 0.1-0.3 mM, and since the half time for degradation of total cellular NAD^+ at this cleavage site is just 1-2 hours, this reaction is a major cellular activity. Despite an extensive research effort from numerous laboratories, the biological purpose for this NAD^+ cleavage reaction remains obscure. The object of this project is to understand the regulation of NAD^+ metabolism and to discover and evaluate the physiological functions for ADP-ribosylation of proteins.

ADP-ribosylation of proteins is primarily nuclear. Thus, this covalent modification may serve to regulate a nuclear function(s). It is our specific goal to evaluate and understand a possible role for ADP-ribosylation in regulation of gene expression.

Methods Employed: Standard culture techniques will be used to grow cells. Thin layer chromatography, high performance liquid chromatography and two dimensional gel electrophoresis will be used to analyze metabolic components and proteins. Standard biochemical techniques will be used to measure NAD^+, RNA metabolism and protein synthesis. mRNA will be detected and quantitated by hybridization to specific cDNA sequences.

Major Findings: Cultured rat pituitary tumor cells (GH_3 cell line) synthesize growth hormone (GH) and prolactin (Prl). When the cells are treated with agents which inhibit ADP-ribosylation, syntheses of these two proteins are increased; syntheses of most other proteins are unaffected. Thyroid hormone is a physiological stimulator of GH synthesis. GH synthesis is increased synergistically when GH_3 cells are treated with both thyroid hormone and ADP-ribosylation inhibitors. GH and Prl mRNAs increase in parallel with the increases in protein syntheses.

In mouse mammary tumor cells, another cell culture system tested, the ADP-ribosylation inhibitors increased mouse mammary tumor virus (MMTV) RNA levels. This treatment decreased endogenous ADP-ribosylation of chromosomal proteins, especially the high mobility group 14 and 17 proteins. Glucocorticoids, which are known regulators of MMTV RNA synthesis, also decreased endogenous ADP-ribosylation of these proteins. The decrease in ADP-ribose

content was observed within 30 min, the time period required for induction of MMTV RNA synthesis. The association between loss of ADP-ribose from chromosomal proteins by inhibitor or steroid treatment and the increase in RNA levels suggest that ADP-ribosylation may influence expression of specific genes.

Significance of Cancer Research and the Program of the Institute: By understanding how metabolism and gene expression are regulated and by understanding differences in this regulation between normal and transformed cells, we may learn to control the growth of certain cancer cells.

Proposed Course: The results obtained thus far are suggestive of a role for ADP-ribosylation in regulation of some genes. In future studies we will attempt to learn how ADP-ribosylation affects this gene expression.

1. In the cell systems discussed above, GH, Prl and MMTV genes are part of nuclear chromosomes. The biochemical complexity of chromosomes makes it difficult to analyze effects of ADP-ribosylation on the expression of specific genes. Thus, it is desirable to have these genes replicated and expressed in simpler genetic systems. In this regard, Drs. Michael Ostrowski and Gordon Hager of the Laboratory of Tumor Virus Genetics, NCI, have succeeded in inserting the MMTV gene within the bovine papilloma virus genome and therefore replicate as an episome. We plan to pursue the effects of ADP-ribosylation on the expression of the MMTV gene in this episomal system in collaboration with these investigators.

2. It is important to establish if ADP-ribosylation inhibitors increase initiation or stimulate ongoing RNA synthesis. Classical "run off" experiments will be done to indicate if initiation is affected. We will work towards developing an in vitro system where RNA synthesis can be initiated under steroid regulation. Hopefully, the episomal system will be useful in this approach.

3. We need to know which chromosomal proteins are ADP-ribosylated in intact cells. Our analysis thus far in mouse mammary tumor cells has been limited to a protein fraction enriched in high mobility group proteins and histone H_1. This fraction was selected because of ease of extraction and also because these proteins are known to be acceptors of ADP-ribose in isolated nuclei. We will expand our analysis to additional chromosomal proteins. We will study endogenous ADP-ribosylation in GH_3 cells where thyroid hormone and glucocorticoid effects on mRNA synthesis can be correlated with ADP-ribosylation of specific chromosomal proteins. Also, the pattern of ADP-ribosylation of proteins in the MMTV-episomal system will be determined since proteins which are involved in regulation of MMTV RNA synthesis should be enriched in this episome.

4. Our initial observations indicated that glucocorticoids decreased ADP-ribosylation by stimulating removal (activation of $(ADP\text{-ribose})_n$ glycohydrolase) rather than by inhibiting ADP-ribose synthesis. Thus, blocking glycohydrolase activity may prevent glucocorticoid induction of MMTV RNA synthesis. At this time there are no specific inhibitors of this enzyme known. We are testing numerous adenine and ribose derivatives in an attempt to discover one.

Publications:

Tamuma, S., and Johnson, G.S.: ADP-ribosylation of nonhistone high mobility group proteins in intact cells. J. Biol. Chem. 258: 4067-4070, 1983.

Johnson, G.S., and Jaworski, C.J.: Glucocorticoids increase GTP-dependent adenylate cyclase activity in cultured fibroblasts. Mol. Pharmacol. 23: 648-652, 1983.

Kimura, N., Kimura, N., Cathala, G., Baxter, J.D., and Johnson, G.S.: Nicotinamide and its derivatives increase growth hormone and prolactin synthesis in cultured GH_3 cells: Role for ADP-ribosylation in modulating specific gene expression. DNA 1983, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CB08710-08 LMB
(Z01CB08711-07 LMB)
(Combined with above)

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
DNA Replication and Recombination In Vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Sue Wickner, Research Chemist, LMB, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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

B

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

The molecular mechanisms involved in DNA replication are being studied. Proteins involved in both phage lambda and E. coli replication are being purified and characterized in vitro. The present emphasis is to gain insight into the process of initiation of chromosome replication. The replication of double-stranded circular lambda dv DNA is being studied in crude extracts of uninfected E. coli supplemented with the two phage proteins necessary for replication.

The biochemistry of lambda site-specific recombination is also being studied. The proteins required for excisive recombination have been purified and are being characterized. The roles of the various proteins in recombination is being pursued.

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

PROJECT DESCRIPTION:

Objectives: The objective is to gain information about basic biochemical mechanisms involved in DNA replication and recombination by purifying and analyzing the proteins required for these reactions and reconstituting the pathways of replication and recombination with purified components.

Methods Employed: Standard microbial genetic and biochemical techniques required for protein purification, enzyme assays, plasmid construction, DNA preparation and nucleic acid sequencing are being used.

Major Findings: (1) Initiation of phage lambda DNA replication. Initiation of lambda and lambda dv plasmid replication in vivo requires two phage proteins, the O and P gene products in addition to several E. coli proteins. Replication is initiated at a unique site, ori, on the lambda chromosome and proceeds bidirectionally. RNA synthesis at or near the origin is also required for initiation although the exact role of transcription is not clear. In vitro the O protein binds specifically to four 19-base-pair direct repeats in the ori region. The purified P protein forms a molecular complex with E. coli dnaB protein. Genetic studies suggest that P also interacts with O, dnaJ, dnaK, grpD and grpE proteins. Most likely, these interactions of phage and host proteins are responsible for the assembly of a replisome structure at ori. Lambda replication also requires E. coli dnaG primase and DNA polymerase III components (dnaE, dnaN, dnaX, dnaY, dnaZ and dnaQ) suggesting that once phage replication is initiated, elongation occurs by the same mechanism as elongation of the E. coli chromosome.

In collaboration with Ken Zahn and Fred Blattner, we have cloned the O and P genes in an expression vector containing the repressible lambda pL promoter. The amino terminal half of the O gene was also cloned. We developed methods for purifying large quantities of these three proteins to homogeneity. The binding of the O protein and amino terminal O fragment polypeptide to lambda ori^+ DNA and several ori^- mutant DNA is being characterized by nitrocellulose filter binding and dimethyl sulfate protection experiments. We found that the O protein binds to the four available ori^- mutant DNAs, suggesting that the block in DNA replication of these mutants is at some step other than O binding. We also found that the amino terminal O fragment polypeptide binds specifically to lambda ori DNA thus confirming the genetic results that the specificity for DNA binding resides in the amino terminal half of O. We are looking for evidence to confirm genetic experiments that suggest that the O and P proteins interact. The results are not conclusive. No enzymatic activities have been found associated with either O or P proteins yet. We are further characterizing these proteins in the expectation of learning more about their roles in replication.

We are also studying the initiation of lambda replication by using an in vitro system that replicates lambda dv plasmid DNA. The system is essentially that of Fuller, Kaguni and Kornberg. Replication requires a crude extract of uninfected E. coli, purified O and P proteins, lambda dv DNA, an ATP regenerating system, polyethylene glycol, 4 rNTPs, 4 dNTPs and Mg^{+2}. Using this system we are attempting to isolate nascent DNA and determine the exact nucleotide in the ori region where DNA synthesis is initiated on each strand and whether or not there is RNA at the 5' ends of the nascent DNA.

(2) E. coli DNA replication proteins. In collaboration with J. Walker and D. Mullen, we are studying several new temperature sensitive E. coli mutants blocked in DNA replication. Our results show the one of these, dnaY, has temperature sensitive DNA polymerase III activity in vitro compared to wild-type when assayed with activated salmon sperm DNA as template. This suggests that the defect is in a subunit of DNA polymerase III core.

(3) Lambda excisive recombination. Lambda inserts into and excises from the E. coli chromosome. Integration requires a specific site on the phage (attP), a specific site on the host chromosome (attB), the lambda Int protein and a host protein (IHF). The reverse reaction, excision, between the left (attL) and right (attR) ends of the prophage requires, in addition to Int and IHF, the lambda Xis protein. In collaboration with J. Auerbach, we have developed methods for extensively purifying the proteins required for these reaction. We are characterizing the individual components and currently focusing on the protein structure of IHF. Our preliminary results suggest that the two subunits can be separated by chromatographic procedures in the absence of denaturing agents and we are studying the two proteins individually.

In collaboration with H. Echols, M. Better and J. Auerbach, we observed by electron microscopy that Int and Xis together produce a stable condensed complex with attR DNA while neither protein alone does. It had been shown previously that Int protein alone forms a stable condensed protein-DNA complex with the attP and attL substrate sites, but not with the attB or attR sites. The attR complex involves the P region DNA to the left of the crossover point (O site). In contrast, the attP complex includes DNA on both sides of the crossover point (P and P') and the attL structure involves P' DNA to the right of O. In the presence of Int and Xis, the attL and attR sites form a paired structure. These results suggest that the role of Xis may be to provide a distinct reactive structure at attR allowing attL and attR to pair efficiently.

In collaboration with Bruce Howard, who is constructing a vector system for shuttling DNA between bacterial and mammalian cells, we are developing in vitro conditions for excising DNA flanked by lambda prophage attachment sites from mammalian chromosomal DNA. Towards this end, we have been successful in excising DNA flanked by the attL and attR attachment sites from E. coli chromosomal DNA using purified proteins.

Significance for Cancer Research and the Program of the Institute: The central process of heredity and cell growth is the replication of the genetic material. Studies of this process in E. coli have been made possible by the availability of (a) mutants defective in DNA synthesis and (b) large amounts of bacteria necessary for biochemical studies. It is expected that the understanding that this work is generating about the biochemical mechanisms of DNA replication in E. coli will shed light on the nature of the same reactions in animal cells. It is also expected that the studies of lambda integration and excision may serve as a model for understanding the integration and excision mechanisms of animal viruses.

Proposed Course: We will continue studying biochemical mechanisms involved in DNA replication and recombination using purified proteins and defined DNA templates.

Publications:

Better, M., Wickner, S., Auerbach, J., and Echols, H.: Role of the Xis protein of bacteriophage lambda in a specific reactive complex at the attR prophage attachment site. Cell 32: 161-168, 1983.

Furth, M. E., and Wickner, S. H.: Lambda DNA replication. In Hendrix, R., Roberts, J., Stahl, F., and Weisberg, R. (Eds.): Bacteriophage Lambda, Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1983, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08712-08 LMB

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Ira Pastan Chief, Laboratory of Molecular Biology LMB NCI

COOPERATING UNITS *(if any)*

Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research

Jimenez Diaz Foundation, Madrid, Spain

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.4	3.4	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We have identified a new organelle, the receptosome, that carries ligands from the clathrin coated pits on the surface of fibroblasts to the Golgi apparatus. Ligands carried by this pathway include α_2-macroglobulin, epidermal growth factor, insulin, low density lipoprotein, β-galactosidase and some viruses. Ligands that eventually end up in lysosomes first traverse the reticular portion of the Golgi and are concentrated in coated pits of the Golgi before they enter lysosomes. We believe that receptosomes form directly from the coated pits of the cell surface and not by uncoating of coated vesicles.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER PRINCIPAL INVESTIGATOR:

Mark C. Willingham	Chief, UCS	LMB	NCI

OTHER INVESTIGATORS:

Robert B. Dickson	Guest Worker	LMB	NCI
Richard Schlegel	Assistant Senior Surgeon	LMB	NCI
John Hanover	Postdoctoral Fellow	LMB	NCI
Laura Beguinot	Guest Worker	LMB	NCI
Raymond Lyle	Guest Worker	LMB	NCI
Nancy Richert	Senior Staff Fellow	LMB	NCI
Elliott Schiffmann	Research Chemist	LDBA	NIDR
José M. Mato	Jimenez Diaz Foundation, Madrid, Spain		

PROJECT DESCRIPTION:

Objectives: To identify the membrane proteins that participate in cell adhesion, cell movement and regulate cell metabolism and growth.

Methods Employed: Cell culture, preparation and analysis of membrane proteins and their effects on cell behavior, isolation of mutants with defective membrane proteins.

Major Findings: R. Dickson and J. Hanover have developed a subcellular fractionation method to analyze the compartments ligands traverse as they enter cells through coated pits and ultmately reach lysosomes. They have used this method to compare the entry of epidermal growth factor (EGF) and transferrin and have found that EGF moves from the plasma membrane to a receptosome-Golgi fraction and then on to lysosomes where it is degraded. Transferrin and EGF enter the cells together from the plasma membrane and rapidly enter the receptosome-Golgi fraction. Transferrin then returns to the cell surface; very little is found in the lysosome fraction. They have confirmed their biochemical analyses using immunofluorescence to detect the location of transferrin and EGF which at early times are found together in receptosomes but at later times diverge.

Using a careful homogenization method to prepare intact receptosomes, R. Dickson, L. Beguinot and J. Hanover have developed a method to purify receptosomes which involves four steps, a Percoll gradient, a Sephacryl S-1000 column and two sucrose gradients. They have characterizd the purified receptosomes and showed that they are rich in cholesterol and have a unique polypeptide pattern when analyzed by SDS gel electrophoresis. In addition using specific antibodies they have shown them to contain transferrin receptors and phosphomannosyl receptors. They do not contain detectable amounts of clathrin.

J. Hanover has developed a method to solubilize the α_2-macroglobulin receptor and has purified this receptor several thousand fold using conventional column chromotography followed by affinity chromotography. The sub-unit molecular weight of the solubilized purified receptor is approximately 85,000 and behaves as a dimer. Cultured cells have two classes of binding sites for α_2-macroglobulin, a high and a low affinity class. Hanover has isolated the high affinity component.

N. Richert has prepared a monoclonal antibody to A431 cell membranes which recognizes the epidermal growth factor receptor. The antibody recognizes a carbohydrate determinant on the receptor of A431 cells but does not react with the receptor of several other cell types. The antibody can be used for isolation of the receptor in a non-activated form. In collaboration with V. Ginsberg, NIAMDD, and co-workers the determinant with which the antibody reacts has been identified. It is the human H Type I determinant and is found on glycolipids and glycoproteins of A431 cells.

Significance for Cancer Research and the Program of the Institute: Understanding how proteins enter cells will help to design better agents for cancer treatment.

Proposed Course: Using purified receptosomes we will try and identify the mechanism by which receptosomes become acid by assaying for a proton ATPase. We will use receptosomes to make monoclonal antibodies to the various components and use these antibodies to establish the function of these proteins. We will attempt to isolate Golgi fractions and study the interaction of receptosomes with Golgi fractions with which they fuse in vivo. We will try and determine how transferrin is sorted in the Golgi and appears at the cell surface.

Publications:

Dickson, R.B., Schlegel, R., Willingham, M.C., and Pastan, I.: Binding and internalization of α_2-macroglobulin by cultured fibroblasts: Effects of monovalent ionophores. Exp. Cell Res. 142: 127-140, 1982.

Via, D.P., Willingham, M.C., Pastan, I., Gotto, A.M., Jr., and Smith, L.C.: Coclustering and internalization of low density lipoproteins and α_2-macroglobulin in human skin fibroblasts. Exp. Cell Res. 141: 15-22, 1982.

Willingham, M.C. and Pastan, I.H.: Image intensification techniques for detection of proteins in cultured cells by fluorescence microscopy. Methods Enzymol. 1983, in press.

Dickson, R.B., Schlegel, C.R., Willingham, M.C., and Pastan, I.H.: Involvement of Na^+ and HCO_3^- in receptor-mediated endocytosis of α_2-macroglobulin, epidermal growth factor, and vesicular stomatitis virus. J. Cell. Physiol. 113: 353-358, 1982.

Cheng, S.-y., Merlino, G.T., and Pastan, I.: A versatile method for the coupling of protein to DNA: synthesis of α_2-macroglobulin-DNA conjugates. Nucleic Acids Res. 11: 659-669, 1983.

Willingham, M.C., Haigler, H.T., FitzGerald, D.J.P., Gallo, M.G., Rutherford, A.V., and Pastan, I.H.: The morphological pathway of binding and internalization of epidermal growth factor in cultured cells: Studies on A431, KB, and 3T3 cells using multiple methods of labeling. Exp. Cell Res. 1983, in press.

Schlegel, R., Dickson, R.B., Willingham, M.C., and Pastan, I.H.: Amantadine and dansylcadaverine inhibit vesicular stomatitis virus uptake and receptor-mediated endocytosis of α_2-macroglobulin. Proc. Natl. Acad. Sci. U.S.A. 79: 2291-2295, 1982.

Dickson, R.B., Willingham, M.C., and Pastan, I.H.: Receptor-mediated endocytosis of α_2-macroglobulin: Inhibition by ionophores and stimulation by Na^+ and HCO_3^-. Ann. N.Y. Acad. Sci. 1983, in press.

Willingham, M.C., and Pastan, I.H.: Receptor-mediated endocytosis of peptide hormones and macromolecules in cultured cells. In Weinstein, I.B., and Vogel, H.J. (Eds.): Genes and Proteins in Oncogenesis. New York, Academic Press, 1983, pp. 183-195.

Horiuchi, R., Johnson, M.L., Willingham, M.C., Pastan, I., and Cheng, S.-y.: Affinity labeling of the plasma membrane 3,3',5-triiodo-L-thyronine receptor in GH_3 cells. Proc. Natl. Acad. Sci. U.S.A. 79: 5527-5531, 1982.

Schlegel, R., Tralka, T.S., Willingham, M.C., and Pastan, I.H.: Inhibition of VSV binding and infectivity by phosphatidylserine: Is phosphatidylserine a VSV binding site? Cell 32: 639-646.

Mato, J.M., Pencev, D., Vasanthakumar, G., Schiffmann, E., and Pastan, I.: Inhibitors of endocytosis perturb phospholipid metabolism in rabbit neutrophils and other cells. Proc. Natl. Acad. Sci., U.S.A. 80: 1929-1932, 1983.

Pastan, I., and Willingham, M.: What receptor-mediated endocytosis tells us about bi-directional processes in cultured cells. In Oplatka, A., and Balaban, M. (Eds.): Biological Structures and Coupled Flows. New York, Academic Press, 1983, pp. 307-316.

Richert, N.D., Willingham, M.C., and Pastan, I.: Epidermal growth factor receptor: Characterization of a monoclonal antibody specific for the receptor of A431 cells. J. Biol. Chem. 1983, in press.

Pastan, I., and Willingham, M.: Receptor-mediated endocytosis: Coated pits, receptosomes and the Golgi. Trends in Biochem. Sci. 1983, in press.

Hanover, J.A., Willingham, M.C., and Pastan, I.: Receptor-mediated endocytosis of α_2M: Solubilization and partial purification of the fibroblast α_2M receptor. N.Y. Acad. Sci. 1983, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08714-06 LMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mode of Action of a Bacterial Function Involved in Cell Growth Control

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Susan Gottesman, Research Chemist, LMB, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	2.6	0.0

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

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

We have been studying the role that protein degradation plays in regulating cell growth control, through the study of a mutant defective in protein degradation, the E. coli lon mutant. This strain is defective in cell division regulation after DNA damage; we have demonstrated that this defect is due to accumulation of a highly unstable cell division inhibitor, the product of the SulA gene. In addition, we have shown that, in vitro, the SulA gene is transcribed from a single promoter which is repressed by the LexA repressor, as part of an integrated response to DNA damage known as the SOS system. lon mutants also overproduce capsular polysaccharide, and we have developed a system for the simple assay of the regulation of the genes necessary for capsule synthesis (cps), using cps-lac operon fusions. Using these strains, we have isolated and mapped mutations in two previously unknown genes which regulate capsule synthesis (cpsR and cpsS). Further work will concentrate on the interaction of lon, the cps regulators, and the structural genes which are regulated.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Patsy Trisler	Research Biologist	LMB	NCI
Angel Torres-Cabassa	Staff Fellow	LMB	NCI

PROJECT DESCRIPTION:

Objectives: We are interested in the role which proteolysis can play in regulating important cell timing functions. As an approach to this, we are investigating the effects on cell growth of a mutation in a gene coding for an ATP-dependent protease.

Methods Employed: Standard microbial genetic and biochemical techniques.

Major Findings: (1) We had previously shown that the product of the sulA gene, genetically identified as an inhibitor of cell division, is highly unstable. This instability is due to the lon-coded protease, since mutations in lon render SulA stable. From the cloned sulA gene, we have constructed appropriate substrates for in vitro transcription experiments. These in vitro experiments have led to the demonstration of a single transcription start for the gene, and a demonstration that transcription is repressed by the LexA repressor.

(2) We have shown that sulA overproduction is sufficient to cause filamentation in cells, supporting the notion that SulA is an inhibitor of cell division.

(3) Using operon fusions of the lacz gene to promoters involved in capsular polysaccharide synthesis, we have identified at least 6 genes whose synthesis is regulated by the lon system. Five of these genes map near 23 minutes on the E. coli map, while the other maps at 90 minutes. Fusions in the galactose operon as well as the manA gene show little if any regulation via lon.

(4) Using the operon fusions described above, in genes we have designated cpsA-F, we have isolated regulatory mutations, in addition to further lon mutations, which regulate capsule synthesis. Two of these regulatory loci, cpsR and cpsS, are being studied in detail. Mapping of the regulatory mutations places cpsR at about 18 minutes on the E. coli map, and cpsS at 47 minutes. Both are far from the structural genes which they regulate. We have cloned the cpsR locus onto a lambda vector, characterized the restriction pattern for the locus, and isolated mutations on the cloned piece. This will enable us to identify the cpsR protein and initiate studies on its mode of action.

(5) Using a previously isolated clone of the lon gene in a λ vector, we are using a mini-Tet insertion element to isolate insertion mutations in the lon gene, which can then be transferred into the chromosome. This type of genetic analysis will both define the functional limits of the lon gene and will help us determine if lon is an essential cell function.

(6) From our results thus far, we have developed a possible model for lon control of capsule synthesis. We believe that lon acts indirectly to control synthesis from the capsular polysaccharide operons, by regulating the half-life of a regulatory protein, possibly CpsR. CpsR may act as a positive regulator of capsule synthesis, possibly through interaction with CpsS.

Significance for Cancer Research and the Program of the Institute: An understanding of the growth control of E. coli may serve as a model for understanding growth control in normal and transformed mammalian cells. Protein degradation is believed to play an important role in the control of mammalian cells; insight into such processes should be gained by further investigation of the process in E. coli.

Proposed Course: Elucidate the mechanism of regulation of capsular polysaccharide synthesis, at the genetic and biochemical level. Analyze the mechanism of lon degradation of cellular substrates and the reasons for protein instability, using the lon substrates as model systems. Use the methods developed in the study of the lon substrates to genetically define other E. coli proteolytic systems and their substrates. Begin a study of the mechanism of cell division regulation, using the cell division inhibitor, SulA, as a probe which will specifically disrupt cell division.

Publications:

Mizusawa, S., and Gottesman, S.: Protein degradation in Escherichia coli. The lon gene controls the stability of sulA protein. Proc. Nat. Acad. Sci. 80: 358-362, 1983.

Mizusawa, S., and Ward, D. F.: A bacteriophage lambda vector for cloning with BamHl and Sau 3a. Gene 20: 317-322, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08715-05 LMB

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Control of Synthesis of a Transformation-Dependent Secreted Glycoprotein

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Michael M. Gottesman Chief, Molecular Cell Genetics Section LMB NCI

COOPERATING UNITS *(if any)*

Laboratory of Experimental Pathology, DCCP, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	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.)*

Cultured mouse fibroblasts which are transformed by RNA viruses, a DNA virus or a chemical agent, all secrete a 35,000 Mr-phosphoglycoprotein (major excreted protein, MEP) in large amounts. Nontransformed murine fibroblasts secrete this protein in much lower amounts but can be stimulated to synthesize and secrete MEP by treatment with tumor promoters or growth factors. We have purified MEP, prepared monospecific antisera against it and cloned a cDNA which codes for MEP. The protein contains mannose 6-phosphate, the lysosomal recognition marker. It is processed intracellularly to give two specific lower molecular weight forms, the lowest of which has a predominantly lysosomal localization. Transformation and tumor promoters stimulate MEP synthesis by increasing levels of MEP specific mRNA. We are studying this system as a model of regulation of lysosomal protein synthesis, processing and secretion as it is affected by transformation and agents which mimic the transformed state, such as tumor promoters.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

George Vlahakis	Research Biologist	LMB	NCI
Margaret Chapman	Research Biologist	LMB	NCI
Ling Hua	Visiting Fellow	LMB	NCI
Paul Doherty	Visiting Fellow	LMB	NCI
Susannah Gal	Chemist	LMB	NCI

PROJECT DESCRIPTION:

Objectives: To determine the mechanism of the control of synthesis, processing and secretion of the major secreted protein of murine fibroblasts.

Methods Employed: Cell culture; radiolabeling of cell proteins, electrophoretic and chromatographic techniques; immunoprecipitation; in vitro translation of mRNAs; recombinant DNA technology including full-length cDNA cloning, hybrid selection techniques and Southern and Northern blots.

Major Findings: (1) We have isolated and characterized the major excreted protein (MEP) of transformed murine cells. Synthesis and secretion of MEP is increased in all transformed murine fibroblasts and is well-correlated with anchorage independence, and hence with tumorigenicity. Rat and hamster cell lines synthesize a similar protein which cross-reacts with MEP. Cultured human cells produce a small amount of protein which cross-reacts with anti-MEP antibody, and this antigen is also increased in amount after TPA treatment. Synthesis of MEP is stimulated by treatment of cells with tumor promoters and growth factors, such as PDGF.

(2) The presence of mannose 6-phosphate on MEP enables it to bind quantitatively to the lysosomal phosphomannosyl receptor. At least 1 in 5 mannose residues on MEP are phosphorylated. MEP made by transformed cells is not handled simply as a lysosomal protein, however, since 60% of it is secreted, rather than quantitatively delivered to lysosomes as are other lysosomal proteins.

(3) In both transformed and nontransformed cells, MEP is processed sequentially into two lower molecular weight forms of 29K and 21K. Analysis by Percoll density gradients of subcellular organelles indicates that the 35K and 29K forms of MEP are in the Golgi-endoplasmic reticulum region of the gradient and the 21K form is in the dense lysosomal fractions. At steady-state, as revealed by immunodetection of MEP on Western transfers of PAGE of unlabeled cell extracts, the lowest molecular weight antigenic form of MEP (21K) is the predominant intracellular form of the protein.

(4) Subcellular localization of MEP has also been determined by light

microscopic indirect immunofluorescence and electron microscopic immunolocalization using affinity-purified antibodies. In both nontransformed and transformed cells, MEP related antigens localize to both the Golgi regions and lysosomes.

(5) We have purified mRNA coding for MEP and prepared a cDNA library from both Chinese hamster ovary and Kirsten virus transformed mouse NIH 3T3 fibroblasts. In both cases, probes prepared from clones identified by hybrid selection techniques recognize a mRNA of approximately 2000 bp on Northern blots. Levels of this putative MEP mRNA are positively regulated by transformation and tumor promoters, consistent with the hypothesis that MEP is regulated at the level of transcription.

(6) We have begun to construct eukaryotic cDNA expression vectors using a promoter from the RSV-LTR, SV40 poly A addition and mRNA processing signals, the pBR322 replicon for growth in E. coli and Okayama-Berg full-length cDNA cloning technology (in collaboration with C. Gorman and B. Howard, LMB/NCI). Full-length cDNA libraries coding for α, β and γ-casein have been constructed using this vector system and will be used as a model to test for casein gene expression after transfection (in collaboration with G. Smith and D. Graham, LMB/NCI). This system can be used to transfer expressible MEP genes into nontransformed cells to test the effect of such expression on the transformation phenotype of the cells, and will allow in vitro mutagenesis of MEP for analysis of sites along the structural gene needed for secretion or lysosomal localization.

Significance of Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 3.5.

MEP is both a marker of transformation and a sensitive indicator of the presence of at least one tumor promoter. The induction of its synthesis by tumor promoters could serve as a screening test for these agents in the environment. If MEP is found to fill an essential role in tumor growth or metastasis, then specific therapy aimed at neutralizing it could be designed as a model for cancer therapy.

Proposed Course: To continue to analyze the molecular mechanism underlying induction of MEP by using cloned MEP cDNAs as probes to measure MEP mRNA levels after transformation and treatment with tumor promoters; to isolate genomic MEP clones in order to study the structure of the gene and the nature of its transformation-sensitive and growth factor responsive promoter; to construct MEP cDNA expression vectors for transfection of nontransformed cells and in vitro mutagenesis studies; to isolate mutant cell lines lacking MEP in order to determine its function within cells; to continue to use MEP as a marker of the molecular events involved in tumor promotion and transformation.

Publications:

Scher, C.D., Hendrickson, S.L., Whipple, A.P., Gottesman, M.M., Pledger, W.J.: Constitutive synthesis by a tumorigenic cell line of proteins modulated by platelet-derived growth factor. Cold Spring Harbor Conferences on Cell Proliferation 9: 289-303, 1982.

Sahagian, G.G., and Gottesman, M.M.: The predominant secreted protein of transformed murine fibroblasts carries the lysosomal mannose 6-phosphate recognition marker. J. Biol. Chem. 257: 11145-11150, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08717-05 LMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Role of Carbohydrates in Protection of Glycoproteins Against Proteases

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Kenneth M. Yamada, Chief, Membrane Biochemistry Section, LMB, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Membrane Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.2	0.6

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The carbohydrate moiety of the transformation-sensitive glycoprotein fibronectin was previously shown to provide protection against proteolytic attack. This protective effect is localized primarily to the collagen-binding domain of the molecule, which is normally rich in carbohydrates. If the glycosylation of fibronectin is inhibited by tunicamycin, a specific tryptic cleavage site in this domain becomes exposed. This site has been localized to a polypeptide loop structure stabilized by disulfide bonding.

The effect of retinoic acid on glycosylation and fibronectin structure is also under investigation. Chondrocytes, in contrast to fibroblasts, synthesize fibronectin that includes oligosaccharides of the high-mannose type. Treatment with retinoic acid results in a conversion to the complex-type structure, with no detectable effects on polypeptide structure. This finding indicates that this vitamin can regulate the glycosylation of a specific glycoprotein.

Our objectives will be to map the carbohydrate residues on fibronectin more accurately, to examine the effect of early proteolytic cleavages on the function of carbohydrate-deficient domains, to compare the effects of proteases on human and chicken fibronectins, and to complete the retinoic acid studies.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Kenneth Olden	Guest Researcher	LMB	NCI
Bruno Bernard	Guest Researcher	LMB	NCI

PROJECT DESCRIPTION:

Objectives: (1) To investigate the mechanisms and effects of carbohydrate stabilization of glycoproteins against proteolysis. (2) To investigate the nature of retinoic acid-induced alterations in the carbohydrate structure of fibronectin.

Methods Employed: Fibronectin was isolated from confluent monolayer cultures of 10 day old chicken embryo fibroblasts by urea extraction after isotopic labeling by ^{35}S-cysteine or ^{3}H-mannose. Yields of nonglycosylated fibronectin were increased by supplementing the medium of tunicamycin-treated cultures with 50μM leupeptin, a nontoxic, specific protease inhibitor. Glycosylated and nonglycosylated fibronectin were digested by the proteases trypsin, chymotrypsin, pronase, or thermolysin in a reaction mixture containing 0.1M NaCl, 10mM $CaCl_2$, and 50mM Tris-HCl (pH 7.0) at 30°C and analyzed by SDS gel electrophoresis with or without reduction by dithiothreitol.

Cultures of 14 day old chick sternal chondrocytes were treated with retinoic acid and labeled with ^{3}H-mannose, ^{14}C-leucine, or ^{35}S-cysteine. Fibronectin was isolated by urea extraction and digested by chymotrypsin or thermolysin. The collagen-binding domain was isolated by affinity chromatography and analyzed for oligosaccharide structure using endoglycosidase H digestion followed by SDS gel electrophoresis or paper chromatography of glycopeptides.

Major Findings: Our previous studies showed that the carbohydrate moiety of fibronectin influences the susceptibility of the protein to proteolytic degradation. Specifically, the absence of carbohydrates results in markedly increased protease susceptibility of the normally carbohydrate-rich, collagen-binding domain of the molecule. To localize more precisely the site(s) of proteolytic cleavage, fragments generated by trypsin digestion of the ^{35}S-cysteine labeled, nonglycosylated collagen-binding domain were examined by SDS gel electrophoresis. Without reduction, the collagen-binding domain had an apparent molecular weight of 53,000, but upon reduction dissociated into two smaller fragments of 27,500 and 25,700. These results indicate that (a) in the absence of carbohydrates, at least one cleavage site in the collagen-binding domain becomes accessible to trypsin and (b) this site is located in a disulfide bond-stabilized loop. In addition, the increased susceptibility

to proteases is not due to the absence of charged terminal sialic acid residues, since their removal by neuraminidase has no effect on proteolysis.

We are also investigating the effect of retinoic acid on the structure of the carbohydrate moiety of chondrocyte fibronectin. We find that the carbohydrate moiety of fibronectin from retinoic acid-treated cells exists primarily as the complex-type structure, in contrast to the high-mannose form found in untreated cultures. This will be the first demonstration that the structure of the carbohydrate moiety of chondrocyte fibronectin differs from that of fibroblasts, and that retinoic acid regulates the glycosylation of a specific protein.

Significance for Cancer Research and the Program of the Institute: Transformed cells often show an increased production of proteases, as well as losses of fibronectin and other extracellular proteins. Proteolytic degradation of the extracellular matrix is thought to contribute to tumor metastasis and invasion. These studies are providing insight into (a) the mechanisms of carbohydrate-mediated protection of glycoproteins from proteolytic attack, (b) the effects of proteolysis on fibronectin, an extracellular protein thought to help regulate cell migration and interactions, and (c) the molecular mechanism of action of retinoic acid, a molecule which may inhibit carcinogenesis.

Proposed Course: We propose to test the hypothesis that the carbohydrate moieties in the collagen-binding domain of fibronectin are located adjacent to critical proteolytic cleavage site(s). The functional sequalae of initial tryptic cleavages will be examined by affinity chromatography, to determine whether one or more cleavages destroys binding activity. To compare the sites of glycosylation and of protease sensitivity in nonglycosylated fibronectin, intact collagen-binding domains will be isolated by gelatin affinity chromatography from brief proteolytic digests of glycosylated and nonglycosylated fibronectins labeled with ^{35}S-cysteine or ^{3}H-mannose. After reduction and alkylation, the peptide maps of both forms of the collagen binding domain will be established, and the carbohydrate moieties localized with respect to the proteolytic cleavage sites. From these studies, we hope to demonstrate the structural relationship between the carbohydrate moieties and the protease-sensitive sites they protect.

Additional experiments will compare the protease-susceptible regions of human and chicken cellular fibronectins by SDS gel electrophoresis and peptide mapping in order to investigate in detail whether there is evolutionary conservation of domains compared to inter-domain regions. We will also complete the retinoic acid studies described above.

Publications:

Olden, K., Parent, J. B., and White, S. L.: Carbohydrate moieties of glycoproteins: a re-evaluation of their function. Biochim. Biophys. Acta 650: 209-232, 1982.

Parent, J. B., Bauer, H., and Olden, K.: Tunicamycin treated fibroblasts secrete a cathepsin B-like protease. Biochem. Biophys. Res. Commun. 108: 552-558, 1982.

Bernard, B. A., Olden, K., and Yamada, K. M.: Carbohydrates protect the collagen-binding domain of fibronectin against proteolytic degradation. In Hawkes, S., and Wong, J. L. (Eds.): Extracellular Matrix. Academic Press, New York, 1982, pp. 225-229.

Olden, K., Bernard, B. A., Turner, W., and White, S. L.: Effect of interferon on glycosylation-comparison with tunicamycin. Nature 300: 290-292, 1982.

Bernard, B. A., Yamada, K. M., and Olden, K.: Carbohydrates selectively protect a specific domain of fibronectin against proteases. J. Biol. Chem. 257: 8549-8554, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08719-04 LMB

PERIOD COVERED

October 1, 1982 through September 30, 1983

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 on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Bruce H. Howard Chief, Molecular Genetics Section, LMB, NCI

COOPERATING UNITS *(if any)*

LMV, DCCP, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	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.)*

We have developed techniques for efficient DNA-mediated transfer of genes into primate cells and used these techniques to search for DNA sequences which regulate mammalian cell DNA replication. Expression vectors based on the Tn9 chloramphenicol acetyltransferase (cat) gene served to optimize conditions for transient expression of exogenous DNA as well as to provide evidence that the promoter in the long terminal repeat of Rous sarcoma virus (RSV) is exceptionally active in primate cells. Based on cat plasmid data we constructed the dominant selectable marker plasmids pRSVgpt and pRSVneo. pRSVgpt was found to mediate stable "transformation" of monkey CV-1 cells with an efficiency of $5\text{-}8 \times 10^{-1}$; pRSVneo proved to be a superior selectable marker for stable transformation of WI38 human embryo fibroblasts and HeLa cells. pRSVneo transfection in combination with genomic DNA from *E. coli*, HeLa and WI38 cells has been used to obtain preliminary evidence for human DNA sequences that inhibit HeLa cell replication. Retrovirus vectors based on these markers will be constructed to facilitate gene transfer into hematopoietic and primary cell types that are inefficiently transformed by conventional vectors. In addition, the potential of novel SV2/cosmid vectors to improve gene shuttling techniques will be further studied.

PHS 6040 (Rev. 1/83)

GPO 895-100

OTHER INVESTIGATORS:

Cornelia M. Gorman	Staff fellow	LMB	NCI
Raji Padmanabhan	Chemist	LMB	NCI
Xu Nai-zheng	Fogarty fellow	LMB	NCI
Mary M. McCormick	Staff fellow	LMB	NCI
Richard B. Stead	Medical staff fellow	LMB	NCI
Max Gottesman	Chief, BGS	LMB	NCI
Mark Willingham	Chief, UCS	LMB	NCI
Glen Merlino	Postdoctoral fellow	LMB	NCI
Sue Wickner	Chemist	LMB	NCI
Ira Pastan	Chief, LMB		
Rudi Pozzatti	Staff fellow	LMV DCCP	NCI
George Khoury	Chief	LMV DCCP	NCI

PROJECT DESCRIPTION:

Objectives: The objectives of this project are at least three-fold. First, we will continue systematic development of vectors and techniques to extend the range of mammalian cell types into which genes can be efficiently transferred. Second, we will place special emphasis on the use of this technology to identify and isolate genes and/or DNA sequences that regulate mammalian cell growth. Third, we will utilize gene transfer in conjunction with analysis of mRNA and protein levels to study mechanisms by which such growth regulatory genes exert their effects.

Methods employed: Vectors designed for amplification of genes in E. coli and subsequent transfer to mammalian cells are crucial to this area of research. In the pSV2 family of vectors the ampicillin-resistance cistron and origin of replication from the plasmid pBR322 are used for selection and propagation in E. coli; simian virus 40 (SV40) early region promoter and mRNA processing signals are used to direct expression in mammalian cells of Tn9 chloramphenicol acetyltransferase (cat), E. coli xanthineguanine phosphoribosyltransferase (gpt), or Tn5 aminoglycoside phosphotransferase (neo) coding sequences. In the pRSV family of vectors the promoter in the long terminal repeat (LTR) of Rous sarcoma virus (RSV) is substituted for the SV40 early promoter to direct expression of cat, gpt, or neo coding sequences.

These vectors are most frequently introduced into mammalian cells using the calcium phosphate-DNA coprecipitation technique. Alternate methods involving DEAE-dextran/DNA complexes or protoplast fusion are investigated where appropriate. During the last months of this reporting period we will construct and test retrovirus vectors for introduction of foreign genes by infection.

Three assays for expression of these vectors in mammalian cells are available. Total transient expression (12-60 hrs after transfection) may be assayed by determining ^{14}C chloramphenicol acetylated by cat in cell extracts. Second, the percentage of cells expressing exogenous DNA may be assayed by detection of intracellular cat with rhodamine-labeled cat antibody or cat antibody in conjunction with indirect rhodamine or peroxidase labeling. Third, the number of cells stably expressing vector genes may be assayed by colony selection using either the gpt or neo markers. Colonies selected may be amplified and analyzed for quantification of integrated exogenous DNA copies (Southern blots), mRNA levels (Northern and S1 nuclease analysis), and cat activity.

Finally, to isolate mammalian DNA sequences detected by these techniques, genomic libraries may be constructed using either bacteriophage lambda vectors or, in principle, newly developed λSV2/cosmid vectors.

Major findings:

I. Mammalian vector development

A. Vectors pRSVgpt and pRSVneo: Experiments comparing pSV2 cat and pRSVcat plasmids demonstrated that the RSV LTR promoter directs 3-10 fold higher transient cat expression levels than the SV40 early promoter in avian and most mammalian cell types. Based on these transient expression results we constructed and tested the vectors pRSVneo and pRSVgpt. pRSVneo (Rous LTR promoter) yielded from equal to 8 fold higher stable "transformation" frequencies than pSV2neo (SV40 early promoter) in all cell lines tested except Chinese hamster ovary cells. Several cell types believed to be refractory to DNA-mediated stable gene transfer were succesfully transformed with pRSVneo: WI38 human embryo fibroblasts ($2x10^{-5}$); SV40-transformed xeroderma pigmentosum cells (10^{-3}); and GH3 rat pituitary cells (10^{-3}). pRSVgpt was found to yield 5- to 10-fold higher stable transformation frequencies than pSV2gpt in monkey kidney CV-1 cells. CV-1 stable transformation frequencies using pRSVgpt were reproducibly $5-8x10^{-1}$, at least 5000 fold higher than previously reported.

B. Other members of the pSV2 and pRSV vector families: Experiments with the vectors pSV2dhfr and pRSVdhfr, which carry sequences coding for the methotrexate-resistant R-388 dihydrofolate reductase (dhfr), have been continued at a low priority level. Unorthodox selective conditions (200 μM aminopterin, 25 μM 5-formyltetrahydrofolate in folic acid-free growth medium) were demonstrated to permit selection of monkey kidney CV-1 cells transfected with pRSVdhfr. Future work with dhfr vectors will be directed towards further optimization of selection conditions and determination of applications for which this system may be uniquely suited. For example, dhfr vectors may provide unique possibilities for amplification of integrated exogenous gene copies to very high levels.

Experiments with the vectors pSVK and BPV/pSV2gpt have been discontinued.

C. λSV2 and derivative vectors: In conventional bacteriophage lambda, cosmid, and plasmid vectors, deletions and rearrangements of insert fragments derived from mammalian genomic DNA, mammalian virues and lower eukaryotic genomic DNA have frequently been observed. Deletions are particularly prone to occur in very large DNA inserts. λSV2, a vector that integrates by site-specific recombination into the chromosome of the specialized lambda lysogen N6106, was developed to minimize these instability problems. Prior to a major effort to clone large or particularly unstable DNA segments, we have concentrated on several remaining technological problems: (1) preparation of minimally aggregated very high molecular weight DNA, (2) construction of new recombination-deficient (e.g. $recA^{-}recBC^{-}sbcB^{-}$) derivatives of N6106 and (3) further improvement in transformation efficiency by construction of λSV2/cos recombinants.

A second reason for development of the λSV2/N6106 vector/host pair was to explore the possibility of using site-specific recombination as a means to rescue integrated recombinant genomes from HMW (high molecular weight) mammalian DNA. As a model system we have studied in vitro site-specific excision of λSV2 integrated in N6106 chromosomal DNA. λSV2 rescue by excision followed by transfection to a non-lysogenic strain was accomplished with an efficiency of 10^3 colonies/μg HMW DNA. Application of this approach to recovery of integrated copies from mammalian DNA will require one or more of the following technical improvements: (1) modification of reaction conditions to increase reproducibility and efficiency in the presence of HMW DNA, 2) identification of a protocol that increases transformation frequencies for large plasmids 10-100X over conventional methods and (3) development of a simple method to separate excised closed-circular λSV2 recombinants from linear HMW DNA.

II. New methods to improve DNA-mediated stable transformation efficiencies

A. Effects of sodium butyrate: We have found that treatment of cells for a 12 hr period immediately following calcium phosphate-DNA transfection frequently results in a 5-10X increase in the efficiency of stable transformation. This increase has been observed with CV-1, NIH/3T3, CHO and WI38 cells, and occurs with either gpt or neo selectable markers. Stable transformation efficiencies of 5-10% have been achieved using butyrate to boost transfection of CV-1 cells with pSV2gpt. Studies on the effects of butyrate treatment on cat plasmid function suggest that this agent elevates stable transformation frequencies by: (1) increasing the percentage of cells that express plasmid functions (from 10% to 30-40% in CV-1 cells), and (2) increasing transcription from the SV40 early promoter via an enhancer-dependent mechanism (up to 40-fold in Hela cells). Butyrate also elevates stable transformation of mouse C127 cells by bovine papilloma virus vectors (10- to 50-fold).

B. Effects of SV40 large T and small t antigens: Preliminary results with WI38 human embryo fibroblasts indicate that SV40/pRSVneo cotransfection yields 5- to 10-fold more aminoglycoside-resistant (neoR) colonies than does transfection with pRSVneo alone. This increase may simply reflect relatively efficient colony formation of SV40-transformed cells under conditions of

low cell density. Alternatively, the effect may indicate stimulation of plasmid integration by large T and/or small t antigens in these non-immortalized cells. Efforts will be made to distinguish these possibilities and to test effects of cotransfection with plasmids in which either large T or small t antigen is expressed under RSV LTR promoter control.

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

A. Malignant transformation of monkey kidney CV-1 cells: The extremely high efficiencies with which CV-1 cells are stably "transformed" using gpt plasmids suggested to us that this cell line may be an excellent recipient in genomic DNA transfer experiments designed to detect new classes of oncogenes. Accordingly, in collaboration with Drs. R. Pozzatti and G. Khoury (NCI, DCCP, LMV) we have constructed CV-1 cell lines that carry mutant human c-ras gene copies. We will determine human c-ras expression levels in these CV-1/c-ras cell lines and determine whether cells that express this gene are malignantly transformed. If such lines fail to manifest a malignant phenotype, they will be tested as recipients in gene transfer experiments using genomic DNA from preneoplastic cell lines (e.g. mouse NIH/3T3 cells) and other tumor cell types.

B. Inhibition of HeLa cell replication: We carried out the following experiment to ask whether human genes that inhibit mammalian cell replication can be detected using our current gene transfer techniques. Hela cells in monolayer culture were transfected with pRSVneo DNA in combination with either E. coli, HeLa or WI38 genomic DNA. Forty-eight hours after transfection, cells from each culture were transferred into suspension medium containing bromodeoxyuridine and Hoechst 33258 dye. After 48 hrs in suspension, cells were exposed to white light for 10 minutes, then plated into neo-selective medium. This protocol is designed to select cells that are prevented by exogenous DNA sequences from replicating in suspension culture and, in addition, stably express the pRSVneo selectable marker. Preliminary results (obtained by counting colonies after 8 days in neo medium) indicate that transfection with WI38 DNA increases cell survival 10-fold more efficiently than HeLa DNA and at least 50-fold more efficiently than E. coli DNA. The average growth rate of WI38/pRSVneo-transfected cells surviving after 18 days in neo medium appears to be 2- to 3-fold lower than HeLa/pRSVneo-transfected cells.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 6, Approach 3

DNA-mediated transfer of genetic material into mammalian cells is a potentially powerful approach for identifying mechanisms that control cell growth and for elucidating how malfunction of those mechanisms leads to malignant transformation. The project described above is directed towards the systematic improvement of this technology, as well as towards its simultaneous application to identify genes and/or DNA sequences that regulate mammalian cell replication.

Proposed course:

Further work will concentrate on refining mammalian vector systems, improving methods for introduction of DNA into mammalian cells, and understanding factors which control stable expression of exogenous genes. More emphasis will be placed on application of gene transfer techniques to primary cells such as murine hematopoietic stem cells and human embryo fibroblasts. If preliminary results on inhibition of HeLa cell replication are confirmed, efforts will of course be made, first, to isolate the DNA sequence(s) that mediate this experimental effect, and then to determine any relationship such sequences may have to malignant transformation.

Publications:

Law, M.F., Howard, B., Sarver, N., Howley, P.M.: Expression of selective traits in mouse cells transformed with a BPV DNA-derived hybrid molecule containing Escherichia coli gpt. Eukaryotic Viral Vectors: 79-85, 1982.

Howard, B.H., Gottesman, M.E.: Vectors that may be propagated by integration into the Escherichia coli chromosome. Eukaryotic Viral Vectors: 211-216, 1982.

Gorman, C. M., Moffat, L.F., Howard, B.H.: Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Molecular Cell Biology 2: 1044-1051, 1982.

Laimins, L.A., Khoury, G., Gorman, C., Howard, B., Gruss, P.: Host specific activation of transcription by tandem repeats from SV40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA 79: 6453-6457, 1982.

Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I., Howard, B.H.: The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eucaryotic cells by DNA mediated transfection. Proc. Natl. Acad. Sci. USA 79: 6777-6781, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08750-03 LMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic Regulatory Mechanisms in Escherichia Coli and Its Bacteriophage

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Sankar Adhya, Chief, Developmental Genetics Section, LMB, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular Biology

SECTION
Developmental Genetics Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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

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

cya, crp, rho and nus genes modulate the expression of a wide variety of bacterial and bacteriophage genes or operons. In order to understand the regulatory processes, we are studying the structure, expression and activity of these genes. We have cloned these genes and characterized them (a) physically by restriction analysis and electron microscopy, and (b) biologically by isolation of mutants, mainly ambers and insertions.

The protein products of the cya, crp and rho genes are autogenously regulated. The autogenous regulation of the rho gene is at the level of transcription. Cyclic AMP modulates rho gene expression in two opposite ways: it enhances rho transcription but inhibits rho translation.

We have isolated new crp mutants which suppress the adenylate cyclase defect of a mutant cell.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Susan Garges	Microbiologist	LMB	NCI
Roberta Haber	Biologist	LMB	NCI

PROJECT DESCRIPTION:

Objectives: Gene expression in E. coli and bacteriophage λ are modulated at the level of transcription initiation, transcription termination and translation. Cyclic AMP, CRP, Rho and Nus gene products of E. coli, and N gene product of phage λ control one or more these regulatory processes. Nus, Rho and N are involved in transcription termination and antitermination. cAMP and CRP either stimulate or inhibit transcription initiation. Frequently, the level of these regulatory proteins are critical in gene control. In order to understand the precise molecular basis of these and other regulatory biochemical reactions, we are studying (a) the structure of these genes, (2) how these genes are themselves regulated to vary their products, (c) their biochemical activity, and finally (d) how these activities are regulated.

Methods Employed: Standard microbial genetic and biochemical techniques. Also employs both in vivo and in vitro recombinant DNA technology.

Major Findings: (1) We have genetically and physically analyzed the cloned cya gene. We have mutagenized this fragment and obtained new cya mutants, including cya amber mutants, and have identified previously isolated mutants as deletion mutants. Comparing the protein products from cya^+ and cya amber containing phages has enabled us to identify with certainty the cya gene product.

(2) We have used the cya-lacZ and the crp-lacZ gene fusion strains which we constructed to follow where these hybrid products are localized in the cell. This has provided strong evidence that adenylate cyclase and CRP are membrane-associated proteins. This is important in realizing how the activity of adenylate cyclase is modulated because of evidence linking other membrane associated proteins with regulation of adenylate cyclase activity.

(3) We have isolated new crp mutants which may either (a) act independently of cAMP or (b) increase the activity of adenylate cyclase. We are currently analyzing these mutants.

(4) We had previously shown that the rho gene of E. coli was autogenously regulated. We have now shown, by measuring in vivo the amount of rho mRNA, that this regulation is at the level of transcription. Previously, we had shown that there is more Rho in strains which lacked CRP or cAMP. We have analyzed the rho mRNA in these strains and have found, surprisingly, that

there is much less rho mRNA than in the corresponding wild type strains, indicating the CRP•cAMP has a positive regulatory role in rho transcription. The increase in rho gene product in these strains, then, may be a result of increased translation when CRP/cAMP are absent. Currently we are investigating in vitro the CRP•cAMP interaction with the rho gene, to determine how the control of rho expression is modulated.

(5) The cloned nusA gene has been genetically and physically characterized: (a) New mutations, including amber mutations, in the nusA gene have been isolated. (b) A restriction map of the nusA gene has been constructed. (c) The cloned DNAs have been translated both in vivo and in vitro. The wild type clone makes a 66 kd protein which is replaced by a 46 kd protein in an amber mutant.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 1.

In cancer cells the expression of some genes are permanently turned on, i.e., expressed constitutively and some genes are permanently turned off, i.e., never expressed. Our studies are aimed to understand the molecular basis of how genes are turned on and off and how genetic regulatory elements interact with each other. This understanding might help to prevent conversion of normal cells to those capable of forming cancers.

Proposed Course: (1) To determine the complete structure of the E. coli rho, cya, crp and nus genes. (2) To determine the regulation of the expression of these genes. (3) To understand the interaction between these gene products.

Publications:

Adhya, S., and Garges, S.: How cyclic AMP and its receptor protein act in E. coli. Cell 29: 287-289, 1982.

Gulletta, E., and Adhya, S.: Cloning of the rho gene of Escherichia coli. Microbiologica 5: 235-243, 1982.

Dambly-Chaudiere, C., Gottesman, M., Debouck, C., and Adhya, S.: Regulation of the p_R operon of bacteriophage lambda. J. Mole. Applied Genetics 2: 45-56, 1983.

Adhya, S., and Gottesman, M.: Promoter occlusion: Transcription through a promoter may inhibit its activity. Cell 29: 939-944, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08751-03 LMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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 on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Sankar Adhya, Chief, Developmental Genetics Section, LMB, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Developmental Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	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.)*

We are studying the modulation of the expression of the gal operon of E. coli. We have so far shown that the operon is controlled by two promoters, which are modulated by cyclic AMP in opposite ways. Both the promoters are under the negative regulation by the gal repressor. The repressor inhibits transcription by binding to two operator sites. One is located at a more conventional position, i.e., near the promoter and the other at an extraordinary position, i.e., within the first structural gene. In order to study the interaction between the gal repressor protein and the two operator DNA elements, we have cloned the repressor gene into a maximum expression vector and have purified the protein. We have also cloned the two wild type and mutant operator elements needed for the repressor binding studies.

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Alokes Majumdar	Visiting Associate	LMB	NCI
Laszlo Orosz	Visiting Associate	LMB	NCI
Susan Garges	Microbiologist	LMB	NCI

PROJECT DESCRIPTION:

Objectives: We believe that the expression of most genes in E. coli is regulated in one way or another. Qualitatively different mechanisms have been discovered, e.g., positive and negative control at the level of both initiation and termination of gene transcription. Control mechanisms also exist at the level of mRNA processing and translation. We have demonstrated many of these control mechanisms using the galactose (gal) operon of E. coli. Using this model system, we are studying the molecular basis of the following regulatory mechanisms: (1) How cyclic AMP and CRP catalyze/inhibit transcription initiation. (2) How Rho protein of E. coli modulates transcription termination. (3) How gal repressor inhibits transcription initiation. (4) The nature of protein-protein and protein-nucleic acid interactions that bring about the above control mechanisms.

Methods Employed: Standard microbiological, genetic, and biochemical techniques. Also employs both in vivo and in vitro recombinant DNA technology and DNA sequencing methods of Maxam and Gilbert.

Major Findings: (1) The two gal promoters, P_{G1} and P_{G2} initiate gal transcription at two sites, separated by five bp. The in vivo start sites were identified by capping the triphosphate ends with labeled ^{32}P-GTP and then sequencing the labeled RNase T1 fragments. The in vivo start sites are identical to the corresponding start points previously shown in vitro.

(2) We have discovered a new control of the gal operon. The expression of the gal operon is derepressed in mutant cells deficient in both cyclic AMP and the transcription termination factor Rho. We have proposed that the cyclic AMP repressible gal promoter, P_{G2}, may also be under Rho dependent transcription attenuation control. We are currently identifying the 5' terminus of the gal transcript and studying the role of gal repressor in rho^- cya^- mutant cells.

(3) The negative control of the gal operon is achieved by an interaction between the gal repressor protein and gal operator DNA. We have previously developed a novel method of identifying the operator DNA segment(s), i.e., by isolating gal operator mutants which are unable to bind the repressor. The mutations have been characterized genetically and their locations determined by DNA sequencing. The following results have been found:

(a) The gal repressor competes with the cyclic AMP receptor protein complex.

(b) The gal operator has two components. One is extragenic and located upstream to the gal promoters and the other is intragenic and located inside the first structural gene, galE.

(4) We have cloned the gal repressor gene into a maximum expression vector, pKC30 (constructed by Dr. N. Rao of Eli Lily). We have also developed a quick procedure to purify the repressor. We are currently studying its binding properties to the gal operator.

(5) We have also cloned the two components of the wild type and mutant gal operators separately and together on plasmid DNA. They will be used for in vitro gal repressor binding studies.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 1. In cancer cells, the expression of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using the gal operon as a model system. This understanding might help to prevent the conversion of normal cells to those capable of forming cancers.

Proposed Course: (1) To determine the complete structure of the gal operon. (2) To identify all the regulatory molecules involved in turning on and off the genes of the gal operon. (3) To understand the biochemistry of each of the regulatory steps. (4) To study the protein-DNA interactions between the repressor and the operator.

Publications:

Irani, M. H., Orosz, L., and Adhya, S.: A control element within a structural gene: The gal operon of E. coli. Cell 32: 783-788, 1983.

Irani, M., Orosz, L., Busby, S., Taniguchi, T., and Adhya, S.: Cyclic AMP dependent constitutive expression of gal operon: Use of repressor titration to isolate operator mutations. Proc. Natl. Acad. Sci. U.S., 1983, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08752-03 LMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Mechanism of the Transport of Thyroid Hormones into Animal Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Sheue-yann Cheng Research Chemist LMB NCI

COOPERATING UNITS *(if any)*

National Biomedical ESR Center, Department of Radiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.1	3.1	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.)*

Affinity labeling techniques were employed to identify and characterize the plasma membrane receptors for 3,3',5-triiodo-L-thyronine (T_3) in cultured Swiss 3T3-4 mouse fibroblasts, GH_3 rat pituitary tumor cells and human epithelioid carcinoma A431 cells. A major specifically labeled protein with an apparent molecular mass of 55 kDalton (kDal) was detected in three cell lines. One-dimensional peptide mapping showed there are structural similarities in the 55-kDal protein from three different species. Thus, the plasma membrane T_3 receptors are highly conserved. Using ^{125}I-labeled L-thyroxine ([^{125}I]T_4), the binding and uptake of T_4 in cultured GH_3 cells and Swiss 3T3-4 cells were shown to be saturable and specific. These results showed that the uptake of T_4 is receptor-mediated. Using affinity labeling and peptide mapping techniques, plasma membrane T_3 and T_4 receptors were shown to have structural similarities. The results from equilibrium binding studies indicate that one plasma membrane thyroid hormone receptor mediates the uptake of both T_3 and T_4 into cells.

Using electron spin resonance (ESR), the dynamic interactions of thyroid hormones with liposomes derived from L-α-dimyristoyl-phosphatidylcholine (DMPC) and plasma membranes of GH_3 cells was studied. Using the motional narrowing formalisms, the rotational correlation times of spin-labeled T_4 in DMPC vesicles at 31°C were estimated at 3.1×10^{-3} and 4.8×10^{-9} sec for the linear term and the quadratic term, respectively. The rate of lateral diffusion of spin-labeled T_4 in DMPC was approximately 5.2×10^{-8} cm^2/sec as determined by the ESR line-broadening method. These results indicate that spin-labeled thyroxine diffuses freely in the DMPC matrix. Studies using spin-labeled T_3 (SL-T_3) in DMPC vesicles gave similar results. However, analyses of the ESR spectrum from the binding of SL-T_3 to the plasma membranes of GH_3 cells indicate that SL-T_3 is highly immobilized. These results suggest that SL-T_3 binds to a protein component. The interaction of SL-T_3 with plasma membranes of GH_3 cells is being characterized.

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER INVESTIGATORS:

Ralph Alderson	Staff Fellow	LMB	NCI
Bernard Rossi	Visiting Fellow	LMB	NCI
Ira Pastan	Chief, LMB	LMB	NCI
Mark Willingham	Chief, UC Section	LMB	NCI
Ching-San Lai	National Biomedical ESR Center, Department of Radiology, Medical Collage of Wisconsin, Milwaukee, Wisconsin 53226		

PROJECT DESCRIPTION:

Objectives: To elucidate the mechanism of the entry of thyroid hormones into cells at molecular level. To study the mechanism of the translocation of thyroid hormone from cytoplasm into nuclei where the initiation of biological actions occurs. To understand the mechanism of thyroid hormone action on cell growth.

Methods Employed: Use radiolabeled T_3 and T_4 to quantify and characterize the binding and internalization of thyroid hormone by cells. Synthesize chemical and photoactivable thyroid hormone affinity labeling reagents to directly identify the plasma membrane receptor which will be purified and characterized. Use immunoprecipitation and metabolic labeling to identify and characterize the biosynthesis of plasma membrane T_3 receptors. High pressure liquid chromatography will be used to isolate and purify the labeled peptides of plasma membrane T_3 receptors.

Thyroid hormones labeled with nitroxide radicals are synthesized. Electron spin resonance spectroscopy is employed to study the dynamic interactions of thyroid hormones with plasma membrane T_3 receptors of highly purified plasma membranes and intact cells.

Major Findings: Affinity labeling of the purified plasma membrane of GH_3 cells with N-bromoacetyl-[^{125}I]T_3 (BrAc[^{125}I]T_3) identified a T_3-specific membrane receptor with a molecular weight of 55,000. Proteins with the same molecular weight were also specifically labeled by BrAc[^{125}I]T_3 in intact GH_3 cells and Swiss 3T3 fibroblasts. A major specifically labeled protein with an apparent molecular weight of 55,000 was also found in human epithelioid carcinoma A431 cells. Peptide mapping of the 55-kilodalton protein from these three species gave identical fragments. These results indicate that plasma membrane T_3 receptors are highly conserved.

Using [^{125}I]T_4 the binding and uptake of T_4 in cultured GH_3 cells and Swiss 3T3-4 cells were shown to be saturable and specific. These results showed that the uptake of T_4 is receptor-mediated. Using affinity labeling, peptide

mapping and equilibrium binding studies, the uptake of T_3 and T_4 was shown to be mediated by one plasma membrane thyroid hormone receptor.

Nitroxide-labeled T_4 and T_3 were synthesized. Electron spin resonance spectroscopy was used to probe the motional characteristics of thyroid hormones in liposomes and plasma membranes of GH_3 cells. The effective rotational correlation time and lateral diffusion coefficient of T_4 and T_3 were determined in liposomes. The values indicate that T_3 or T_4 are freely diffuse in liposomes. However, analyses of the spectrum from the binding of spin-labeled T_3 to the plasma membranes of GH_3 cells show characteristics of binding to a protein component. The interaction of spin-labeled T_3 with plasma membranes of GH_3 cells is being characterized. The information derived from this technique will provide new insights into the nature of the hormone-receptor dynamic interactions at molecular level.

Significance of Cancer Research and the Program of the Institute: National Cancer Plan Objective 5, Approach 5.

Transformation of cultured cells appears closely linked with the ability to form malignant tumors in vivo. The understanding of the basic mechanisms in hormone-receptor interactions which results in controlled or uncontrolled cell growth are likely to be of great value in understanding the basic mechanisms leading to the formation of cancer cells. The knowledge gained from such studies is likely to have great impact on the ability to design or modify therapeutic procedures.

Proposed Course: We will continue to study the molecular mechanism of the transport of thyroid hormones into animal cells. We will isolate and purify the plasma membrane T_3 receptors from human placenta. The purified receptors will be characterized. Polyclonal and monoclonal antibodies against the purified receptors will be produced and purified. The antibodies will be used to localize the receptors in cells, thereby gaining understanding of the precise mechanism by which thyroid hormones translocate extracellularly to the nucleus where initiation of biological activities occurs.

Using anti-T_3 antibody and [^{35}S]methionine labeling, the kinetics of the biosynthesis of the 55-kDal receptor protein will be examined. Furthermore, the 55-kDal receptor protein will be subjected to proteolysis. The labeled peptides will be isolated, purified and sequenced. The nucleotide sequences corresponding to the amino acid sequences in the peptides will be synthesized and used as a probe to clone the genes of the receptor proteins. This should lead to the understanding of structure and function of the plasma membrane thyroid hormone receptors.

Publications:

Horiuchi, R., Johnson, M.L., Willingham, M.C., Pastan, I., and Cheng, S.-y.: Affinity labeling of the plasma membrane 3,3',5-triiodo-L-thyronine receptor in GH_3 cells. Proc. Natl. Acad. Sci. U.S.A. 79: 5527-5531, 1982.

Cheng, S.-y., Merlino, G., and Pastan, I.: A versatile method for the coupling of protein to DNA: Synthesis of α_2-macroglobulin-DNA conjugates. Nucl. Acids Res. 11: 659-669, 1983.

Hanover, J.A., Cheng, S.-y., Willingham, M.C., and Pastan, I.H.: Alpha$_2$-macroglobulin binding to cultured fibroblasts: Solubilization and partial characterization of binding sites. J. Biol. Chem. 258: 370-377, 1983.

Lai, C.-s., and Cheng, S.-y.: Rotational and lateral diffusions of L-thyroxine in phospholipid bilayers. Biochim. Biophys. Acta 692: 27-32, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08753-01 LMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Immunotoxin Therapy of Cancer Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Ira Pastan Chief, Laboratory of Molecular Biology LMB NCI

COOPERATING UNITS *(if any)*

The Salk Institute, San Diego, California
The University of Kansas Medical School, Kansas City, Kansas
U.S. Army Medical Research Institute of Infective Diseases, Fort Detrick, MD

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	2.3	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.)*

Using Pseudomonas exotoxin (PE) prepared by Dr. Stephen Leppla, we have developed a method to modify the toxin so that it no longer binds to cellular receptors but is fully enzymatically active. We have coupled this modified toxin to hormones such as epidermal growth factor (EGF) and antibodies such as that to the human transferrin receptor. Both of these conjugates are specifically cytotoxic for cells with the appropriate recognition markers. The conjugates are about equally potent to conjugates with ricin A chain. When these toxins enter cells they do so through coated pits and receptosomes, a pathway used by many ligands including adenovirus. Dr. D. FitzGerald has found that adenovirus enhances the toxicity of these conjugates by up to 10,000-fold. It does this by disrupting the receptosome that contains the virus and toxin giving the toxin free access to the cytosol. Enhancement by adenovirus has been observed with PE conjugates of EGF and PE conjugates with monoclonal antibodies for the human transferrin receptor.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER PRINCIPAL INVESTIGATORS:

Mark C. Willingham	Chief, UCS	LMB	NCI
Michael M. Gottesman	Chief, BGS	LMB	NCI

OTHER INVESTIGATORS:

David J.P. FitzGerald	Staff Fellow	LMB	NCI
Prem Seth	Visiting Fellow	LMB	NCI
Shin-ichi Akiyama	Visiting Associate	LMB	NCI
Ian S. Trowbridge	The Salk Institute, San Diego, California		
R. Padmanabhan	University of Kansas School of Medicine, Kansas City, Kansas		
Stephen Leppla	U.S. Army Medical Research Institute of Infective Diseases, Fort Detrick, Maryland		

PROJECT DISCRIPTION:

Objectives: To develop methods of selectively killing human cancer cells.

Methods Employed: Preparation of toxins, antibodies and toxin-antibody conjugates; growth and purification of adenovirus; chemical modification of toxins and virus, protein purification and cell culture.

Major Findings: Using Pseudomonas exotoxin (PE) prepared by Dr. Stephen Leppla, we have developed a method to modify the toxin so that it no longer binds to cellular receptors but is fully enzymatically active. We have coupled the modified toxin to hormones such as epidermal growth factor (EGF) and antibodies such as that to the human transferrin receptor. Both of these conjugates are specifically cytotoxic for cells with the appropriate recognition markers. The conjugates are about equally potent to conjugates with ricin A chain. When these toxins enter cells, they do so through coated pits and receptosomes, a pathway used by many ligands including adenovirus. D. FitzGerald has found that adenovirus enhances the toxicity of these conjugates by up to 10,000-fold. It does this by disrupting the receptosome within the cytoplasm giving the virus and the toxin free access to the cytosol. Enhancement by adenovirus has been observed with PE conjugates of EGF and PE conjugates with monoclonal antibodies for the human transferrin receptor. P. Seth has begun to examine the components of adenovirus responsible for vesicle lysis. S.-i. Akiyama has begun to isolate mutant cells resistant to killing by PE-EGF.

Significance for Cancer Research and the Program of the Institute: Increasing the entry of toxins into cells will aid in designing better biological agents for cancer treatment.

Proposed Course: To determine the components of adenovirus that are responsible for vesicle lysis, to determine if adenovirus enhances the activity of other cytotoxic conjugates, to determine the mechanism of action of adenovirus and to test these agents on human tumors growing in nude mice.

Publications:

FitzGerald, D.J.P., Padmanabhan, R., Pastan, I.H., and Willingham, M.C.: Adenovirus-induced release of epidermal growth factor and pseudomonas toxin into the cytosol of KB cells during receptor-mediated endocytosis. Cell 32: 607-617.

FitzGerald, D.J.P., Trowbridge, I.S., Pastan, I., Willingham, M.C.: Enhancement of toxicity of anti-transferrin receptor antibody-pseudomonas exotoxin conjugates by adenovirus. Proc. Natl. Acad. Sci., U.S.A. 1983, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08754-01 LMB

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Genetic Analysis of the Multiple Drug Resistance Phenotype in Tumor Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Michael M. Gottesman Chief, Molecular Cell Genetics Section LMB NCI

COOPERATING UNITS *(if any)*

Division of Cancer Therapy, NCI

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

During the course of chemotherapy of human cancers, variants which are resistant to multiple drugs frequently arise. We have been investigating the genetic and biochemical basis for this pleiotropic resistance of human tumor cells to chemotherapeutic agents. A model system using the cultured KB cell, a human tumor cell line, has been developed in which mutant cells selected for resistance to high levels of colchicine have also been found to be resistant to adriamycin, vincristine, vinblastine, puromycin and actinomycin-D. These drugs are chemically and mechanistically unrelated to each other but may share a common mode of entry, metabolism or exit from cells. The mechanism of this multiple drug resistance is being studied by analysis of cell surface glycoproteins which might be involved in uptake of the drugs, dominance and complementation analysis of mutant cell lines and gene transfer of drug resistance genes from human tumor cells.

PHS 6040 (Rev. 1/83) GPO 895-100

PHS-NIH
Individual Project Report
October 1, 1982 through September 30, 1983

OTHER PRINCIPAL INVESTIGATOR:

Ira H. Pastan	Chief, Laboratory of Molecular Biology	NCI

OTHER INVESTIGATORS:

Shin-ichi Akiyama	Visiting Associate	LMB	NCI
Gregory Curt	Clinical Associate	DCT	NCI
Neil J. Clendeninn	Clinical Associate	DCT	NCI
Irene Abraham	Expert	LMB	NCI
John Hanover	Guest Researcher	LMB	NCI

PROJECT DESCRIPTION:

Objectives: To use genetic and biochemical analysis of human drug resistant cell lines and tumor tissues to understand the molecular basis of resistance to chemotherapy in tumors.

Methods Employed: Somatic cell genetic analyses including tissue culture, mutant isolation, cell hybridization and gene transfer and standard biochemical techniques including gel electrophoresis and chromatography.

Results:

1. We have screened a large number of human tumor lines for the following properties: high cloning efficiency, rapid doubling time, and ability to generate variants resistant to multiple drugs. Two cell lines, the breast cancer cell line MCF-7 and the KB cell line (probably a derivative of HeLa), satisfied these requirements. Drug resistant derivatives of a subclone of KB (KB-3-1) have been extensively analyzed. After ethanemethane sulfonate mutagenesis, it is possible to isolate colchicine resistant (col^R) variants, and these single-step mutants show different patterns of cross-resistance to adriamycin, vincristine, vinblastine, actinomycin-D and puromycin indicating that there may be multiple mechanisms for generating pleiotropic drug resistant human cells. Mutants resistant to higher levels of colchicine (up to 20-fold resistant) have been isolated in independent, sequential single steps from the original col^R KB-3-1 cell line and these also show increased resistance to the drugs listed above. These human cell lines appear phenotypically very similar to Chinese hamster ovary (CHO) cell mutants which have been isolated and characterized as having increased expression of a cell surface phosphoglycoprotein, termed p170 (Riordan and Ling, J. Biol. Chem. 254, 12701, 1979).

2. We have purified p170 from CHO cells with the multiple drug resistant phenotype and prepared anti-sera against this surface antigen. These sera will

be used to screen human multiple drug resistant cell lines for the presence of p170.

3. We have metabolically labeled with ^{14}C-glucosamine a large series of human tumor cell lines, some of which come from tumors known to be drug resistant. Although the pattern of surface glycoproteins differs dramatically among the cell types, there is no consistent alteration seen in drug-resistant lines compared to drug-sensitive lines of cells from the same type of tumor.

4. We have established a system for gene transfer of drug resistance genes from human cultured cells and tumor material to drug sensitive human recipient cells. Rodent cell lines such as mouse L cells and CHO cells, which are usually used as recipients in gene transfer experiments, are naturally more resistant to the drugs than human cells and cannot be used as recipients for DNA transfer. Drug-sensitive human lines were tested for ability to serve as recipients for DNA transfer using the vector pSV_2 neo which carries a dominant selectable marker coding for resistance to the aminoglycoside antibiotic G418. Several potential human cell recipients, including HeLa S3 and HTD-114 have been identified. In addition, we have successfully prepared transforming DNA from frozen human tumor material.

Significance of Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 3.5.

The goal of these studies is to understand the basis of multiple drug resistance in chemotherapy-resistant human tumors so that rational therapy can be devised to prevent or circumvent the multiple drug resistance phenotype. Dramatic success in treatment of some human tumors with chemotherapy suggests that chemotherapy can be curative in many more cases if steps can be taken to reduce the appearance of drug resistant tumor cells. One possible approach, if a cell surface protein is responsible for multiple drug resistance, would be to use toxin-antibody complexes to kill cells with the antigen in question on their surfaces. Another approach would be to use cloned cDNA probes for drug resistance genes to detect their expression before treatment and modify treatment modalities accordingly.

Proposed Course: To continue to isolate human drug resistant cell lines and characterize them with respect to their drug permeability and cell surface properties; to determine whether resistance to multiple drugs is linked in mutant cells by somatic cell hybridization and segregation analysis and by gene transfer; to isolate human genes coding for multiple drug resistance by gene transfer and rescue techniques.

Publications:

None to date.

SUMMARY

Annual Report of the Laboratory of Biochemistry, National Cancer Institute
October 1, 1982 to September 30, 1983

I. INTRODUCTION

The Laboratory has had a busy year pursuing our mission to investigate fundamental biochemical processes. As in the past, a range of organisms has been used, reflecting the basic unity of biological mechanisms and the special opportunities provided by different systems. Moreover there is a notable diversity of interests within the laboratory. The problems being investigated cover many areas: the control of gene expression by chromatin structure and by environmental, hormonal and developmental signals, the organization of genomes, moveable eukaryotic elements, the regulation of physiological processes by calcium ions, the mechanism of muscle contraction, cellular immunology, nutrition, and improved methods for separation of proteins. Although a variety of methods are used, a great deal of the work is made feasible by the extraordinary power and versatility of recombinant DNA techniques and other recent improvements in the analysis of nucleic acid structure. In addition, there is a continually increasing application of computer technology to various areas of our research. During this year an additional young investigator of great promise, Paul Wagner, joined the Laboratory and has begun independent work.

We have continued with an active program of seminars and Laboratory research reports. The Laboratory "Show and Tell" has a new format and is livelier and more productive than in the past. Our Wednesday guest seminars attracted substantial numbers of colleagues from all over NIH. The speakers included NIH scientists as well as guests from other institutions and the topics covered a broad sample of current research. Even more importantly, the work of the entire Laboratory staff benefits from the very active exchange of information, advice, materials and techniques within the Laboratory itself.

As in the past, the Laboratory was enormously enriched by the presence of an excellent group of postdoctoral fellows and other trainees. Whatever they gained in the way of training and expertise was more than repaid in their contributions to our research. We also acknowledge the skill and dedication of the supporting staff of technicians, aides and office personnel; their efforts were also critical to our achievements.

II. BIOCHEMISTRY OF GENE EXPRESSION (Dr. E. Brad Thompson, Chief)

The section made considerable progress towards the goals set last year. The following summary mentions a few of the highlights.

They constructed a variety of vectors containing all or part of the rat growth hormone (RGH) gene, including: (1) The entire gene, plus flanking regions extending about 2 kb in the 5' direction and about 1.5 kb in the 3' direction, linked in a modified pBR-SV40 plasmid containing either the bacterial xanthine-guanine phosphoribosyltransferase (*XPT*) or bacterial amino-glycoside phosphotransferase (*neo*) genes; (2) 650 bases comprising the the promoter region of the gene linked to the *E. coli* galactose kinase gene. The former vectors were successfully transfected and expression of RGH was observed in GH x L cell

hybrids which contain but do not otherwise express, the RGH gene. Notably, these transfectants are the first to process RGH transcripts to proper mature mRNA size, perhaps because of some informational molecules supplied by the GH_3 cell (a rat anterior pituitary adenoma) portion of the hybrid. One of these transfected clones is inducible. The initial problem with the galactose kinase-linked RGH gene, that of host cell galactose kinase activity, is being overcome by selection of appropriate cell variants.

Initial studies on cross-linking glucocorticoid receptors with DNA look promising. Evidence that DNA methylation has little to do with expression, inducibility or hybrid cell extinction of the RGH gene continues to build; this question is now being examined with methylation-sensitive restriction endonucleases which have sites in the 5' - proximal end of the gene. To find whether RGH induction in GH_3 cells is a primary consequence of steroid treatment or requires intervening protein induction(s), studies of induction in the presence of cycloheximide are being carried out. Initial results suggest that blockage of protein synthesis does not block induction of GH transcripts.

Human (and rat) glucocorticoid receptors labeled with [^{3}H]-dexamethasone mesylate have been further purified and shown to have a molecular weight of ~ 90,000g. The group has begun proteolytic analysis of the receptors and studied their immunoreactivity with a polyclonal anti-human glucorticoid receptor antiserum. They hope to accumulate enough purified receptor to begin amino acid sequencing and if successful with that, to synthesize the corresponding oligodeoxynucleotides to use as probes for the receptor gene in recombinant DNA libraries. In another approach to finding the human glucocorticoid receptor gene, rat r cells are being transfected with human r^+ DNA and screened for restoration of receptor specific functions.

A set of somatic cell hybrids between various combinations of wild-type and receptor-mutant CEM cells have been prepared. These show wild-type cells to be dominant, i.e. there is no negative complementation between mutant and wild type receptors and the mutants contain no elements that prevent normal responses to glucocorticoids in the presence of normal receptors. The group intends to prepare a CEM cell DNA library and examine these cells for their "domain" of transcriptional responses to glucocorticoids, using the computer-assisted screening methods developed previously.

Using nude mice bearing intrathecal CEM cells, the group observed that cortivazol is a more effective treatment than the standard therapeutic glucocorticoid, prednisone. In CEM cells, ^{3}H-cortivazol not only binds the glucocorticoid receptor with great avidity, but also binds to a second, weaker site, not seen with standard, potent glucocorticoids.

III. BIOSYNTHESIS SECTION (Dr. E. L. Kuff, Chief)

The Section has three independent research groups.

A. (Dr. Beverly Peterkofsky an coworkers). This group has continued its studies on the regulation of collagen biosynthesis in animals and in normal and transformed cells. During the year, they further substantiated the hypothesis that the specific decrease in collagen synthesis in the bone of scorbutic guinea pigs is related primarily to the decreased food intake and weight loss associated

with scurvy. Thus, there was a linear relationship between the percentage of collagen synthesis and the extent of weight loss in both scorbutic and paired, food-restricted controls receiving vitamin C, as compared to animals fed ad libitum. This decrease in collagen synthesis occurred independently of the effects of ascorbate deprivation on hydroxylation of proline in collagen. The specific effect on collagen synthesis was duplicated in acutely fasted guinea pigs provided with supplemental ascorbate. Collagen synthesis in other connective tissues was equally susceptible to the effect of fasting, regardless of whether the rates were determined (by radiolabeling) in vivo or in vitro. There was no change in the vitamin C content of tissues from the fasting animals, nor was proline hydroxylation affected. The data suggest an entirely new concept for the mechanism of ascorbate deprivation on collagen synthesis in connective tissue. The effect of fasting on collagen production is not degradation. Preliminary experiments suggest that the concentration of procollagen mRNA is decreased in tissues of fasted animals.

Studies on the relationship between cAMP levels and collagen production in cultured cells were resumed. It was found that cAMP levels within normal and Kirsten sarcoma virus - transformed 3T3 cells were similar, contrary to the widely held belief that transformation is regularly associated with decreased intracellular cAMP. Thus, in these cells, cAMP may not be involved in maintaining the transformed phenotype or regulating collagen production (which is decreased on transformation).

B. (Dr. Samuel Wilson and coworkers). This group is investigating enzymes and accessory protein factors involved in DNA synthesis. Their recent results on the structure of the mammalian α-polymerases emphasize a 195 kilodalton (KDa) polypeptide that appears to be an abundant yet previously unrecognized α-polymerase constituent in calf and monkey cells. This protein is obtained from crude soluble extracts of growing cells by immunoprecipitation with a monoclonal antibody to calf α-polymerase. The 195 KDa protein is being compared with other α-polymerase constituents, such as the 120 KDa polypeptide which represents a distinct but quantitatively minor species of α-polymerase in growing cells. Experiments were conducted to validate and improve a rapid new method of gel analysis for DNA polyymerase activity in crude cell extracts. The method was used to show that increased levels of the 120 KDa α-polymerase are induced in resting monkey cells by infection with SV40. The procedure was also used to examine the levels of β-polymerase in cultured cells from patients with ataxia telangiectasia (AT), a disease characterized by defects in DNA repair synthesis. This enzyme species, which is thought to function in DNA repair, was found to be equally abundant in AT cells and those of normal individuals. Additonal studies are in progress to obtain cDNA clones of calf thymus α-polymerase gene sequences and to localize an essential deoxynucleotide triphosphate - binding site in the primary sequence of E. coli DNA polymerase I.

C. (Drs. Edward Kuff and Kira Lueders, and coworkers). These investigators continued their studies on intracisternal A-partical genes, which they had previously shown to make up a family of extensively reiterated retrovirus-like elements in the genomic DNA Mus musculus and some other rodent species. They have now shown by nucleotide sequence analysis that one randomly cloned IAP gene has a retroviral form of long terminal repeat unit (LTR) and is bracketed by short duplications of cellular sequence. The results indicate that this particular IAP gene copy and by inference others, were originally integrated in a

manner ascribed to retroviral proviruses and certain other transposable elements. It was also shown that the IAP LTRs can effectively promote gene expression in mammalian cells when introduced in an expression vector carryng the bacterial gene for chloramphenicol acetyl transferase (CAT). Thus, IAP genes have a number of attributes associated with the conventional integrated proviruses, even though IAPs themselves are not known to have an infectious extracellular phase. The appearance of IAP gene copies in novel genomic locations was observed in the past year. The instances involved established mouse myeloma or derived hybridoma cell lines, which express abundent IAPs in their cytoplasm. In one case, the group here collaboratd with investigators at the University of Toronto in identifying and mapping IAP insertions in mutant κ-light chain genes. In another, comparison between the NCI nucleotide sequences and other published data established that an IAP gene insertion was responsible for the rearrangement and activation of a c-mos oncogene described by workers at the Weizmann Institute of Science. This is the first demonstration of cellular oncogene activation by insertion of an endogenous retroviral element. The results indicate that IAP genes can function as movable elements in the mouse genome and may occasionally have a role in cell transformation and/or tumor progression.

Characterization of a mouse repetitive gene family, the so-called "R-sequence" family, was also continued. These interspersed sequence elements are about 400 bp long and represent 1-2% of the mouse genome. Computer analysis of the sequence has revealed RNA polymerase II promoter and polyadenylation signals and a region of homology with the enhancer/activator region of the SV40 72 bp repeat. Functional aspects of the R-sequence elements are being studied by incorporating them into an expression vector carrying the bacterial CAT gene.

IV. CELLULAR REGULATION SECTION (Dr. O. Wesley McBride, Chief)

The work of the Section is carried out by three independent groups.

A. (O. Wesley McBride and coworkers). Analysis of DNA isolated from somatic cell hybrids that segregate human chromosomes. has been used to localize a variety of human genes to specific human chromosomes using molecularly cloned DNA probes. Many independent somatic cell hybrid lines and subclones were isolated, and the human chromosome content of each cell line was determined by isoenzyme analysis and karyotyping. Some human parental donor lines also contained specific, well-characterized chromosome translocations or deletions, and this permitted regional localization of many genes on human chromosomes. This method was previously used to map the human immunoglobulin genes and pseudogenes in collaboration with Dr. Philip Leder and colleagues. Collaborated efforts with Dr. Stuart Aaronson and coworkers have now assigned several human cellular onc genes (c-H-ras-1, N-ras, c-K-ras-1, c-K-ras-2, c-sis, c-mos, c-myc, and c-myb) to specific human chromosomes and subchromosomal regions. Many of these c-onc genes map to the same sites as non-random chromosome rearrangements in specific human cancers, supporting the assumption that these genes may be involved in human oncogenesis. The α, β, and Γ fibrinogen genes have been mapped to human chromosome 4 in collaboration with Drs. J. A. Kant and G. R. Crabtree. Chromosomal mapping of the multigene metallothionein family with Dr. D. Hamer indicates that these genes are located on at least four different human chromosomes.

The human thymidine kinase gene has been cloned from a recombinant DNA library containing this gene in a mouse background using plaque hybridization

with a repetitive human DNA probe. The entire human DNA segment containing tk and as well as other unique sequences has been subcloned into plasmid vectors for further analysis.

B. (Dean Hamer and coworkers). The objective of this group is to understand eukaryotic gene regulation and they have used heavy metal induction of metallothionein (MT) as a model system. MT genes from mice and humans have been cloned and characterized. Presumptive control sequences of the mouse MT-I gene have been analyzed by in vitro mutagenesis and gene transfer techniques. An upstream activator region and a more distal control region required for heavy metal inducibility were each identified. Although heavy metals and glucocorticoids both induce MT-I expression, gene transfer experiments indicate that they regulate this gene by independent mechanisms. Gene dosage experiments suggest that MT induction requires a positive transcriptional activator, and approaches to isolate this factor are being explored. This group constructed a versatile set of mammalian cell expression vectors that allow essentially any coding sequence to be placed under the control of the MT promoter. This has allowed an exceptionally high level of production of several appropriately processed and post translationally modified polypeptides which are biologically active.

At least 12 different fragments with homology to MT probes have been found in the human genome. Analysis of DNA from human/rodent somatic cell hybrids indicates that the MT genes are located on at least four different human autosomes but not on the X-chromosome. Cultured fibroblasts from Menke's patients (X-linked human genetic disease with copper deficiency) exhibit elevated MT levels but no detectable rearrangement of MT genes. These facts suggest that Menke's Syndrome results from a mutation that effects MT synthesis in trans, and the possible mechanism is being examined.

Recombinant DNA techniques have also been used to develop a yeast expression vector containing a yeast metallothionein-like control region. This provides a model system for analysis of these regulatory genes, their products, and interaction with control sequences in a simple eukaryotic organism that possesses great technical and genetic advantages.

C. (Martin Rosenberg and coworkers.) The goal of this group has been to understand the relationship between function and DNA structure in the control of initiation and termination of RNA synthesis, as well as in the translational expression of mRNAs, in both prokaryotic and eukaryotic systems. Recombinant DNA techniques have been used to develop plasmid, phage, and bacterial vector systems which allow the isolation, characterization, and comparison of procaryotic transcriptional regulatory systems. A variety of defined prokaryotic genes have been examined in these systems thereby permitting identification, characterization, and sequencing of transcriptional initiation and termination sequences. A vector system containing a strong promoter, which can be regulated effectively in high copy number, has been used to overproduce a λ phage encoded polypeptide (cII) which serves as a positive activator of transcription by RNA polymerase. Purification of the cII protein has now permitted detailed physical and chemical characterization. In vitro transcription experiments indicate that the cII protein interacts with repeat DNA sequences on both sides of any phage promoter which is selectively activated.

An RNA-dependent E. coli S30 translation system has been used to examine the translational efficiencies of in vitro synthesized mRNAs. This has permitted analysis of discoordinate expression of two proteins encoded by the E. coli galactose operon. These studies have been extended to examine coupled expression of the two distal genes of the gal operon; consequences of upstream translation on the expression of an adjacent gene have been assessed using in vitro mutagenesis.

Previous work demonstrated that under appropriate conditions prokaryotic genes could be expressed efficiently in eukaryotic cell-free systems and in mammalian cells. Dr. Rosenberg and his colleagues have constructed an SV40-plasmid vector system carrying two independent gene transcription units which function in eukaryotic cell systems. Each transcription unit controls the expression of a different selectable (galactokinase and xanthine phosphoribosyltransferase) gene function which can be easily quantified in cell extacts. The modular design of the vector system allows selective replacement of each regulatory element controlling galk expression with alternative DNA segments. The resulting changes in galk expression can be quantified using Xanthine-guanine phosphoribosyl transferase expression as an internal control. The system is amenable to analysis of both transient and stable expression; it can be used to evaluate transcriptional initiation and translational regulatory sequences in eukaryotes.

V. DEVELOPMENTAL BIOCHEMISTRY SECTION (Dr. Maxine Singer, Acting Chief)

This section is composed of two independent groups.

A. (Bruce Paterson and colleagues). Using cloned cDNA probes this group has isolated the genomic sequences (from the chicken) for the following proteins: alpha skeletal muscle actin, alpha cardiac actin, beta cytoplasmic actin, myosin light chains 1 and 3, vimentin (a major intermediate filament protein), pyruvate kinase, and GAPDH (glyceraldehyde phosphate dehydrogenase). These have been defined by DNA sequence analysis. The various actin genes have been subcloned into the eukaryotic expression vector, pRSV-CAT, in order to study the developmental regulation of these genes after transfection (calcium phosphate precipitation) into the C2 mouse muscle cell line. Probes specific for the various chicken actin genes have been used to monitor expression during differentiation in vitro. In vivo transcription of the vimentin gene, a single copy gene, yields two distinct mRNA transcripts by Northern analysis. The coding potential and formation of these transcripts has been determined. The myosin light chains 1 and 3 are encoded by a single gene. The structure and regulation of this expression is under study. The mouse histone H4 gene is expressed in a cell-cycle dependent fashion. The structure of the gene has been altered to determine, in transfection studies with L-cells and C2 cells, which regions of the gene confer this regulation. Pyruvate kinase undergoes an isoform shift during myogenesis. The structure and regulation of the pyruvate kinase gene is under investigation. The three Acantha amoeba myosin polypeptides have been synthesized in vitro in preparation for the isolation of the three genes. The genes will be used in structural comparison of the myosins.

B. (Carl Wu and colleagues). The sequential arrangement of nucleosomes along the chromatin fiber is punctuated by sites that are highly sensitive to nuclease attack. This group previously mapped such sites to the 5' terminus of several heat shock genes in Drosophila by a novel indirect end-labeling technique. Such

preferentially accessible sites in chromatin may function as points of entry to the DNA for RNA polymerase and control proteins. In order to locate specific sequences to which these proteins might be bound, they have recently used indirect end-labeling in a new way to identify short, nuclease-resistant DNA sequences, and have found such "footprints" within hypersensitive sites in chromatin. To determine the functional relationship of 5' terminal hypersensitive sites in chromatin to gene activity, they have made a promising start in the development of an in vitro transcription system from Drosophila nuclei.

VI. MACROMOLECULAR INTERACTIONS SECTION (Dr. Claude Klee, Chief)

This section is composed of three independent groups.

A. (Dr. Cary Queen and coworkers). This group is studying the regulation of expression of the immunoglobulin gene family by attempting to answer two questions: (1) is synthesis of immunoglobulins restricted to cells of the B-lymphoid lineage, (2) how do these limit transcription to only one or a few immunoglobulin genes, while leaving hundreds of other, similar immunoglobulin genes inactive? The approach to these questions is to insert a cloned, rearranged kappa light chain gene into a plasmid in various configurations, to transfect the plasmid into various types of cells, and to determine whether the transfected gene is transcribed. They have already shown that the complete kappa gene is transcribed after transfection into antibody-producing myeloma cells but not in nonlymphoid 3T3 or L cells. Hence the lymphoid cells appropriately regulate the kappa gene even when it is not in its usual chromosomal environment. By deleting different parts of the cloned gene, they have shown that certain sequence elements downstream of the promoter are necessary for gene transcription in myeloma cells. Investigations are under way to characterize these sequence elements further and to study their mode of action.

B. (Dr. Paul Wagner and coworkers). Dr. Wagner is studying the regulation of cellular motility by Ca^{2+}. One problem under study is the role of Ca^{2+} dependent phosphorylation of myosin light chains in the assembly of myosin filaments in smooth muscle and non-muscle cells. Although non-phosphorylated myosins are assembled into thick filaments "in vivo", in vitro studies suggested that assembly of myosin filaments was regulated by myosin phosphorylation. This apparent discrepancy was shown to be due to the fact that the equilibrium between monomeric and filamentous myosin is dependent not only on phosphorylation but also on the nature of the anions used for the experiments. In the presence of propionate as opposed to chloride, non-phosphorylated smooth muscle myosin is filamentous. Dr. Wagner has also initiated a study on the roles of fodrin and calmodulin in the regulation of the actin-myosin ATPase system. Fodrin is a widely distributed, membrane associated protein that is analogous to erythrocyte spectrim and binds actin and calmodulin. Calmodulin and fodrin form a one to one complex with a binding constant of $5 \times 10^6\ M^{-1}$. Fodrin also interacts with actin and exerts complex effects on the actin-stimulated myosin ATPase. At high fodrin concentration (0.1 μm), an inhibition is observed whereas low concentrations (0.01 μm) have a stimulatory effect. No effects of actin on fodrin-calmodulin interaction or of calmodulin on fodrin modulation of actin-stimulated myosin ATPase have yet been detected.

C. (Dr. Claude Klee and coworkers). This group is concerned with the mechanism of the coordinated regulation of cellular processes by Ca^{2+} and cAMP. In most, if not all, eukaryotic cells the action of Ca^{2+} is mediated by the Ca^{2+}-

receptor protein, calmodulin. Previous work in this laboratory had shown that Ca^{2+} binding to calmodulin is sequential and accompanied by stepwise conformational transitions. These separate states would allow calmodulin to transfer quantitative changes in Ca^{2+} concentration into qualitatively different cellular responses if different enzymes recognize different calmodulin conformers. Using calmodulin fragments, this group has now identified the high and low affinity Ca^{2+} binding sites as sites III and IV, and sites I and II respectively (the numbering starts at the NH_2 terminus). The conformation of the isolated calmodulin fragments is very similar to that of these fragments in the native protein. The peptides can therefore be used to study the interaction of calmodulin with its target proteins and with anti-calmodulin drugs. The carboxyl terminal fragment (residue 78-148), which contains an interacting site for phosphorylase kinase, also contains an interaction site for cAMP phosphodiesterase and the anti-calmodulin drugs, norchlorpromazine and trifluorperazine. However, this fragment, which fully activates phosphorylase kinase, has a low intrinsic activity with phosphodiesterase. Thus, fragment 78-148 can act as an agonist with one enzyme and as an antagonist with another enzyme. Since the activation of phosphorylase kinase required only partial occupancy of the Ca^{2+} sites (2 Ca^{2+} per mol) as opposed to full occupancy for phosphodiesterase, the amino terminal fragment (residue 1-77) may be necessary to confer upon calmodulin its ability to activate a class of enzymes which requires fully liganded calmodulin. Fragment 1-77 contains a second phenothiazine binding site suggesting that this particular class of enzymes may interact with calmodulin at two distinct sites. These studies may therefore provide a general molecular mechanism for the mode of action of agonist and antagonist ligands.

Another aspect of Ca^{2+}·calmodulin regulation of cellular processes is its coupling with that of cAMP. Most calmodulin regulated enzymes are also regulated by cAMP-dependent phosphorylations which enhance or inhibit their stimulation by calmodulin. Conversely, calmodulin and Ca^{2+} modify cAMP dependent responses both directly at the level of cAMP metabolizing enzymes and indirectly by activating Ca^{2+}-regulated kinases and phosphatase(s). In collaboration with P. Cohen's laboratory, this group has characterized a major calmodulin binding protein, calcineurin, as a calmodulin regulated phosphoprotein phosphatase. This enzyme belongs to a specific class of calmodulin regulated proteins which contains a Ca^{2+} binding protein as an integral subunit. The small subunit of calcineurin has been sequenced and revealed the existence of four Ca^{2+} binding loops analogous to the Ca^{2+}-loops of calmodulin. In addition, the amino terminal blocking group has been identified as myristic acid. This hydrophobic substituent may serve to anchor the enzyme reversibly in the membrane. This calmodulin stimulated phosphoprotein phosphatase may serve as an important link between Ca^{2+} and cAMP mediated regulations of cellular processes.

VII. NUCLEIC ACID ENZYMOLOGY SECTION (Dr. Maxine Singer, Chief)

Two different projects engage the interest of this group.

A. A range of interactions occur between permissive cells and infecting viruses. Using simian virus 40 (SV40) as a model, this group is studying one aspect of viral-host interaction, namely, the use of common regulatory elements. Some years ago, segments of the African green monkey genome that are homologous to the control region of the SV40 genome (SV40-ori) were cloned from a monkey library. Three separate genomic segments, each with short (a few hundred base

pairs) and scrambled regions of homology were isolated although approximately 80 such segments can be counted in the monkey genome.

The work this year concentrated on studying whether the monkey segments, in addition to being homologous in structure, are similar to the SV40-ori in function. One of the monkey segments designated clone 7, was used. The SV40-ori-like region in clone 7 is 450 bp long and is embedded in genomic region that is rich in interspersed repeated sequences. It is flanked by two members of the Alu family. One of these Alu segments had an unusual primary structure and was shown to be a member of a novel Alu subfamily whose members contain a potential Z-DNA forming segment. The SV40-ori-like region in clone 7 serves as a promoter for RNA transcription; earlier experiments had already shown that the region is hypersensitive to DNase I in monkey chromatin. The promoter capacity of the segment was demonstrated in several ways. 1) Cellular transcripts originate in this region and their start sites were mapped by the S1 procedure. The nature of the gene encoded in these transcripts is unknown. However like the SV40-like itself, transcription from this region proceeds in both directions. 2) Recombinant vectors were constructed in which the clone 7 region was placed upstream of an E. coli gene, xanthine-guanine phosphoribosyl transferase (XGPRT). When the vector is inserted into monkey cells, transcripts of the bacterial gene are detected and the gene is expressed, regardless of the direction of insertion of the monkey segment relative to the XGPRT gene. The starting points of the transcripts map within the monkey segment. Moreover, both the level of transcription and the start sites vary when monkey sequences that flank the SV40-ori-like region are included in the construction. Thus, the SV40-ori-like region in clone 7 is an efficient cellular promoter; it does not include enhancer activity, however, and an enhancer must be separately supplied in the vector. The cellular promoter is notable for the absence of the TATAAA box typical of RNA polymerase II transcription units. It is possible that it represents a rare class of polyII promoters that play a special regulatory role in cellular metabolism.

B. The group is also investigating various aspects of the organization and function of highly repeated sequences in primate genomes. During the past year work focused on the deca-satellite whose discovery was reported earlier, and on the KpnI family of long interspersed repeated sequences. The most striking feature of the deca-satellite is the fact that its long range organization is not fixed; no two monkeys thus far investigated show the same set of deca-satellite containing restriction fragments. In order to try to estimate the frequency with which deca-satellite organization changes, two types of experiments were carried out. First, the inheritance of organizational pattern was studied in monkey families. The results failed to give any indication that deca-satellite is reorganized in the course of a single generation. However the second type of experiment, in situ hybridization to endoreduplicated chromosomes, indicated that in most somatic all replications at least one chromosome undergoes an unequal-crossing over resulting in a major reorganization of deca-satellite.

The abundant KpnI family (on the order of 10^4 copies) is dispersed in primate genomes. The characterization this year of several cloned monkey family members at the sequence level, confirmed that family members vary as to total length and internal arrangement of family sequences. Consistent with this, the copy number in the genome of subsegments from within long family members, varies. In one case, a KpnI family member was found to be flanked by direct repeats of the genomic

sequence into which it was inserted. Therefore at least some family members are moveable within the genome. These moveable KpnI elements appear to belong to a distinctive class of eukaryote moveable elements that are characterized by the absence of long terminal repeats and the presence of a 3'-terminal polyA stretch. It has been proposed that such elements move by the intermediary formation of transcripts and reverse transcripts. For this reason, the transcription of KpnI family members in human and monkey cells was investigated; RNA molecules homologous to KpnI family probes were found in the size range from 400 to 9000 nucleotides. Homologous cDNA clones were then isolated and are being characterized. Finally, primary sequence data that was accumulated in the laboratory during the past year (about 1800 bp) was compared with recently published sequences for an analogous interspersed rodent DNA family. The two were found to be between 60 and 70 percent homologous over at least 1400 bp. Thus the sequences appear to have had a common ancestor and to have been conserved in evolution.

VIII. PROTEIN CHEMISTRY SECTION (Dr. Albert Peterson, Chief)

This section is composed of three independent groups.

A. (Michael Mage and colleagues). Immunization of CTL precursors in vivo is a complicated process that involves adherent accessory cells (macrophages), T helper cells, Class I MHC antigens (H-2 in mice), Class II MHC antigens (Ia), and at least two different nonspecific helper factors (IL1 and IL2). Simplification of this system by use, in vitro, of highly purified $Lyt2^+$ lymphocytes as a source of CTL precursors and by substituting the helper factors IL1 and IL2 for adherent accessory cells and T helper cells, respectively, has led to the following:

1. CTL precursors probably do not need to have antigen presented to them by adherent accessory cells (macrophages), because successful immunization of CTLp has been accomplished in cultures depleted of accessory cells.

2. Class II MHC antigens are apparently not necessary for presentation of Class I (Ia) MHC antigens to CTLp, because successful immunization has taken place when Class I antigens were furnished by a tumor cell line (EL4) that is Ia^- when tested by immunofluorescence.

Ia^+ and Ia^- variants of an immunogenic tumor cell line (P815) have been obtained by limiting-dilution cloning, and these should permit direct examination of the possible role of Ia antigen in immunization of CTLp by this tumor cell line.

B. (Warren Evans and colleagues). The new HPLC assay developed by this group for detecting and quantifying granulocyte differentiation proteins has been applied to the study of leukocytes obtained from CML patients known to have the Philadelphia chromosome defect in the hope of finding a protein marker that would facilitate early diagnosis and clinical staging of this disease. The HPLC patterns of acetonitrile-trifluoroacetic acid extracts of mature granulocytes from patients in the stable phase of CML revealed marked variations from the highly reproducible normal pattern. These variations, which differ considerably from patient to patient, apparently reflect elevated levels of some of the normal granule proteins and thus a defect in the regulation of granule protein synthesis rather than the synthesis of abnormal proteins. Analysis of CML granulocytes

from two patients in the accelerated phase of the disease yielded HPLC patterns that were clearly distinct from those obtained from patients in the stable phase.

In continuing efforts to identify the humoral factors that induce the synthesis of differentiation proteins in granulocytes of guinea pigs, the incubation of partially purified serum protein fractions (isolated by chromatography on DEAE-cellulose and phenyl-Sepharose) with normal immature granulocytes showed that most of the serum proteins are inactive with respect to differentiation activity, as tested by the HPLC assay. The active fraction, which still contains numerous proteins, retains the ability to induce the synthesis of all the differentiation marker proteins under investigation.

The GL-13-BC guinea pig model for human CML developed earlier by this group not only makes possible the study of molecular aspects of this disease, which is the main emphasis, but offers a means of rapidly screening new therapeutic regimens that clinicians might otherwise be reluctant to test on patients who are in the stable, chronic phase of the disease. Studies during the past year have shown that this model responds essentially the same as human CML to Busulfan or hydroxyurea. These drugs are used in the conventional treatment of CML to control the overproduction of granulocytes during the stable phase, though they produce only a slight increase in the mean survival time. Two other alkylating agents, cyclophosphamide and Alkeran, which are not generally used in the treatment of CML, were also tested in both early and late stages of the disease. The most striking therapeutic effect was observed when cyclophosphamide was given during the early stage. In two ongoing experiments, all of the guinea pigs are still alive and symptom-free (without maintenance therapy) 271 and 100 days, respectively, beyond the mean survival time (35 days) of the untreated controls. These findings emphasize the need for a routine diagnostic method, unavailable at present, for the early detection of CML.

C. (E.A. Peterson). Earlier concern that the use of carboxymethyldextrans (CM-Ds) for the displacement chromatography of proteins might result in the removal of required metal ions from enzymes has been eased somewhat by the ready assay of fractions of a commercial preparation of the alkaline phosphatase of calf intestine, the activity of which depended on retention by the enzyme of Zn^{++} and a more loosely bound secondary metal such as Mg^{++} or Mn^{++}. Similarly the complex of α-lactoglobulin with Ca^{++} and that of conalbumin with iron retained their metal ions when chromatographed with CM-D.

Significant new simplifications of the procedures for purifying and fractionating the CM-Ds have not only facilitated their preparation but improved resolution of CM-Ds in the low to moderate range.

Displacement chromatography of proteins on DEAE-cellulose (DE-52) requires several times as much CM-D as there is protein if the protein peaks are to be well-spaced. However, a study of several other hydrophilic anion exchangers has indicated that the same spacing can be obtained with amounts of CM-D comparable to the amount of protein. Even the CM-D requirements of DE-52 can be dramatically reduced if the molarity of the background buffer is raised moderately.

β-Lactoglobulins A and B, which differ in isoelectric point by only 0.1 pH unit, were separated on several anion exchangers differing widely in matrix and charge density by displacement with the same narrow-range CM-D in the same buffer.

It is too early to conclude that such consistency is the general rule but there are theoretical reasons to expect it.

In a test of the applicability of the displacement system to high performance liquid chromatography (HPLC), a very good separation of the β-lactoglobulins (0.4 mg) was obtained in 35 minutes on a 0.1-ml column of a silica-based anion exchanger (sold as a microbore guard column), using the narrow-range CM-D mentioned above.

IX. OFFICE OF THE CHIEF

Dr. Cecil Fox carries out his research at the Armed Forces Institute of Pathology where he has access to the two million specimens of human tissues contained in the AFIP's Tissue Repository. The goals of this program are to develop measures of malignancy in human cancers by improving grading of prognostic features in human cancer. Grading is a method of estimating the potential of human cancers for growth, invasion and metastasis, all of which are important to clinicians in treating human cancer. The measures of malignancy being studied are morphometric parameters of tumor cell populations, standardization of histopathological preparatory technique for image analysis, computerized nuclear morphometry and cytogenetic homogeneity of tumor tissue. In addition, he is studying tumor cell invasion, growth and cell attachment to purified basement membrane proteins using reflection contrast microscopy, scanning integrating interferrometry, low light video and fluorescence microscopy. Tumor systems being studied are endometrial hyperplasia/endometrial carcinoma, Bowenoid hyperplasia, lobular hyperplasia/lobular carcinoma of the breast and treated and untreated head and neck tumors. In vitro studies use cell lines from high and low metastatic potential cell lines from humans and selected murine tumors. A retrospective study is being initiated to detect oncogene product in formalin fixed tissues in the repository.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB00333-20 LB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biochemical Basis for Defective Differentiation in Granulocytic Leukemia

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
W. H. Evans Research Chemist, LB, NCI

COOPERATING UNITS *(if any)*
Hematology, Oncology Section, Walter Reed Army Medical Center

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	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 main thrust of this work is to develop biochemical methods for the early diagnosis of granulocytic leukemia and methods for inducing leukemic cells to develop some or all of their functional properties as a means of partially or completely restoring host defense mechanisms in leukemia patients. Work is first aimed at establishing which of the many biochemical steps involved in normal granulocyte differentiation are controlled by humoral regulators. The results will be compared with those obtained from similar studies on leukemic cells at corresponding stages of maturity in order to determine the nature and potential reversibility of the arrested differentiation steps. Biochemical analyses are carried out on mature and immature granulocytes isolated from blood and bone marrow and the effects of external cell regulators on granulocyte differentiation, as measured by changes in the synthesis of specific cellular components, are studied in a defined culture system previously developed in this laboratory.

PHS 6040 (Rev. 1/83) GPO 895-100

OTHER INVESTIGATORS:

E.A. Peterson	Chief, Protein Chemistry Section	LB	NCI
S. Wilson	Biologist	LB	NCI
M. Mage	Immunochemist	LB	NCI
V. Alvarez	Expert	LB	NCI

Methods Employed: Leukocytes are isolated from bone marrow, blood and inflammatory exudates by a variety of methods. Subcellular fractions are prepared by differential centrifugation. Proteins extracted from leukocytes and serum proteins are fractionated by column chromatography, high performance liquid chromatography (HPLC), and gel electrophoresis. Glycolipids are separated by thin layer chromatography and analyzed by gas-liquid chromatography and mass spectrometry. Immunological reagents, including fluorescent antibodies, are prepared against granulocyte proteins and used to follow their formation during granulocyte maturation. Stationary tissue culture methods are employed to study the synthesis of specific proteins from radioactive precursors. The distribution of radioactive proteins in electrophoretic gels is determined by slicing the gels in a gel fractionator and counting the slices in a liquid scintillation counter. Densitometric patterns of gels stained for glycoproteins are obtained by scanning the gels in a spectrophotometer equipped with a gel scanner accessory.

Major Findings:

A. HPLC assay for granulocyte differentiation proteins - application to normal and leukemic human granulocytes

Previously, we described studies which indicated that reverse phase HPLC could be used to rapidly and simultaneously screen extracts of granulocytic leukocytes for a variety of protein markers of granulocyte differentiation. (A paper describing these initial studies has been completed and submitted for publication). This new assay has opened up several new avenues of research in our laboratory on the problem of granulocyte differentiation. Though the HPLC assay was originally developed using a guinea pig model for granulocyte differentiation, one major focus of attention during the past year has been the application of this assay to the study of granulocyte differentiation markers in normal and leukemic human granulocytes. One question we are attempting to answer is whether or not mature granulocytes obtained from CML patients, known to have the Philadelphia chromosome defect, have any abnormalities in their phenotypic pattern of differentiation proteins. It is postulated that the chromosomal abnormality in CML cells might be associated with an altered polypeptide composition in the CML granulocyte. Such abnormalities might serve as useful markers for the clinical staging of this disease. For these studies highly purified populations of mature, normal and leukemic granulocytes are isolated from either blood or bone marrow by Ficoll density centrifugation. The HPLC assay developed for studying guinea pig granulocyte differentiation proteins was modified in several ways for the studies with human granulocytes. These modifications included the use of 10% acetonitrile in 0.1% trifluoroacetic acid in place of nonidet P-40 buffer as the cell extracting medium and the use of a new, reverse phase HPLC gradient with greater resolving capability than

that used previously. These changes make it possible to obtain a more comprehensive phenotypic protein pattern for normal and leukemic granulocytes. Using these methods we find that a highly reproducible HPLC elution pattern of granulocyte differentiation markers can be obtained with normal cells. Studies with subcellular fractions of normal granulocytes and purified standards of known granulocyte proteins indicate that many of the proteins detected in this assay are associated with the cytoplasmic granules and plasma membrane of these cells. We have also compared the HPLC profiles of normal cells with those of leukemic cells obtained from ten patients in the stable phase of CML and one patient in the accelerated phase. The data indicate that the HPLC patterns of the CML granulocytes show marked variations from the normal pattern in most of the CML patients. SDS gel electrophoretic analysis of the proteins in one of the HPLC peaks which was elevated in granulocytes from the stable phase patients showed that the change in the HPLC peak height was due to elevated levels of several normal cytoplasmic granule proteins rather than to the appearance of a new abnormal protein. The data tenatively suggest that the regulation of granule protein synthesis in granulocyte precursors is altered in some CML patients and that this regulatory defect varies considerably from patient to patient. The nature of the other changes detected in the HPLC patterns of CML granulocytes remains to be determined. We are also periodically repeating the HPLC assay on the ten stable phase CML patients in order to determine whether any significant changes in the phenotypic protein pattern of their granulocytes can be detected that might herald the onset of the accelerated phase of this disease. Analysis of CML granulocytes from two patients already in the accelerated phase of the disease showed that the HPLC patterns were clearly distinct from those of granulocytes from patients in the stable phase.

B. Studies of the induction of maturation in normal and leukemic immature granulocytes.

Work continues on the problem of identifying the humoral factors that induce the synthesis of differentiation proteins in normal immature granulocytes. (Note: progress in this area of our research was slowed greatly by the fact that normal strain 13 guinea pigs, which are used as the model in these studies, were unavailable for the last six months of 1982 due to a dietary mishap in the NIH guinea pig production unit). As reported previously we have shown that normal dialyzed guinea pig serum can serve as a source of physiological factors, for inducing granulocyte maturation in vitro. These studies indicate that dialyzed normal serum appears to be capable of inducing the synthesis of a variety of granulocyte differentiation proteins some of which are associated with cytoplasmic granules and others with the plasma membrane of mature cells. (A paper on this completed work has recently been submitted for publication). One of the questions we are now attempting to answer is whether the synthesis of these various proteins is induced by a single factor in normal serum or whether multiple factors are involved. In order to answer this question we are currently isolating various protein fractions from dialyzed normal serum by chromatographic methods and testing these fractions for their ability to induce the synthesis of the various differentiation markers. Results obtained, thus far, using partially purified serum protein fractions isolated by DEAE-cellulose and Phenyl Sepharose chromatography, show that most of the serum proteins (e.g., albumin, gamma globulins) are inactive with respect to differentiation activity. The active fraction, which still contains numerous serum proteins, retains the

ability to induce the synthesis of all the differentiation proteins detected by the HPLC assay.

Studies of the biochemical mechanisms involved in the arrested maturation of leukemic granulocytes are being pursued with the aim of determining the potential reversibility of the cell maturation defects in leukemia. Recent studies in other laboratories have suggested that the vitamin A derivative, retinoic acid, is capable of reversing the arrested maturation of a human promyelocytic leukemia cell line (HL-60) and thereby induces these cells to form mature granulocytes in vitro. The conclusion that mature granulocytes are produced in these cultures is based primarily on subjective morphological evidence that is inconclusive since many of the morphological features described are also present in macrophages, which are also produced from these leukemic cells under certain conditions. In order to test the conclusion that retinoic acid induces the formation of mature granulocytes from HL-60 cells in vitro, we compared the HPLC pattern of differentiation markers in normal human mature granulocytes formed in vivo with the pattern obtained with the putative mature granulocytes produced from HL-60 cells in vitro. We found that these patterns showed little resemblance. This finding is in keeping with studies in other laboratories which show that lactoferrin, a major marker of granulocyte differentiation, is absent in HL-60 cells exposed to retinoic acid in vitro. On the basis of the evidence now available we conclude that retinoic acid induces either the formation of highly defective granulocytes or, more likely, the formation of a macrophage-like cell from HL-60 cells in vitro.

Significance to Cancer Research: (Objective 2, Approach 3). Granulocytic leukocytes are produced in the bone marrow by a complex, multistage process of cell differentiation whereby proliferating, nonphagocytic, precursor cells are converted to nonproliferating phagocytic cells that play a crucial role in the body's defense against microbial invasion. Granulocytic leukemias can be thought of as diseases in which this program for differentiation is arrested at various stages, resulting in the overproliferation of immature cells lacking, in varying degrees, the capacity to carry out their normal function in the body's defense against infections. At present, the mechanisms involved in the initiation and modulation of the various steps in the differentiation program are poorly understood. Our research is aimed at identifying the humoral factors and clarifying the molecular processes that control the appearance of specific subcellular components during granulocyte differentiation. Such information should be useful in developing therapeutic approaches for reversing the arrested differentiation of leukemic cells.

Proposed Course of Research: The rate of differentiation of granulocytic leukocytes in the bone marrow is regulated by a feedback control mechanism which responds to the rate of destruction of these cells in the peripheral tissues of the body. Feedback information could be of a positive (stimulator) or negative (inhibitor) type transmitted by humoral and/or cellular factors, but at present the nature of these factors is unknown. The humoral hypothesis for regulation will be examined by studying the effect of normal and inflammatory sera on granule-specific protein synthesis by granulocyte precursors in vitro. This approach is analogous to the use of hemoglobin as a marker for erythrocyte

differentiation in assays for erythropoietin. Unlike erythrocytes, which contain only one major specific protein, granulocytes contain many potential marker proteins in their granules. Looking at this problem from a broader perspective, it is believed that the system we are developing for the study of the coordinated regulation of macromolecular synthesis associated with secondary granule formation in myelocytes could serve as a useful model for studies of coordinate gene expression in mammalian cells, just as the study of hemoglobin formation in erythrocyte differentiation has provided much information about the regulation of single genes. Our research plans are as follows:

(1) To isolate, purify and eventually test in vivo any humoral regulators of granulocyte differentiation detected by our newly developed combined morphological and biochemical assay for granulocyte differentiation in vitro.

(2) To further develop the use of HPLC as a method for phenotypically characterizing the degree of differentiation of purified populations of normal and leukemic granulocytes from guinea pigs and humans. This rapid screening method might be useful in classifying or staging AML and CML patients for various types of therapy.

(3) To investigate various potential agents for inducing leukemic cells to mature either partially or completely in vitro. Such agents could serve as attractive adjuncts, or in some cases, as alternatives to cytotoxic therapy, for they would inhibit the expansion of the leukemic population while leaving normal granulocytes unharmed.

(4) The standardized guinea pig model for human CML will be used to test the effectiveness of conventional and potentially new therapeutic regimens for delaying or preventing the onset of blast crisis and in increasing the survival of leukemic guinea pigs. In this connection, studies in collaboration with Dr. C.K. Hsu are underway to develop monoclonal antibodies against the guinea pig leukemic cells for the purpose of testing the efficiency of using such antibodies, either alone or as conjugates with various therapeutic agents (drugs, toxins, isotopes), for eradicating leukemic cells in vivo or from syngeneic bone marrow preparations to be used as grafts for repopulating irradiated leukemic hosts.

Publication:

Evans, W.H. and Miller, D.A.: Blast crisis associated with granulocytic leukemia in strain 13 guinea pigs. Leukemia Res. 6, 819-825, 1982.

Appendix: NIH Contract No. 1-60-25423

Funding: General NCI Contract for FCRC - No individual project funding breakdown available.

Man Years Purchased: 1

Major Findings

Characterization of the GL-13-BC leukemia

The GL-13-BC strain of guinea pig leukemia developed under this contract has been shown previously to exhibit many hematological and pathological similarities to chronic myelocytic leukemia in humans. It is well known that the overproduction of granulocytes which characterizes this disease in humans can be controlled in most CML patients by treatment with either busulfan or hydroxyurea. These treatments are chiefly for relief of symptomatic aspects of the disease since they produce little benefit with respect to long term survival in most patients with CML. The median survival of these patients is about 3 years from diagnosis and the main cause of death is myeloblastic transformation, for which combination chemotherapy is usually ineffective. If the survival of these patients is to be improved, then new forms of therapy must be developed to delay or prevent the onset of blast crisis. With the GL-13-BC leukemia model it should be possible not only to study the molecular aspects of this disease, which is the main emphasis of our work, but also to rapidly screen new therapeutic regimens that clinicians might otherwise be reluctant to test on CML patients who are in the stable, chronic phase of the disease. During the past year we have been collaborating with Dr. Jack Pearson and Dr. Richard Smalley at FCRF on the problem of characterizing the response of the GL-13-BC leukemia to the two conventional drugs, hydroxyurea and Busulfan, used to treat this disease. For comparison with Busulfan, a known alkylating agent, we also tested two other alkylating agents, Alkeran and cyclophosphamide, which are not generally used in the treatment of CML but are effective in treating other forms of cancer. Guinea pigs were injected subcutaneously with leukemic cells under the standardized conditions previously reported. Treatment was initiated in one group of animals at an early stage (day 14) before any change in the absolute or differential count of the blood leukocytes occurred. In another group of animals treatment was initiated at a time corresponding to the chronic phase of human CML when a marked elevation in the granulocyte count in the blood is observed. The results obtained in our studies indicate that the response of the GL-13-BC leukemia is essentially the same as that of human CML with respect to treatment with hydroxyurea and Busulfan. Both of these drugs produced a marked reduction in the leukocyte count in the chronic phase. A small increase in the mean survival time of the leukemic animals was observed when they were treated in the early stage of the disease. Among the three alkylating agents, the most striking effect was observed with cyclophosphamide given at the early stage of the disease. Two separate experiments in which leukemic guinea pigs were treated with this drug are still ongoing. In the first experiment all of the guinea pigs are still alive without maintenance therapy and symptom-free 271 days beyond the mean survival time of the controls (35 days). In the second experi-

ment, started more recently, all of the guinea pigs are still alive without maintenance therapy and symptom-free 100 days beyond the mean survival time of the controls. The data indicate that the life span of guinea pigs with CML can be greatly extended if these animals are treated with cyclophosphamide at an early stage of the disease. These studies dramatically emphasize the need for a diagnostic method which would permit the early detection of CML. At present this disease is generally not detected in humans until it is well advanced and beyond the reach of any curative chemotherapy. We are currently attempting to determine whether any specific antigenic markers exist in leukemic cells or serum which might serve as early diagnostic indicators of the presence of this disease in guinea pigs and humans.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB00366-13 LB

PERIOD COVERED: October 1, 1982 to September 30, 1983

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Biosynthesis and Assembly of Intracellular Components

PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.)

(Name, title, laboratory, and institute affiliation) E. L. Kuff Chief, Biosynthesis Section LB NCI

K. K. Lueders Chemist LB NCI

COOPERATING UNITS (if any): Department of Medical Biophysics, University of Toronto

LAB/BRANCH: Laboratory of Biochemistry

SECTION: Biosynthesis Section

INSTITUTE AND LOCATION: DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2.5	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)

We have previously characterized intracisternal A-particle (IAP) genes as retrovirus-like elements and shown that they are extensively reiterated in the cellular DNA of Mus musculus and some other rodent species. IAPs are not known to have an infectious extracellular phase and are genetically distinct from the commonly recognized murine leukemia and mammary tumor viruses. Recently, we have confirmed by nucleotide sequence analysis that IAP genes have a retroviral form of long terminal repeat unit (LTR) and are bracketed by short duplications of cellular sequence, suggesting that these genes have been integrated in a manner ascribed to retroviral proviruses and certain other transposable elements. We have also found that cloned IAP LTRs can promote gene expression when introduced in an expression vector carring the bacterial gene chloramphenicol acetyl transferase . The appearance of IAP gene copies in novel genomic locations has been directly demonstrated in the past year. In one case, insertions into κ-light chain genes resulted in defective gene function. In another, an IAP gene insertion resulted in rearrangement and activation of a cellular oncogene. Thus IAP genes constitute a source of genetic variability in the mouse and may have a role in cell transformation and/or tumor progression.

OTHER INVESTIGATORS:

A. Feenstra	Visiting Fellow	LB	NCI
R. Hawley	Not NIH		
N. Hozumi	Not NIH		
M. Shulman	Not NIH		

Project Description:

Objectives: To study the mechanisms for coordinately regulating the synthesis of individual macromolecules and their assembly into complex intracellular components (organelles and viruses); specifically, to study the regulation of endogenous viral gene expression in relationship to normal development and neoplastic transformation.

Methods Employed: Culture of animal and bacterial cells; recombination and cloning of specific eukaryotic and viral DNA sequences in plasmids, lambda phage and retrovirus vectors; analysis of DNA components by restriction endonuclease cleavage, electrophoresis, and blot hybridization; chromosomal localization of specific DNA sequences by in situ hybridization; electron microscopy of DNA heteroduplexes and DNA:RNA hybrid molecules; nucleotide sequencing by procedure of Maxim and Gilbert; chromatographic and electrophoretic analysis of cellular and viral proteins; radio-immunoassay and immunoprecipitation of specific antigens; immunofluorescent staining.

Major Findings: We have continued our studies of intracisternal A-particle (IAP) genes, a distinctive class of endogenous retrovirus-like elements that are extensively reiterated in the cellular DNA of Mus musculus and certain other rodent species.

1. IAP genes are mobile elements in the mouse genome.

   a. Both long terminal repeat units (LTRs) of a randomly selected "typical" IAP gene designated M1A14, have been sequenced. They show many structural features associated with LTRs of the infectious avian and mammalian retroviruses, including appropriately positioned promoter and polyadenylation signals and short inverted repeats at the ends of each LTR. Direct duplication of cellular sequences flanking the IAP gene were also found, suggesting that this particular gene copy - and by inference others - had been integrated in a manner ascribed to retroviral proviruses and certain other transposable elements.

   b. In collaboration with investigators at the University of Toronto (Hawley, Hozumi, and Shulman) we studied cloned DNA from two mutant hybridoma cell lines in which defective κ-light chain production was associated with insertion of novel DNA sequences in the corresponding immunoglobulin genes. By heteroduplex analysis and restriction site mapping, we definitively identified the inserted sequences as IAP gene elements and showed that the insertional junction were very close to, if not precisely at, the ends of IAP LTRs. Preliminary nucleotide sequence data also indicat-

ed a clean transition between light chain gene and IAP LTRs at the insertion points. Structural differences between the inserted elements in the two mutants showed that a different IAP gene had been involved in each event. The data were consistent with the insertion of IAP provirus, but it is not clear whether these were new DNA copies generated in the IAP-rich parental hybridoma line, or transpositions of copies already present in the genomic DNA.

c. A group at the Weizman Institute in Rehovot(Rechavi, Givol, and Canaani) found that the mouse myeloma cell line XRPC-24 contained an activated and rearranged cellular oncogene c-mos. They reported the sequence of a new DNA element which had displaced the 5' one-fourth of the coding sequence in the rearranged gene (designated rc-mos). Comparison of this sequence with that of the LTR from the IAP gene M1A14 revealed nearly 90% homology. The inserted LTR is in head-to-head orientation with respect to the mos coding sequences in rc-mos. It appears that transcription is somehow inititated on the antisense strand of the IAP LTR and extends "outwards" into the retained oncogene sequence. The resulting transcript apparently codes for an active product since the cloned rearranged gene induces transformation when transfected into NIH3T3 cells. This is the first demonstration of cellular oncogene activation by insertion of an endogenous retroviral element.

2. Regulation of IAP Gene Expression

a. As mentioned above, nucleotide sequencing of the LTRs from a randomly selected IAP gene M1A14 revealed typical RNA polymerase II control signals. To determine whether these LTRs are actually capable of functioning in cells, we introduced them into expression vectors carrying the bacterial gene chloramphenical-acetyl transferase (CAT). Constructs containing the 5' and 3' LTR sequences in both orientations with respect to the CAT gene were tested for CAT expression following transfection into monkey cells. When the 5' LTR was present upstream from the CAT gene in the same transcriptional orientation as in the IAP gene, promotion of expression of the CAT gene was measured at levels comparable to that seen with the SV40 early promoter. The 3' LTR was also active, but at lower levels. Neither LTR was active in promoting CAT expression when inserted in the opposite orientation. S1-mapping experiments are in progress to locate precisely the transcriptional start.

b. Hypomethylation has been shown to be associated with gene activity in a number of instances. We are examining the state of methylation of IAP genes in particle-producing vs particle-negative mouse cells and tissues. Dr. Feenstra has mapped the MspI/HpaII sites in cloned IAP genes and identified a specific fragment which is generated by cleavage at one site close to the putative transcriptional initiation point in the 5' LTR and another about 1 Kb downstream in the body of the IAP gene. With the appropriate hybridization probe, this fragment is detected as a strongly reacting band in Southern blots of mouse DNA digested with Msp I (an enzyme insensitive to methylation of cytosine within the recognition sequence CCGG). When cellular DNA from various sources is digested

with HpaII(sensitive to C-methylation), the relative intensity of this band in Southern blots is an index of the fraction of IAP genes that are hypomethylated at their ends. Several IAP-negative cells such as liver, F9 embryonal carcinoma and NIH3T3 cells show varying degrees of hypomethylation of internal Msp I/Hpa II sites but little or no 5' demethylation. However, in particle-rich neuroblastoma and myeloma cells, there is extensive demethylation at both internal and 5' sites. In the N4 neuroblastoma line, for example, as many as 100 IAP gene copies are demethylated at the MspI/HpaII sites in their 5' LTRs. It may be that multiple gene copies are activated in IAP-producing cells. It should also be noted that proviral DNA that has been newly generated by reverse transcriptase activity and integrated into cellular DNA could also contribute to the pool of hypmethylated gene copies.

c. A thymidine-kinase minus(TK^-) rat liver cell line has been cotransfected with the cloned herpes TK gene and mouse IAP sequences (M1A14), and several HAT-resistant clones (TK^+) have been isolated. In the most thoroughly studied case, the transformed rat cells contain 4-5 integrated mouse IAP elements. All of these copies are demethylated at their 5' ends, and IAP-related transcripts have been detected by RNA dot-blot analysis.

3. Comparative Studies

Sequences related to mouse IAP genes have been isolated from rat and Syrian hamster gene libraries as recombinants in λ phage. The sequences are moderately reiterated in both of these species but their sequence organization in the hamster genome is different from that in the rat genome. Restriction analysis and electron microscopy indicate that the Syrian hamster IAP sequences represent a family of relatively homogeneous well-conserved units; in this they resemble the mouse IAP genes. The rat sequences, in contrast, are heterogenous. Both the hamster and rat IAP sequence units contain regions homologous to mouse IAP genes interspersed with regions of apparent non-homology. The interspersed regions range in size from 0.5-1.0 Kb. The regions of homology among the mouse, rat and Syrian hamster IAP sequences have been mapped to a 5-6 Kb internal region on the mouse IAP genes. Mouse IAP long terminal repeat (LTR) sequences were not detected in the rat and Syrian hamster genomes.

We used the thermal stability of hybrids between cloned and genomic IAP sequences to measure family homogeneity. Mouse and Syrian hamster IAP sequences are homogenous by this criterion, but the rat IAP sequences are heterogenous with a T_m 6°C below the self-hybrid. The contrasting organization of IAP-related elements in the genomes of these rodents indicates that amplification or homogenization of this sequence family has occurred independently and at different periods of time during their evolution.

Proposed Course of Research: We will continue to investigate the possible effects of IAP genes on the structure, position and activity of other genetic elements. We will search for other instances of mutagenesis resulting from the movement of IAP genes into novel locations. We will examine the activity of IAP genes introduced by transfection into cultured somatic cells and (in collaboration with other laboratories) by microinjection into early mouse embryos.

Significance for Cancer Research: The extensively reiterated IAP genes in mice and Syrian hamsters may provide a significant source of genetic variation in these species and could occasionally have a role in trnsformation or tumor progression. IAP genes may provide a model for the activation of cellular oncogenes by insertion of endogenous transposable elements.

Publications:

Kuff, E.L., Feenstra, Lueders, K., Smith, L., Hawley, R., Hozumi, N., and Shulman, M.: Intracisternal A-particle genes as movable elements in the mouse genome. Proc. Natl. Acad. Sci. U.S.A. 80:1992-1996, 1983.

Kuff, E.L., Feenstra, A., Lueders, K., Rechavi, G., Givol, D., and Canaani, E.: Homology between an endogenous viral LTR and sequences inserted into an activated cellular oncogene. Nature 302:547-548, 1983.

Lueders, K.K. and Kuff, E.L. Comparison of the sequence organization of related retrovirus-like multigene families in three evolutinarily distant rodent species. Nucleic Acids Res. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB00375-21 LB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Homogeneity and Structure of Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

E. A. Peterson Chief, Protein Chemistry Section, LB, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Protein Chemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Methods for the fractionation and analysis of proteins are developed and applied to the purification of specific proteins for the study of their function and structure. Displacement chromatography is being developed for the fractionation of macromolecules and particles of biological interest, employing polyanions differing in number of charges per molecule as displacers. The procedure is particularly advantageous when large amounts of source material must be used to obtain sufficient amounts of a minor component, since the resolving power of the system can be focused on the narrow range of affinity represented by the protein of interest and its nearest neighbors.

PHS 6040 (Rev. 1/83)

GPO 895-100

OTHER INVESTIGATOR:

N. Najam Forgarty Visiting Fellow LB NCI

Project Description

Objectives

To develop methods for the separation and analysis of proteins that will overcome present limitations and to apply these methods to the purification of specific proteins for the study of their functions.

Methods Employed

Protein fractions separated by ion-exchange displacement chromatography are evaluated by gel electrophoresis, using both denaturing and nondenaturing conditions. Carboxymethyldextrans (CM-Ds) having a variety of degrees of substitution are prepared by reaction of alkaline dextran (nominally 10,000 M.W.) with chloroacetic acid.

Major Findings

In previous years, the use of carboxymethyldextrans (CM-Ds) having different numbers of charges per molecule to fractionate proteins by ion-exchange displacement chromatography has been reported, offering high resolution as well as the high capacity and band-sharpening that are characteristic of displacement chromatography of small molecules. One concern was the possibility that even the low level of metal-binding affinity to be expected of such substances might be amplified by the relatively large amounts employed in chromatography to the point where the activity of metal-dependent enzymes might be seriously impaired unless compensating concentrations of the appropriate metal ions were provided. However, in recent experiments with a commercial preparation of the alkaline phosphatase of calf intestine, the presence of CM-D in the fractions did not interfere with the assay, though the activity depended upon retention by the enzyme of Zn^{++} and a more loosely bound secondary metal such as Mg^{++} or Mn^{++}. A relatively low affinity of CM-Ds for metal ions has also been shown by the fact that the complex of α-lactalbumin with Ca^{++}, which can readily be separated from the Ca^{++}-free form of the protein by displacement chromatography, is not converted to the Ca^{++}-free form when chromatographed alone in a CM-D train at pH 7.5. Similarly, the complex of conalbumin with iron does not lose its iron (as shown by retention of its pink color) when it is chromatographed with CM-D at the same pH.

Simplification of the purification and fractionation procedures involved in the preparation of narrow-range CM-Ds has been pursued, not only to facilitate preparation for use in this work but also to encourage the production of these displacers by others. The number of precipitations required has now been cut in half by using small amounts of ion-exchange resin to remove the last of the contaminating chloride, and preparations are being made for testing an all ion-exchange (no precipitations) procedure for the purification of the CM-Ds from reaction by-products and residual reagents.

The fractionation of the CM-Ds by displacement chromatography has, in the past, required the use of low-affinity CM-Ds as counter ions in the initial preparation of the column in order to avoid the disturbing effects of such ions as phosphate or chloride, which compete effectively with low-affinity CM-Ds for sites on the adsorbent, especially at the concentrations attained when these ions are displaced by higher-affinity CM-Ds. It has now been found that acetate ion can be used as the initial counter ion without causing these difficulties since it emerges at the beginning of the displacement train. This permits better fractionation of the low-affinity CM-Ds and reuse of the column without repacking. Also, along with the prospective all ion-exchange purification, it opens up the possibility of purification and fractionation of the product in one continuous stream as it emerges in a timed program from the reaction vessel. Such a procedure would further enhance the efficiency of the CM-D fractionation since it would preserve, in the liquid entering the final column, the partial segregation of affinities that is inherent in the continuous withdrawal of the crude product from the reaction vessel. When the reaction product is collected in several portions before purification, as in the present procedure, much of this pre-segregation is lost.

Displacement chromatography of proteins on DEAE-cellulose (DE-52), using CM-Ds as displacers, generally requires several times as much CM-D as the amount of protein being separated when well-separated peaks of closely similar proteins are required. The intervals on the column between the protein bands must be saturated with CM-D having intermediate affinities, and DE-52 (among others) appears to have a substantial number of sites that are capable of binding CM-D but not proteins. Presumably, this is due to the ability of the flexible CM-D chains to accommodate to patterns of charges on the adsorbent that do not match the more rigid charge patterns on the protein molecules. Recent experiments show, however, that a moderate increase in the concentration of the background buffer (which can be introduced with the final displacer) strikingly increases the spacing produced by a given amount of CM-D, reducing the CM-D requirement several fold in the low to moderate affinity range. Apparently, marginal CM-D-binding sites on the adsorbent are rendered ineffective.

DEAE Bio-Gel A, with a low incorporation of basic groups in a beaded agarose matrix, has a low capacity for CM-D relative to its capacity for proteins. It requires only 1/4 to 1/5 of the amount of narrow-range CM-D needed with DE-52 to produce the same spacing of proteins. DEAE-Trisacryl M, a beaded adsorbent with a very high content of basic groups in a modified polyacrylamide matrix has been shown to have a very low requirement for spacer CM-D in the separation of β-lactoglobulins A and B at pH 6, but that is a consequence of its special sensitivity to increase in pH in that range. Its capacity for CM-D at pH 7.5 is higher than that of DE-52.

DEAE-Sephacel, which has a moderate content of basic groups in a beaded matrix of regenerated cellulose, has been studied extensively with a mixture of model proteins. Taking advantage of the fact that the column bed does not shrink during chromatography and cleaning, we have used the same column for many experiments and have found that its requirement for CM-D, initially well below that of DE-52, undergoes a substantial further decrease with repeated use of the column.

Titrations of fresh adsorbent and one that had been used 10 times show no differences in the shape of the titration curve or the amount of basic groups. It is suspected that the cleaning operation is responsible for the change in CM-D requirement, but the absence of change in the titration curves leaves the effect unexplained. In any case, it appears at this point to be an advantageous, if mysterious, one. If there is an accompanying decrease in the capacity of the column for proteins, it is not apparent under the conditions obtaining in these experiments.

It is of interest that when β-lactoglobulins A and B, which differ in isoelectric point by only 0.1 pH unit, were subjected to displacement chromatography on DE-52, DEAE Bio-Gel A, DEAE-Trisacryl M, and DEAE-Sephacel in 20 mM sodium phosphate at pH 6.0, the same narrow-range spacer CM-D readily separated them on all of these columns, whereas in elution chromatography they emerged from different columns at substantially different salt concentrations. Since this is the only protein pair that has been studied in this way, it is too early to conclude that such consistency is the general rule, though there are theoretical reasons to expect it. If true, even with exceptions, it will be of considerable practical importance.

Preliminary trials, in collaboration with Dr. Vernon Alvarez, exploring the application of narrow-range CM-Ds to displacement chromatography in HPLC have been promising. Thus, a very good separation of the A and B forms of β-lactoglobulin was achieved on a commercial 0.1-ml silica-based anion-exchange column (sold as a microbore guard column) in 35 minutes, applying solutions containing 0.4 mg of the protein, 3 mg of the CM-D spacer, and 8 mg of the final displacer at 5.5 ml per hour with a peristaltic pump. The entire chromatogram encompassed only 3.2 ml and the concentrations of the bands were high. This illustrates the simplicity of the apparatus required for displacement HPLC as well as its high capacity. However, future work with complex protein mixtures and multiple spacers is expected to require more careful application of sample and spacers, somewhat longer columns, and a syringe pump for pulse-free flow.

Significance to Cancer Research (Objective 2, Approach 3)

Displacement chromatography of proteins promises to be of value at any scale of operation and therefore has significance to all research that involves the isolation of such substances. The high capacity, resolving power, and convenience of these systems offer to expedite the recognition and isolation of minor protein components such as regulating factors and marker proteins of interest in disease. The anticipated development of a systematic, general procedure for the purification of nonhistone nuclear proteins that participate in the regulation of the transcription of genetic information would be of substantial significance to cancer research since defective control of these processes appears to be involved in cancer.

Proposed Course of Research

Development of displacement systems for the separation of nonhistone nuclear proteins will be continued. A large scale isolation of HMG-1 and HMG-2 will be

undertaken and the LMG proteins will be fractionated into several groups with selected narrow-range spacers.

Displacement chromatography will be applied to the purification of enzymes and other proteins of interest to members of this Laboratory. Preliminary trials on tissue cytosols have been very promising. Among these applications will be the isolation of serum and cytoplasmic factors mediating bone marrow response to inflammation in the guinea pig. Possible application in the fractionation of leukocyte granules and cell populations will be explored, using appropriate adsorbant matrices. Also the narrow-range CM-Ds will be further tested as spacers in analytical separations on HPLC ion-exchange columns in the hope that they will improve resolution as well as eliminate the need for gradients. They will also be tested in thin layer chromatography of enzymes.

A system of CM-Ds will be prepared from 5000 MW dextran instead of the usual 10,000 MW material. Anticipated advantages, especially for the low-affinity range, are less heterogeneity in the initial preparations, less mass required for spacing, and a possible improvement in resolution.

Publications:

Torres, A.R. and Peterson, E.A.: Solubilization of nonhistone chromosomal proteins by carboxymethyldextran for chromatography. Biochim. Biophys. Acta 698: 303-306, 1982.

Peterson, E.A. and Torres, A.R.: Ion-exchange displacement chromatography of proteins, using narrow-range carboxymethyldextrans and a new index of affinity. Anal. Biochem. 130: 271-282, 1983.

Peterson, E.A. and Torres, A.R.: Displacement chromatography of proteins. In Jakoby, W.B. (ed.): Methods in Enzymology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CB00945-10 LB

PERIOD COVERED
October 1, 1982 to September 30, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
B. Peterkofsky, Ph.D. Research Chemist LB NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Biosynthesis Section

INSTITUTE AND LOCATION
DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.8	3.0	1.8

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

B

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

The hypothesis that the specific decrease in collagen synthesis in bone of scorbutic guinea pigs is due mainly to the decreased food intake and weight loss of scurvy was further substantiated. There was a linear relationship between the percentage of collagen synthesis and the extent of weight change in scorbutic and food-restricted controls receiving vitamin C, compared to ad libitum fed controls. The fact that this decrease occurs independently of the effects of scurvy on hydroxylation of proline in collagen, represents a new concept for the mechanism of action of ascorbate on connective tissue. Four days of acutely fasting guinea pigs receiving ascorbate specifically decreased collagen production in guinea pig calvaria and other connective tissues by 70-80%. Collagen synthesis in other connective tissues was equally susceptible to the effect of fasting. The mechanism of this effect is being investigated.

It also has been found tht the intracellular levels of cAMP in a series of normal and Ki-MSV transformed BALB 3T3 cells are similar, although the rates of collagen production are decreased in the transformed cells. Those studies suggest that cAMP is not involved in maintaining the transformed phenotype in BALB 3T3 denved cells.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Investigators:

G. Majmudar, Ph.D.	Visiting Fellow	LB NCI
R. Spanheimer, M.D.	Guest Worker	LB NCI

Objectives: To elucidate the basic mechanisms of collagen synthesis and secretion and to investigate the factors which regulate these processes in normal and transformed cells.

Methods Employed:

1. Proteins of whole chick embryos, isolated bones of these embryos, or cell-free systems derived from these bones or cultured fibroblasts are labeled with ^{14}C-proline. The proteins are precipitated with trichloroacetic acid, redissolved and then assayed for radioactivity in collagen and noncollagenous proteins by a method involving specific digestion of collagen in a mixture of proteins using highly purified collagenase. This method was developed in our laboratory. The relative rate of collagen synthesis can be calculated from data obtained by this method and the specific rate of synthesis in tissues or cells is calculated by determining the DNA content and expressing the rate as cpm/μg DNA. The proteins of the cell and medium fractions of cultured fibroblasts are analyzed separately in order to study secretion. Morphological changes in cultured cells are recorded by polaroid photomicroscopy.

2. The level of proline hydroxylation in collagen of cells or tissues is measured by a new dual labeled proline method which was devised in this laboratory and eliminates the necessity for hydrolysis. To measure the level of lysine hydroxylation, cells or tissues are labeled with radioactive lysine, and hydrolyzed collagenase digests are chromatographed on Dowex-50 in 2N HCl, which separates lysine and hydroxylysine.

3. Prolyl and lysyl hydroxylases are measured by $^{3}H_2O$ release from ^{3}H-proline or ^{3}H-lysine labeled unhydroxylated collagen prepared by incubating chick embryo frontal bones with the labeled amino acid in the presence of the iron chelator α,α-dipyridyl, which inhibits hydroxylation.

4. Collagen types synthesized in cultured cells are determined by analysis of ^{14}C-or ^{3}H-proline labeled, denatured collagen using electrophoresis in sodium dodecyl sulfate-polyacrylamide slab gels. Disulfide bonds are detected by running samples with and without dithiothreitol and observing alterations in the positions of α chains. Radioactive proteins are detected by fluorography.

5. Cyclic 3', 5' adenosine monophosphate(cAMP) is measured by a competitive binding assay. Cells are extracted with trichloroacetic acid, the cAMP purified by passing the extract through a cation exchange resin column and the extent of binding to a purified cAMP-dependent protein-kinase is measured by competition against ^{3}H-cAMP.

Cell lines used in these studies:

Chick embryo fibroblasts: prepared by digesting frontal bones from 15 day chick embryos with crude collagenase and culturing the released cells. Subcultures in the second to fifth passage are used.

L-929--a line of mouse embryo fibroblasts established about 30 years ago by use of a chemical carcinogen.

BALB-3T3--a contact inhibited line of mouse embryo fibroblasts isolated by Todaro and Aaronson and subclones of this line, P3 and 714, which are more stringently contact-inhibited than the original culture, and P13, which has lost density dependence.

Ki-3T3-234--a line established by Aaronson by transformation of BALB 3T3 cells with Kirsten sarcoma virus. These cells do not produce viral particles unless super-infected with murine leukemia virus.

Mo-3T3--BALB 3T3 cells transformed with Moloney sarcoma virus.
SV-3T3--BALB 3T3 cells transformed with SV-40 virus.
NQT-3T3-714--BALB 3T3 cells transformed with a chemical carcinogen, 4-nitroquinoline-1-oxide.

Embryonic and adult human diploid fibroblasts are purchased commercially.

BALB 3T3 subclones 3 and 714 were transformed with a temperature sensitive mutant of Kirsten sarcoma virus (KSV) containing Moloney leukemia virus (MLV) helper to give a mass infected and transformed line, tsKi(MLV)-3T3. Control lines infected with only MLV were also produced (MLV-3T3). A nonproducer subclone, tsKi-3T3-714 was derived from the transformed culture of 3T3-714.

Major Findings:

I. The role of ascorbic acid in collagen metabolism.

A. Influence of decreased food intake and body weight on collagen synthesis in scorbutic animals.

Previous results had shown that ascorbate levels in cultured calvaria bone from scorbutic guinea pigs had decreased almost maximally by the end of 1 week of ascorbate deficiency and that proline hydroxylation was maximally decreased after 2 weeks on this diet. In contrast, there was little effect on collagen synthesis by the second week but thereafter it decreased dramatically to a level about one-third of the control level by the end of 4 weeks. These and other results suggested that there was a dissociation between the effect of ascorbate deficiency on proline hydroxylation and collagen synthesis, contrary to the hypothesis previously favored. Preliminary results suggested that the weight-loss characteristic of scurvy was related to the decrease in collagen synthesis and

further experiments were carried out to verify this conclusion. Normally, guinea pigs begin to lose weight after 2 weeks on a scorbutogenic diet By carefully following the weights of guinea pigs on this diet, we were able to select a small group which continued to gain weight at the same rate as vitamin C supplemented controls during the third week. Other experimental groups included scorbutics rapidly losing weight during the third and fourth weeks, control animals fed ad libitum and receiving vitamin C but having food restricted to induce weight loss at times coinciding with the points when scorbutics started to lose weight and at approximately the same rates. Collagen synthesis in calvarial bones bones of the various groups was measured at several points during the third and fourth week of ascorbate deficiency. Regardless of the length of time on the scorbutogenic diet beyond two weeks, animals losing weight showed decreased collagen synthesis. The rates of synthesis in bones of animals still gaining weight at the same rate as ad libitum fed controls, were comparable to the control rates. Animals induced to lose weight by food restriction, but receiving vitamin C, also showed decreased collagen synthesis. For both the scorbutics and food-restricted controls, there was a direct relationship between the percentage of collagen synthesis and the extent of weight change(+ or -), giving a linear plot with a good correlation (R=0.89). These results suggest the hypothesis that ascorbate deficiency leads to decreased food intake which results in either loss of another nutritional factor specifically required for collagen synthesis or alteration of the level of a hormone involved in specific regulation of collagen synthesis. Fasting is known to cause decreases in insulin and somatomedins and an increase in cortisol in the rat.

B. Effect of acute fasting on collagen production in normal guinea pigs supplemented with vitamin C.
We investigated in more detail the phenomenon of decreased collagen synthesis in calavaria bone of animals losing weight because of food-restriction Guinea pigs were acutely fasted for 96 hr, except that they received vitamin C orally . Body weight decreased markedly during this period. Animals then were re-fed for 96 hr and weight was regained. Controls were ad libitum fed animals recieving vitamin C throughout the 192 hr period Collagen and noncollagen protein production in cultured calvaria bones from these animals was determined each day. The rate of collagen production relative to total protein production had decreased by 24 hr of acute fasting and there was little effect on noncollagen protein production. Collagen production continued to decrease so that by 96 hr its' relative rate of production was 30% of the control level. The percentage of collagen production gradually returned to control levels by 96 hr after refeeding. Tissue levels of ascorbate, DNA, and proline hydroxylation were not affected by fasting, The fasting-induced decrease in the relative rate of collagen production was independent of age and sex and was observed in other connective tissues of the guinea pig. A similar effect was observed in calvaria bone of the rat, which synthesizes ascorbic acid, confirming that this effect is not mediated directly through ascorbic acid. Pulse-chase experiusing cartilage organ cultures suggest that decreased collagen production

is not due to a degradative mechanism. Preliminary experiments on cell free translation of total RNA extracted from cartilage of control and fasted animal indicate that there is a specific defect at the level of procollagen mRNA.

C. Regulation of secretion of non-interstitial collagen types of ascorbic acid We have shown that the rate of secretion of the most abundant types of collagen, I and III, are stimulated by ascorbic acid in cultured cells. The reason for this stimulation is that ascorbate allows formation of hydroxyproline which stabilizes the triple helical form of procollagen required for secretion. Reports in the literature have suggested that the secretion of type IV, or basement membrane, collagen, may not require hydroxylation of proline for maximal secretion, and thus its secretion would not be regulated by ascorbate. We have initiated a program to determine whether proline hydroxylation, and consequently ascorbate, is required for maximal secretion of type IV and type V collagens. Both of these collagens do not form fibers characteristic of the interstitial collagen types I, II, and III. We had previously found that a chemically transformed 3T3 cell line, NQT3T3-714, produced significant amounts of types IV and V collagens and this line was used for the initial experiments in this program. Analysis of the collagens produced by these cells revealed another hitherto undescribed type of collagen, and its secretion was not dependent on hydroxylation of proline.

III. Alteration of collagen phenotype in transformed cells

A. Role of cAMP

We had previously reported that total collagen producton in transformed cells was lower than in the parent 3T3 cells. In one Ki-MSV transformant Ki-3T3-234, dibutyryl cAMP(dbcAMP) specifically increased collagen production 2-3-fold. It was generally assumed at that time that cAMP levels in transformed cells was lower than in normal cells and therefore the induction of collagen production by dbcAMP was thought to result from elevation of intracellular cAMP. We reinitiatied this project with the objective of determining the molecular mechanism of this inductive process. In addition, we wished to determine whether the increased collagen production which occured after tsKi-3T3-714 cells were shifted to a nonpermissive temperature also resulted from elevation of intracellular cAMP and whether the mechanism was for dbcAMP induction of collagen production. Suprisingly, we found that dbcAMP did not increase collagen production in tsKi 3T3 cells, although the effects on growth and morphology were Ki-MSV. This led us to determine intracellular cAMP levels in a series of normal and transformed 3T3 lines and the results showed that the levels were similar in all of the lines. Furthermore, there was no significant change in cAMP when tsKi-3T3-714 was grown at the nonpermissive temperature nor was there an increase in a flat revertant which lacked viral RNA and

transforming gene DNA. Therefore, we conclude that the maintenance of the transformed phenotype with respect to growth, morphology and collagen production is not related to intracellular cAMP concentrations. In addition, dbcAMP caused changes in some transformed properties but this may occur by a pathway different that the one affected by transformation.

Proposed Course of Research

I. Role of ascorbate in collagen metabolism

A. Validation of the hypothesis that decreased food intake induced by ascorbate deficiency is the major cause of defective collagen synthesis in scurvy.

1. We showed that acute fasting inhibits collagen synthesis in cartilage and bone. Therefore, we will examine the effects of ascorbate deficiency on cartilage collagen(type II) synthesis and proline hydroxylation and correlate these effects with the extent of weight loss by techniques used for the analysis of calvarial bone.

2. If these experiments show similar results in cartilage as in bone, i.e. correlation of decreased collagen synthesis with weight loss rather than with defective proline hydroxylation, then a long-term cartilage culture system will be established for studying factors required to reverse the inhibition of collagen synthesis. Epiphyseal cartilage is especially amenable to such culturing techniques since it is a rapidly growing tissue.

3. A profile of hormonal changes in the serum of acutely fasted, vitamin C supplemented guinea pigs and in scorbutic animals will be determined. If the patterns are similar, this would be further evidence that scurvy is equivalent to fasting. Secondly, if the levels of specific hormones are found to be altered, these can be tested for reversal of the inhibition of collagen synthesis in the long-term cartilage culture system.

B. Molecular mechanism of inhibition of collagen synthesis by acute fasting and ascorbate deficiency.

Experiments on the molecular mechanism for the fasting effect will be completed and extended to scorbutic tissue. Although the results of preliminary experiments indicate that there is a preferential decrease of approximately 65% in the concentration of procollagen mRNA in cartilage of fasted animals, the recovery of purified RNA was poor A procedure which gives more quantitative recovery is needed in order to have greater confidence in the results. Several different extraction procedures and the use of RNAase inhibitors will be tested.

C. Effect of scurvy and fasting on synthesis of non-interstitial types of collagen

Our results thus far indicate that type I(bone) collagen synthesis is inhibited by scurvy and fasting. Type II(cartilage) collagen synthesis also is inhibited by fasting and our proposed studies will establish whether scurvy effects synthesis of this collagen. In line with our project on whether the secretion of non-interstitial types of collagen is regulated by ascorbate through proline hydroxylation, we will also evaluate the in vivo effect of ascorbate deficiency on these collagen types. A guinea pig hepatoma which grows in the strain used by us and which synthesizes type IV collagen, will be established. We will determine whether scurvy or fasting affects the synthesis of collagen by the tumor. It will also be of interest to determine whether vitamin C affects the growth rate of the tumor.

D. Regulation of secretion of non-intersititial collagen types by ascorbic acid.

The requirement for ascorbate in secretion of types IV and V collagens will be determined in NQT-3T3 cells as well as in other cell lines which synthesize specific collagens. The new collagen synthesized by NQT-3T3 will be purified and further characterized by peptide mapping.

II. Alteration of collagen phenotype by transformation

The mechanism by which collagen production in Ki-3T3 cells is increased by dbcAMP will be investigated. It will be determined whether ths effect occurs via a change in the level of mRNA for types I and III procollagens. In addition, we will test the possibility that failure of dbcAMP to induce collagen production in tsKi-3T3 cells is due to a difference in the relative amounts of cAMP-dependent protein kinases I and II, as compared to Ki -3T3 cells.

Significance to Biomedical Research and the Program of the Institute:

Determining the precise mechanism by which vitamin C affects collagen synthesis should lead to a more accurate use of this vitamin. In addition, our studies with transformed cells indicate that, at least under some conditions, animal cells may be able to synthesize a substitute for the function of vitamin C in connective tissue. Our proposed studies also should give some insight into the question of whether vitamin C specifically affects tumor growth.

The alteration of the collagen phenotype by transformation provides further information on biological changes caused by the carcinogenic agents as well as useful models for studying cellular differentiation.

Publications:

Chojkier, M., Spanheimer, R., and Peterkofsky, B.: Specificallly decreased collagen biosynthesis in scurvy dissociated from an effect on proline hydroxylation and correlated with body weight loss. *In vitro* studies in guinea pig calvarial bones. J. Clin. Invest. in press, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05202-16 LB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

O. Wesley M Bride, Chief, Cellular Regulation Section, LB, NCI

COOPERATING UNITS *(if any)*

Drs. David Swan & Stuart Aaronson, LCMB, NCI
Drs. Gerald R. Crabtree & Jeffrey A. Kant, LP, NCI
Drs. Y. T. Chen & D. Nebert, DP, CH

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	2.5	2.8

CHECK APPROPRIATE BOX(ES)

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

B

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

The purpose of this project is to develop methods for gene transfer to mammalian cells and to use these techniques for gene mapping, analysis of gene expression, and cloning eukaryotic genes. Many independent somatic cell hybrid lines segrating human chromosomes have been isolated and the human chromosome content of each line determined. Analysis of these lines with isotopically labeled cloned DNA probes has allowed assignment to specific human chromosomes, and sometimes regional localization, of human cellular onc genes including N-ras, c-myb, c-kis-1, c-kis-2, c-mos, c-myc, c-bas-1, and c-sis. Many of these c-onc genes map to the same sites as non-random chromosome rearrangements in specific human cancers. Similar procedures were used to map α, β, and Γ fibrinogen genes to human chromosome 4; chromosomal mapping of a multigene metallothionein family and an aryl hydrocarbon hydroxylase gene are in progress. The human thymidine kinase gene has been molecularly cloned and fragments subcloned for analysis of its structure and regulation.

OTHER INVESTIGATORS:

Usha Kasid	Fogarty Fellow	LB NCI
Adina Breiman	Fogarty Fellow	LB NCI
Carolina Parisi	Fogarty Fellow	LB NCI

PROJECT DESCRIPTION:

Objectives:

Development of methods for gene transfer to mammalian cells and use of these procedures for chromosomal and regional gene mapping, analysis of gene expression in normal and neoplastic cells, and cloning eukaryotic genes.

Methods Employed:

Tissue culture procedures, including plating in selective media, cloning, selection of cell mutants, and isolation of somatic cell hybrids after interspecific fusion of microcells with whole cells or cell/cell fusion. Metaphase chromosome isolation and purification by isopycnic and velocity sedimentation; gene transfer to mammalian cells by uptake of purified metaphase chromosomes, high molecular weight, eukaryotic DNA, or cloned recombinant DNA with subsequent isolation of biologically transformed colonies in selective media. Isolation and characterization of gene products by chromatography, electrophoresis, isoelectric focusing, and immunochemical procedures. Karyological analysis of hybrid cells and transformed cells by fluorescence, phase, and bright-field photomicroscopic techniques. Cloning of eukaryotic genes in prokayotic vectors and analysis of cloned genes by standard recombinant DNA techniques.

Major Findings:

I. Somatic Cell Hybridization. Analysis of somatic cell hybrids segregating human chromosomes permits the localization of human genes to specific chromosomes. Several large series of independent hybrid cell lines were isolated in selective medium containing HAT (hypoxanthine, amethopterin, and thymidine) and 100 μM ouabain after polyethylene glycol 1000 induced fusion of human cells with $hprt^-$ and tk^- mutant rodent fibroblasts. The human parental cells included Hela, normal WI38 cells, peripheral leukocytes from a patient with Chronic Myelogenous Leukemia (CML), and well characterized fibroblast lines (from Camden Institute for Medical Research) containing normal karyotypes except for the presence of specific reciprocal translocations [t(2; 6) = GM2658; t(X; 14) = GM0073; t(17; 22) = GM119 and GM3196; t(13; 17) = GM1663] or deletions [del(13) (pter > q14 :: q22 > qter) = AG1142]. Cloned hybrid lines were subcloned once or more to obtain segregant hybrid cell populations containing a reduced, and relatively homogeneous, content of specific human chromosomes. The specific human chromosomes present in hybrid cell lines and subclones were determined by isoenzyme analyses and sometimes confirmed by karyotyping. In collaboration with Dr. David Swan, DNA was simultaneously isolated from these same hybrid cell populations, and DNA fragments were transferred to nitrocell-

ulose after restriction endonuclease digestions and agarose gel electrophoresis. Hybridization of the transferred DNA with isotopically labeled, cloned DNA probes thereby permitted assignments of genes to specific human chromosomes. Analysis of somatic cell-hybrids containing well-characterized human chromosome translocations or spontaneous breaks frequently permitted regional localization of genes on a chromosome.

A. Chromosomal Localization of Human Proto-Oncogenes. Several laboratories have identified transforming genes in various types of human tumor cells. These genes induce morphological transformation when used to transfect NIH/3T3 cells. Three of these transforming genes have been isolated recently by molecular cloning techniques and shown to be closely related to the onc genes of BALB, Harvey, and Kirsten murine sarcoma viruses which arose in nature by transduction of evolutionarily highly conserved cellular genes from mouse (v-bas) and rat (v-H-ras and v-K-ras) cells. Dr. Stuart Aaronson and colleagues have used cellular transforming genes and v-onc genes as probes to molecularly clone several cellular proto-oncogenes from normal human DNA. These cloned genes were employed to prepare isotopically labeled probes for Southern blotting analysis of DNA from our somatic cell hybrid lines. Using this method, we have now assigned the following proto-ocogenes to sepcific human chromosomes: N-ras (chr. 1), c-kis-1 and c-myb (chr. 6), c-mos and c-myc (chr. 8), c-bas-1 (chr. 11), c-kis-2 (chr. 12), and c-sis (chr. 22). Moreover, analysis of hybrid cell lines containing human chromosome translocations or spontaneous breaks has permitted regional localization of several onc genes. Their regional assignments include c-kis-1 ($6q_{13}$-6qter), c-myb ($6q_{15}$-6qter), c-bas-1 (11p), and c-kis-2 (12q).

B. Chromosomal Mapping of Human Fibrinogen Genes. Genes coding for the three polypeptide chains (α, β, and γ) of the complex coagulation protein, fibrinogen, have been cloned by Drs. G. R. Crabtree and J. A. Kant of the Laboratory of Pathology, NCI. These cloned genes, and appropriate subclones, have been radiochemically labeled and used as probes to chromosomally map these genes. There is known close linkage of α-Fib and Γ-Fib in rats and α-Fib and β-Fib in humans. Our chromosomal mapping quickly established that all three Fib genes are linked in humans and Dr. Kant confirmed this conclusion by recombinant DNA "walking". Our current results strongly indicate that the Fib genes are located on human chromosome 4. Studies are in progress to confirm these results and possibly regionally localize these genes.

C. Mapping of the Human Metallothionein (MT) Multigene Family. The metallothioneins are low molecular weight proteins with high cysteine content which are involved in heavy metal detoxification. Drs. D. H. Hamer and C. J. Schmidt have cloned and sequenced two monkey MT c-DNA's encoding MT-I and MT-II. We are collaborating with them in mapping MT genes in the human genome using these monkey MT c-DNA probes. The MT genes present some unusual problems in chromosomal mapping since they represent a multigene family and they are highly conserved in all mammals. Southern analysis of human DNA after digestion with restriction endonucleases which do not cleave MT genes internally, demonstrates 10 or 12 fragments which hybridize with the monkey MT c-DNA probe. Analysis

of our human/rodent somatic cell hybrid DNA's shows chromosomal linkage of several of these MT bands and it indicates that MT genes are represented on at least 4, and posibly 6, different human chromosomes including antosomes 1 and 18. Identification of the human chromosomal location of each MT gene and pseudogene will help to understand the mechanisms involved in the evolution of this multigene family.

D. Aryl Hydrocarbon Hydroxylase (AHH) Gene Mapping. Aryl hydrocarbon hydroxylases convert aromatic polycyclic hydrocarbons to their toxic and carcinogenic form. Hence, the location of these genes and relationship to other nucleic acid sequences might have considerable importance. Drs. Y. T. Chen and D. Nebert have cloned a human AHH and have untaken preliminary studies to map this gene. Subclones retaining only unique sequence DNA are being prepared to complete this project. Previous studies elsewhere (based on AHH expression) have indicated that an AHH structural or regulatory gene may be located on chromosome 2p (i.e. short arm).

II. Molecular Cloning of Human Cytoplasmic Thymidine Kinase (TK) gene. After serial transfer of human DNA to tk^- mouse cells, a mouse transformant containing rare human DNA sequences, including the human tk gene, was isolated in selective medium. Size fractionated, partial Mbo I digests of transformant DNA were used to prepare a recombinant DNA library in a λ phage vector and the library was screened by plaque hybridization with human repetitive DNA, human Alu, and mouse repetitive DNA probes. Two human DNA recombinants were detected and plaque purified from a library of 350,000 recombinants. Restriction enzyme mapping and heteroduplex analysis indicated a region of homology within these two inserts and reverse orientation within the phage vector. Both inserts originate at a common Mbo I site within a 7.5-9Kbp homologous segment and terminate in a 4-5.5Kbp non-homologous region. Human repetitive DNA sequences are present in the region of homology but they are not found in the non-homologous region of one recombinant. A probe (4Kbp) was prepared from this segment and Southern analysis indicates that it represents a unique sequence mouse fragment which may be contiguous to an integration site of the donor DNA. Transfection experiments demonstrate that both recombinants contain a tk gene. The TK activity in all transformants following recombinant DNA transfer has the same electrophoretic mobility in gels but it differs from the mobility of either human or mouse cytoplasmic or mitochondrial TK or Herpes Simplex viral TK activities. Potential mechanisms for this surprising observation are under investigation and include the possibility that a truncated human tk gene is present in the recombinant DNA region of homology. The entire insert from phage recombinants has been cloned into a plasmid vector in both orientations and several insert fragments have been subcloned into plasmids for further analyses.

Proposed Course of Research:

Chromosomal assignment and regional localization of additional human cellular proto-oncogenes and other genes of interest will continue. Hybrid cell lines retaining specific human chromosomes which contain putative proto-oncogenes (i.e. chr. 3p in small cell carcinoma of lung) and lacking those

chromosomes with known proto-oncogenes will be analyzed with onc gene probes to detect, clone, and analyze any additional proto-oncogenes from the same onc gene families.

Unique sequence, subcloned fragments of our phage recombinants containing the human tk gene will be used as probes for Southern analysis of DNA from appropriate human/rodent cell hybrids and mouse transformants containing only the human tk gene. This will allow verification that the human tk gene is present in these recombinants. These probes will also be used to isolate human tk from a c-DNA library. This will permit identification of the tk gene within the cloned insert, analysis of its structure by heteroduplex formation between c-DNA and the cloned genomic DNA, and nucleic acid sequencing of the coding region.

Efforts will resume to purify the autonomously replicating X-chromosomal fragment from our mouse transformant line which contains no other detectable human chromosome fragments. This fragment represents the distal third of the X-chromosome long arm and contains the hprt and g6pd genes; loci for anti-hemophilic factor A and a fragile site associated with mental retardation have been mapped to this same segment. Purified DNA from this fragment will be cloned into phage and cosmid vectors.

Significance to Cancer Research:

Non-random chromosomal rearrangements previously have been associated with specific human cancers. Chromosomal and regional localization of proto-oncogenes to many of these same sites strongly suggests that this small group of evolutionarily conserved genes may be involved in human oncogenesis. This interpretation is supported by many other recent studies including cloning of rearranged c-mys genes in Burkitt's Lymphona and mouse plasmacytonas and demonstration of specific mutations in c-onc genes resulting in neoplastic transforming activity in vitro.

Publications:

Battey, J., Max, E.E., McBride, O.W., Swan, D. and Leder, P.: A processed human immunoglobulin epsilon gene has moved to chromosome 9. Proc. Natl. Acad. Sci. USA 79: 5956-5960, 1982.

Swan, D.C., McBride, O.W., Robbins, K.C., Keithley, D.A, Reddy, E.P. and Aaronson, S.A.: Chromosomal mapping of the simian sarcoma virus onc gene analogue in human cells. Proc. Natl. Acad. Sci. USA. 79: 4691-4695, 1982.

Prakash, K., McBride, O.W., Swan, D.C., Devare, S.G., Tronick, S.R. and Aaronson, S.A.: Molecular cloning and chromosomal mapping of a human locus related to the Moloney murine sarcoma virus transforming gene. Proc. Natl. Acad. Sci. USA 79: 5210-5214, 1982.

McBride, O.W., Swan, D.C., Robbins, K.C., Prakash, K. and Aaronson, S.A.: Chromosomal mapping of tumor virus transforming gene analogues in human cells. Plenum Press, 1982.

Peterson, J.L. and McBride, O.W.: Close Linkage of Transferred Galactokinase and Thymidine Kinase Genes in a Transformant After DNA-Mediated Gene Transfer. In Genetic Engineering in Eukaryotes (P.F. Lurquin and A. Kleinhofs, eds.), Plenum Press, in press, 1982.

McBride, O.W., Swan, D.C., Santos, E., Barbacid, M., Tronick, S.R., and Aaronson, S.A.: Localization of the normal allele of T24 human bladder carcinoma oncogene to chromosome II. Nature 300: 773-774, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB05203-15 LB

PERIOD COVERED
October 1, 1982 to September 30, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
M. G. Mage Immunochemist, LB, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 4.0	PROFESSIONAL: 3.0	OTHER: 1.0

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

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

Our goal is the development of cell separation methods for the specific isolation of immune cells, particularly for varieties of antigen-reactive cells (ARC) involved in cellular immune reactions, and for their subcellular fractionation in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. Populations of cells containing ARC are tested for binding to the cell surface antigens of target cells attached to insoluble supports. Separated populations are tested for cytotoxic effector cells (CTL) and their precursors, for activity in allograft rejection and graft-versus host reaction and in the mixed lymphocyte reaction. T cell subpopulations from thymus and spleen are also separated by and characterized with specific reagents such as peanut aggulutinin and antibodies to the Lyt and CTL differentiation antigens. Surface molecules of target cells are isolated to test their binding to ARC. Monoclonal antibodies are prepared against CTL and CTL-derived cell lines in order to characterize their surface antigens.

PHS 6040 (Rev. 1/83) GPO 895-100

OTHER INVESTIGATORS:

V. Alvarez	Expert	LB	NCI
L.L. McHugh	Biologist	LB	NCI
L. Romani	Forgarty Visiting Fellow	LB	NCI

Objectives

The goal of this project is the development of methods for the specific isolation of immune cells, particularly for different types of antigenreactive cells (ARC) involved in cellular immune reactions to alloantigens, to study the ontogeny of these cells, to study their roles, alone and in combination, in several in vitro and in vivo cellular immune reactions, and for their immunochemical characterization in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. In particular, we study the differentiation of cytotoxic effector cells (CTL) from precursors (CTLp) in thymus, the molecular reactions between immune cells and antigens bound to target cell surfaces, and the role of T cell subpopulations in in vivo cellular immune phenomona such as graft rejection and the graft-versus host reaction.

Methods Employed

ARC, obtained from normal mice or from mice immunized with allogeneic tumor cells or normal cells are incubated on surfaces coated with allogeneic or syngeneic target cells or on surfaces coated with antibodies to cell surface molecules. Graft-versus host activity (GVH) of nonadherent cells or of released adherent cells is measured by the Simonsen spleen weight assay in neonatal F1 mice. CTL activity is measured by ^{51}Cr release from target cells. Stimulation by alloantigen is measured by the mixed lymphocyte reaction and by generation of CTL in vitro.

T cell subpopulation from thymus and spleen are separated and characterized by their reactions with specific reagents such as peanut agglutinin and antibodies to the LyT and CTL differentiation antigens. Cell surface molecules of target cells are isolated to test their binding to ARC. In collaboration with Dr. K. Ozato (PR, NICHD) monoclonal antibodies are prepared that react with CTL surface antigens.

Major Findings

1. Purified CTL precursors have little if any need for adherent accessory cells to present them with antigen.

In collaboration with Dr. Y. Shu (NIDR) we have looked at the dual role of adherent accessory cells (macrophages) in the immunization of CTL precursors. We have found (in keeping with previous reports on soluble antigen stimulation of unfractionated populations) that purified Lyt2+ CTLp require more adherent accessory cells for provision of the monokine growth factor IL1 than they do for presentation of antigen, and suggest that CTLp may not need adherent accessory cells at all for antigen presentation. However, because it is difficult to

deplete spleen cell populations of the last few adherent accessory cells, we have looked at the CTL response to tumor cells grown in vitro in suspension, where after repeated passages such cell populations are devoid of any adherent accessory cells.

2. Some tumor cell lines can immunize CTL precursors, apparently in the absence of adherent accessory cells or of Ia (Class II MHC) antigens.

Experiments on the specificity of CTL using target cells from different strains of inbred mice have long indicated that these CTL react with the Class I MHC antigens (e.g. murine H-2 K and D). Nonetheless, it has been difficult to rule out the participation of Class II (Ia) antigens in the immunization of CTL precursors, although if the antigen-specific receptors of the precursors are the same as those of their mature descendants,(in keeping with what has long been established for B cells), it would follow that class II antigens are not a specificity-determining part of the molecule or molecular complex that immunizes the CTLp. Part of the difficulty in learning the role (if any) of class II antigens is that the usual systems for immunizing the CTLp are not devoid of Class II molecules, because unfractionated cell populations, be they stimulators or responders, contain variable numbers of adherent accessory cells which have Ia antigen molecules on their surfaces, as do B cells. We have attempted to get around this problem by using in vitro passaged tumor cells as stimulators (i.e. immunizing cells) and using highly purified Lyt2+ T cells as responder cells, supplementing the medium with T cell growth factor to replace the nonspecific function of the adherent accesory cells and T helper cells that are no longer present. In this system, we have found three tumor cell lines that immunize CTLp. Two of them are T cell tumors and one is a mastocytoma. Because of their origins, we expected all three to be lacking Ia antigens, but unexpectedly, two of the three lines clearly had such antigens when examined by indirect immunofluorescence. However, one of the T cell lines clearly is immunogenic in the absence of Ia antigen molecules. We have also obtained (by limiting dilution cloning) a subline of one of the other tumors which is also Ia^-, giving us the opportunity to compare Ia^- and Ia^+ variants of the same cell line.

Significance to Cancer Research (Objective 2, Approach 1)

The T cells that undergo maturation in the thymus (i.e. Cytotoxic T cell precursors, and helper and suppressor T cells) are thought to be directly involved in anti-tumor immunity, by means of cell-mediated tumor rejection, and its augmentation and suppression. Knowledge of the antigen-binding characteristics, differentiation antigens, and reactivities of immune cells at different stages of differentiation may help in developing specific purifications of various types of immune lymphocytes reactive against tumor antigens and in specific suppression of the graft versus host reaction, which is a serious iatrogenic complication of cancer therapy with bone marrow transplantation.

Proposed Course of Research

We plan to (1) continue to develop the general separation methodology based on specific binding of Ig-coated cells to anti-Ig-coated surfaces, (2) develop further separations of T cell subtypes, particulary CTLs, their precursors,

helpers, and suppressors, and study their differentiation, (3) continue to study the contributions of these cells in vivo to immune reactions such as graft rejection, GVH reaction, and tumor rejection, (4) study the macromolecules involved in the binding of CTL to target cells, and (5) study the molecular requirements for immunogenic antigen presentation to CTLp.

Publications:

Scher, I. and Mage, M.: Cellular Identification and Separation. In Paul, W. (Ed.): Fundamental Immunology, Raven Press, NY,NY, in press 1983.

Mage, M.: Cell Separation on Antibody Coated Plates. In di Sabato, G. (Ed.): Immunochemical Techniques Part G, Chapter 2.8 in a volume of Methods in Enzymology, in press 1983.

Mage, M: Cell Separation on Cellular Monolayers. In di Sabato, G. (Ed.): Immunochemical Techniques Part G, Chapter 2.9 in a volume of Methods in Enzymology, in press 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB05210-15 LB

PERIOD COVERED: October 1, 1982 to September 30, 1983

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

Cellular Controls over Growth and Inducible Processes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

E. B. Thompson Chief, Biochemistry of Gene LB NCI

COOPERATING UNITS *(if any)* P. Dannies, S.S. Simons; L. Mercier; H.J. Eisen; R. Simon; M. Rosenberg; D. Schümperli; B. Howard; R. Padmanabhan; M. Zasloff; L. Zwelling; M. Costlow; T. Antakli; D. Biswas; J. Harmon.

LAB/BRANCH: Laboratory of Biochemistry

SECTION: Biochemistry of Gene Expression Section

INSTITUTE AND LOCATION: DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12	11	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.)*

Vectors containing the entire rat growth hormone gene have been transfected into GH^3 x L cell, non-growth hormone-expressing somatic cell hybrids. Growth hormone expression is thereby reestablished in the hybrids, and in some is under glucocorticoid control. Human glucocorticoid receptor, covalently labelled with ^3H dexamethasone mesylate has been characterized by physical methods and by its' reactions with anti-human glucocorticoid receptor antiserum. Rat and human glucocorticoid receptors have been purified more thoroughly. Somatic cell hybrids have been made to analyze cells bearing various mutations in the human glucocorticoid receptor. ^3H cortivazol binds to sites in wild-type CEM cells; one appears to be the glucocorticoid receptor. Hybrid genes between the promoter region the rat growth hormone gene and the E. coli galactose kinase gene have prepared, put in vectors and transfected into various cells.

PHS 6040 (Rev. 1/83) GPO 895-100

Project Description

Objectives

In general, our objective is to understand the mechanism(s) of action of steroid hormones: how they act to kill some cells and not others, and how they induce specific proteins. When possible, we aim to apply our findings to clinical situations. We have achieved or made significant progress in most of the goals we set in our Proposed Course of Research last year. The list below summarizes those goals and our progress.

Specific sub-objectives are as follows:

A. The growth hormone/prolactin system.

1. Objective one: To transfect GH_3 x L cell hybrids and other cells with vectors containing the rat growth hormone genes, or parts of it, alone or fused to the E. coli galactase kinase genes and to there by examine whether the extinction seen in the hybrids could be over some and where in the growth hormone gene the regions are which are responsive to hormonal control.

2. Objective two: To further explore the methylation state of growth hormone genes in GH_3 cells and GH_3 x L cell hybrids.

3. Objective three: To try to use a cell-free transcription system to study control of the growth hormone gene.

4. Objective four: To study cell-free binding of purified glucocorticoid receptors to specific hormonally regulated genes, as growth hormone, mouse mammary tumor virus and prolactin by covalently cross-linking the protein to the DNA.

5. Objective five: To complete restriction endonuclease analysis of prolactin genes in GH_3 x L cell hybrids.

6. Objective six: To determine whether growth hormone induction is a primary or secondary event. control of expression of these genes.

Methods

In addition to the methods outlined in these reports earlier, we have added: transfection of somatic cells with specfic DNA vectors and semi-quantitative assay of mRNA by hybridization to specific probes after fixation to nitrocellulose filters.

B. The CEM cell system.

We have shown before that this human, T cell derived, acute lymphoblastic leukemia cell line contains glucocorticoid receptors whose saturation by active glucorcorticoids is required for cell death, which occurs in G_1. Spontaneous mutations in receptor occur at a rate of 10^{-5}/cell/genera-

-tion and lead to a unique, "activation labile" $r^{act/l}$ phenotype. One unselected clone from the wild-type CEM population was found to have apparently normal receptors and yet to resist lysis. The structurally unusual, extremely potent glucocorticoid, cortivazol, was found capable of killing these latter cells, as well as other resistant cell types, including the $r^{act/l}$ class and enven cells which had <10% of wild-type dexamethasone receptor sites (r^{o}). These results made us conclude that it was essential to further purify and characterize the human glucocorticoid receptor (HGR).

1. Objective one: To purify and characterize the HGR (and the rat GR) utilizing covalent labelling with dexamethasone mesylate, antisera, proteolytic mapping and amino acid analysis and sequencing.

2. Objective two: Carry out somatic cell hybridizations of various classes of steroid-resistant and steroid sensitive CEM cells.

3. Objective three: To use our newly-synthesized ^{3}H-cortivazol to examine directly the binding site(s) for this compound in various classes of CEM cells.

Major Findings

A. The growth hormone/prolactin system.

1. Objective one: We have successfully prepared vectors containing the entire rat growth hormone gene, plus either the "XPT" or "neo" genes preceded by SV40 origin sequences, and a portion of the PBR genome. These have been transfected into GH_3 x L cell hybrids which contain, but do not express, the rat growth hormone gene. In a number of these transfected cells we have demonstrated rat growth hormone transcripts. These have included transcripts corresponding to the sizes of known growth hormone mRNA precursors and to mature mRNA. The latter is significant, since other labs' transfections of other cells, incluiding L cells, resulted in shorter-than-mature rat growth hormone mRNAs. This may have to do with the peculiar structure of the second'intron of this gene in this species, and we suggest that other GH_3 cell genes in the hybrid are responsible for factors which allow the correct transcription/processing of the growth hormone. One of the transfected clones shows growth hormone inducibility by glucocorticoids/triiodothyronine. Thus, we have overcome or bypassed "extinction".

   Rat growth hormone gene expression is also being studied using an expression vector in which the rat growth hormone promoter region is fused to the E. coli galactokinase gene. However, the rat growth hormone gene promoter is too weak to be analyzed by the standard starch gel assay. An in vitro filter assay has proved to be sufficiently sensitive to analyze low enzyme activities, but only eukaryotic cell lines lacking any endogenous gal K can be used. At the present time only one such cell line from hamster - is available. Since growth hormone is expressed and regulated by glucocorticoids in GH_3 cells we are mutagenizing them and selecting mutants on the basis of resistance to 2 deoxygalactose. The first step in the process has been achieved, and several

clones which show greater resistance to 2 deoxygalactose have been isolated. However, they still retain considerable gal K activity. Presumably this is due to mutation in one gal K allele only. Thus, a second step of mutogenesis and selection is currently in progress.

In addition, we have constructed similar vectors containing all or part of the MMTV LTR fused to gal K. Preliminary experiments show that with the entire LTR as a promoter for gal K, the enzyme activity is inducible 10 fold with dexamthasone. However, no induction is observed when only 100 basis upstream of the TATA box is present. The above involve collaborations with B.H., R.P., M.R., and D.S.

2. Objective two: The methylation state of the rat growth hormoe gene in GH_3 cells and GH_3 x L cell hybrids has been extensively examined by use of the isoschizomer pair of enzymes, MspI and HpaII. No correlation between methylation and growth hormone gene expression was found. However, the sites cut by these enzymes are rather far from the 5' and 3' ends of the gene. Results with 5 azacytidine have been ambiguous. Therefore, we have begun to restudy this question with other methylation - sensitive enzymes whose sites lie near the 5' end of the gene.

3. Objective three: We have not been able to sustain our initial success with growth hormone transcription in the "Manley" system. Therefore, we are collaborating with M. Zazloff's lab to try to obtain correct transcription of rat growth hormone in Xenopus oocytes. A preliminary experiment was encouraging.

4. Objective four: In collabortation with L. Zwelling, we are developing methods to covalently but reversibly attach steroid receptors and DNA. We have obtained preliminary evidence that we are sucessful in the covalent attachment between partially purified rat glucocorticoid receptor and non-specific DNA.

5. Objective five: Only limited data has been obtained about the state of the prolactin gene in the cell hybrids, due to the fact that Dr. S. Ayukawa, who was working on the project, left for a job in industry.

6. Objective six: This project is proceeding steadily. We have shown that growth hormonespecific transcripts are induced by glucocorticoids despite the presence of cycloheximide.

C. Miscellaneous

1. Objective one: We have shown that cortivazol extends the life of nude mice bearing intrathecal CEM cell tumors beyond that of predisone-treated animals.

2. Objective two: Preliminary experiments with monkey glucocorticoid receptors show them to be of the same molecular weight as human or rat.

3. Objective three: Collaborations with T. Antakly and M. Costlow are encouraging that using our antisera successful immunocytochemical identification of human glucocorticoid receptors will be possible.

Proposed Course of Research

As the above relates, we have made good progress in most of the goals set for this year , and it is our intention to continue to pursue these goals. In addition, if we are successful in getting some amino acid sequences of the rat and/or human glucorcorticoid receptors, we intend to try to prepare the corresponding DNA sequeces and use them as probes in existing libraries to look for receptor genes. We also have initiated experiments attempting to try to isolate the human receptor gene by transfection experiments utilizing r° cells.

Publications:

Original Papers

Strobl, J.S., Dannies, P.S., and Thompson, E.B.: Somatic cell hybridization of growth hormone-producing rat pituitary cells and mouse fibroblasts results in extinction of growth hormone expresion via a defect in growth hormone RNA production. J. Biol. Chem. 257: 6588-6594, 1982.

Taub, F. and Thompson, E.B.: An improved method for preparing large arrays of bacterial colonies containing plasmids for hybridization: In situ purification and stable binding of DNA on paper filters. Anal. Biochem. 126: 222-230, 1982.

Harmon, J.M., Schmidt, T.J., and Thompson, E.B.: Non-glucocorticoid receptor-mediated effects of the potent glucocorticoid deacylcortivazol. Cancer Res. 42: 2110-2114, 1982.

Mercier, L., Thompson, E.B., Simons Jr., S.S.: Dissociation of steroid binding to receptors and steroid induction of biological activity in a glucocorticoid-responsive cell. Endocrinology 112: 601-609, 1983.

Reviews

Thompson, E.B. and Strobl, J.S.: Regulation of hormonally controlled mRNA synthesis and gene expression in variant somatic cells and cell hybrids. In Molecular and Genetic Neuroscience, Schmitt, F.O., Bird, S.J., and Bloom, F.E. (eds.), Raven Press, New York, 1982, pp. 307-320.

Thompson, E.B. and Harmon, J.M.: A novel synthetic glucocorticoid, with therapeutic potential in acute lynphoblastic leukemia. Progress in Research and Clinical Application in Corticosteroids, Proceedings of the Sixth Annual Clinical Symposium of the School of Pharmacy, Florida A & M University, in press, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB05214-12 LB

PERIOD COVERED: October 1, 1982 to September 30, 1983

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

DNA Synthesis in Mammalian Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

S. H. Wilson Medical Officer LB NCI

COOPERATING UNITS *(if any)*

J. Mitchell, NCI; J. D. Minna, NCI;
M. Smulson, Georgetown University;
W. Brown, Carnegie-Mellon University

LAB/BRANCH: Laboratory of Biochemistry

SECTION: Biosynthesis Section

INSTITUTE AND LOCATION: DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We continued an investigation of the structure of mammalian DNA polymerase α. Our current results emphasize a 195,000-M_r polypeptide in growing calf and monkey cells that appears to be an abundant α-polymerase constituent. This protein is obtained directly from crude soluble extracts of growing cells by immuno precipitation with monoclonal antibody to calf α-polymerase. Further validation and improvement of the activity gel method for study of DNA polymerase catalytic polypeptides as conducted, and this method was used to demonstrate induction of the ~ 120,000 - Mr α-polymerase polypeptide in resting monkey cells by SV40 infection and for analysis of β-polymerase levels in human cells in culture. Efforts were continued to obtain plasmid cDNA clones with sequences homologous to αpolymerase genes.

Other Investigators:

E. Karawya	Visiting Fellow (11 mo.)	LB NCI
S. Detera	Visiting Fellow (7 mo.) Guest Worker (2 mo.)	LB NCI
B. Zmudzka	Visiting Associate (8 mo.)	LB NCI
R. Karpel	Guest Worker (2 mo.)	LB NCI

Project Description:

Objective: The ultimate objective of this research program is to understand mechanisms of DNA synthesis in mammalian cells. Our approach is the examination of DNA replication in vitro using purified DNA polymerases and other required proteins.

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

Methods Employed: Procedures for assay and purification of DNA polymerases and other DNA proteins from mouse and other tissues are being used. These procedures involve subcellular fractionation, various types of ionic separation, gel filtration, immunobinding, HPLC, and affinity chromatography, and assays for DNA polymerases using a variety of methods. Cell fractionation procedures and characterizations of reaction products are performed using conventional techniques of rate-zonal centrifugation, scintillation spectrophotometry, thin-layer chromatography, and gel electrophoresis. Recombinant DNA technology is being used for preparation of cDNA libraries and plasmid clones containing gene sequences of DNA enzyme.

Major Findings:

I. Mechanism of DNA Polymerase

a. Studies with E. coli DNA polymerase I (Pol I) large fragment

Earlier we had shown that pyridoxylation resulted in covalent incorporation of ~ 3 mol of pyridoxyl phosphate (PLP) per mol of enzyme. This incorporation was at lysine residues, and our data suggested that a lysine in an essential binding site for dNTP was among the targets. Experiments to localize the PLP-modified lysine residues in the primary sequence of the enzyme are underway in collaboration with William Brown, Carnegie-Mellon University. We have found thus far that all of the modified lysine residues are in a large tryptic fragment (~ 30,000-M_r) corresponding to the N-terminal portion of the enzyme. CNBr cleavage of this fragment and resolution by HPLC yielded about 10 peptides, only three of which contained PLP-modified lysine residues. The placement of these peptides along the published amino acid sequence of the enzyme has not yet been accomplished.

b. Studies with α-polymerases

The initial rate of poly[d(A-T)] replication by purified calf α-polymerase was stimulated several fold by calf helix destabilizing protein-1 (HDP-1). Similar results were obtained with HDP-1 and α-polymerase from mouse cells. Using kinetic methods we found that stimulation involved a change in K_m for the template•primer, yet direct binding measurements failed to reveal any differences in enzyme binding to poly [d(A-T)] in the presence or absence of HDP-1. We have sugggested from these results that HDP-1 acts by increasing productive association between the enzyme and the template•primer structure required for initiation of DNA synthesis.

II. Structure-Function Relationships of Mammalian α-Polymerases

DNA polymerase α heterogeneity in mammalian systems is well-known, yet analysis at the polypeptide level has failed to explain adequately functional and biochemical reasons for this property. Our current approach toward study of this problem has been (1) characterization of α-polymerase genes and (2) analysis of proteins recognized in crude extracts of cultured cells by monoclonal antibodies to α-polymerse.

a. Identification of target proteins of monoclonal antibodies against α-polymerase

Monoclonal antibodies to calf α-polymerase described in last year's report were used. Two of these antibodies were selected for studies of immunoreactive polypeptides in crude soluble extracts from growth phase calf EBT cells and monkey BSC-1 cells. Immunoprecipitated targets of both antibodies included the familiar α-polymerase polypeptides of ≈ 160, ≈ 110, ≈ 70, and ≈ 57 KDa. A major polypeptide component of 195 KDa also was specifically immunoprecipitated, and a 81 KDa component was observed in monkey cells, but not in the calf cells. The immunoprecipitated polypeptides of 160 and 195 KDa contained immunobinding determinants as revealed by Western blotting analysis. The 195 KDa protein was heavily labeled when cells were exposed to [^{35}S]methionine for 30 min. During a 4.5 h chase with BSC-1 cells, label in this 195 KDa species declined markedly, whereas label accumulated in several of the immunoprecipitated polypeptides of lower M_r. The results suggest that the 195 KDa polypeptide is an abundant α-polymerase species of growing mammalian cells. We have suggested that some of the lower M_r polypeptides commonly observed in purified α-polymerases may be degradation products of this 195 KDa polypeptide.

b. Identification of α-polymerase mRNA and cDNA clones

In vitro translation of calf thymus RNA results in synthesis of ~ 70,000- and ~ 120,000-M_r polypeptides with DNA polymerase activity, as revealed by activity gel analysis. The molecular weights of these catalytic polypeptides are identical to those of two of the α-polymerase catalytic polypeptides observed in crude extracts of calf cells. Sucrose gradient centrifugation of total RNA resulted in resolution of the mRNAs encoding these two DNA polymerase polypeptides. In addition, immunobinding experi-

ments with one of our α-polymerase specific monoclonal antibodies revealed that antigenic polypeptides at these molecular weights had been synthesized in vitro. Using the hybrid selection method to screen a cDNA library, we have identified one cDNA clone that appears to contain sequences complementary to α-polymerase mRNA. Characterization of this clone is still preliminary.

c. Regulation of α-polymerase in monkey cells in culture

In DNA collaboration with E. Kuff and J. Fewell, we continued an examination of levels of DNA polymerase activities in monkey cells in culture. Tightly confluent cell lines BSC-1 and CV-1 held in serum-depleted medium for several days exhibited an extremely low level of thymidine incorporation into cellular DNA. Yet, these cells still were capable of supporting replication of SV40 DNA, and they contained a level of α-polymerase equal to about 15% of the level in rapidly dividing cells. SV40 infection in serum-depleted medium resulted in a four-fold induction of α-polymerase in CV-1 cells, whereas no change in α-polymerase level was observed in BSC-1 cells. Characterization of α-polymerase partially purified from infected CV-1 cells revealed that the enzyme was aphidicolin resistant; it was distinguished on this basis from α-polymerase isolated from mock infected CV-1 cells. Purified calf αpolymerase used as a reference enzyme in this work was inhibited by aphidicolin. Using activity gel analysis, we found that two α-polymerase catalytic polypeptides (Mr = 82,000 and 120,000) were induced strongly in CV-1 cells by SV40 infection. We conclude that SV40 infection may induce an aphidicolinresistant species of α-polymerase or lead to a modified α-polymerase.

III. Regulation of β-Polymerase in Human Cells in Culture

a. In collaboration with James Mitchell, NCI, we have examined β-polymerase activity levels in a number of normal skin fibroblast cell lines and in cell lines from patients with ataxia telangiectasia (AT), an inherited disorder associated with deficiencies in DNA repair. Our results indicate that β-polymerase levels in AT cells and normal cells are about the same and, in addition, levels are constant from early logarithmic phase of growth to late stationary phase. Damage to the DNA of normal cells by exposure to ionizing radiation results in induction of β-polymerase levels. This is the first observation to our knowledge of induction of β-polymerase.

b. Subchromatin localization of HeLa cell DNA polymerase β

In collaboration with M. Smulson and associates, Georgetown University, we found that DNA polymerase β was bound in native chromatin from logarithmically growing HeLa cells, and after brief digestion with staphylococcal nuclease most of the enzyme activity was found in associatin with monomeric, dimeric and trimeric nucleosomes. Longer digestions, up to 16 minutes, resulted in βpolymerase-associated monomeric nucleosomes, but no release of β-polymerase in soluble form. From these results, we concluded that chromatin-bound β-polymerase is associated predominantly with nucleosomes that are in metabolically active regions of the genome.

IV. Methods for Analysis of DNA Polymerases in Crude Extracts

a. Activity gel analysis

During the past year we continued application and validation of the activity gel method for analysis of DNA polymerase catalytic polypeptides. The sensitivity and reproducibility of the method were improved through systematic evaluation of the individual steps. Further, we observed that purified E. coli DNA polymerase I, purified mammalian β-polymerase, and purified ~ 120,000-M_r mammalian α-polymerase exhibited enzymatic turnover numbers in the activity gel that were equal to the highest turnover number obtained by routine solution assays of each enzyme. Some species of purified mammalian α-polymerase, however, failed to produce signals in the activity gel assay. It now appears that the α-polymerase species which are not active in the gel assay represent the predominant α-polymerase activities routinely observed by ion-exchange chromatography (and solution assay) of crude mammalian cell extracts.

b. Development of immunological reagents for α-polymerase

In collaboration with William Brown, Carnegie-Mellon University, we found that a rabbit antiserum to E. coli DNA polymerase I cross-reacted with calf α-polymerase. Results from radioimmunoassays indicated that about 1/20 of the IgG molecules immunoreactive against Pol I also are reactive against α-polymerase. The Pol I antibody is capable of neutralizing α-polymerase activity.

Significance to Cancer Research:

Detailed knowledge of the mechanisms of DNA synthesis is vital to our understanding the molecular biology of neoplasia. Many approaches are being used to investigate DNA synthesis in normal and neoplastic cells, and advances on all levels will prove useful in preventing, treating, and ultimately controlling cancer. Activity of DNA synthesizing proteins is necessary for maintenance of a rapid rate of cell division, and in some cases there is evidence that these proteins may play an important role in the development of the neoplastic state.

Proposed Course of Research:

1. To investigate replication activity of purified DNA replication proteins in vitro using as template either a single-stranded closed circular viral DNA, SV40 or polyoma replication intermediates, or various plasmids containing SV40 or polyoma DNA sequences.

2. To further characterize mammalian DNA replication proteins and their genes.

3. To further study the enzymatic mechanism of DNA polymerases and the properties of single-stranded DNA specific binding proteins of interest.

Publications:

Becerra, S.P., Detera, S.D., and Wilson, S.H.: Anomalous electrophoretic migration of oligodeoxynucleotides with terminal-OH groups: applications for DNA exonuclease characterization. Anal. Biochem. 129:200-206.

Becerra, S.P. and Wilson, S.H.: Properties of a novel oligonucleotide-releasing bidirectional DNA exonuclease from mouse myeloma. Biochemistry, in press, 1983.

Lambrianidou, A., Smulson, M.E., Karawya, E., and Wilson, S.H. Subchromatin localization of HeLa cell DNA polymerase β. Nucleic Acids Res., in press, 1983.

Detera-Wadleigh, S., Karawya, E., and Wilson, S.H.: Synthesis of catalytically active DNA polymerase α by in vitro translation of calf RNA. J. Biol. Chem., in press, 1983.

Tanabe, K., Karawya, E., Fewell, J., Kuff, E.L., and Wilson, S.H.: DNA polymerase and simian virus 40 infection of resting monkey cells: induction of aphidicolin resistant α-polymerase. Nucleic Acids Res., in press, 1983.

Karawya, E., Albert, W., Swack, J., Fedonko, J., Minna, J.D., and Wilson, S.H.: Characterization of monoclonal antibodies to calf DNA polymerase α : identification of target polypeptides in soluble extracts of calf and monkey cells. J. Biol. Chem., submitted, 1983.

Karawya, E., Swack, J., and Wilson, S.H.: Improved conditions for activity gel analysis of DNA polymerase catalytic polypeptides. Anal. Biochem., submitted, 1983.

Hazra, A., Detera-Wadleigh, S., and Wilson, S.H.: Site specific modification of E. coli: DNA polymerase I large fragment with pyridoxal 5'-phosphate. Biochemistry, submitted, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05231-09 LB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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 on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Claude B. Klee
Chief, Macromolecular Interactions Section, Laboratory of Biochemistry, NCI

COOPERATING UNITS *(if any)*

J. Schiloach, NIAMMD: Mr. Richard Feldman, CR-CCB; Dr. P. Cohen, University of Dundee, Scotland; Dr. L. Heppel, Cornell University, Ithaca, NY; Dr. S. Forsen, Lund University, Sweden

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Macromolecular Interactions Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	3	3.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The interaction of calmodulin with Ca^{2+} and its target proteins has been studied in order to understand the mechanism of the regulation of cellular processes by Ca^{2+}. Using large calmodulin fragments obtained in highly purified states by HPLC, we have identified the two high affinity Ca^{2+} binding sites as sites III and IV. Calmodulin fragment 78-148 (sites III and IV) interacts with two different enzymes and with anticalmodulin drugs. The NH_2 terminus fragment 1-77 also interacts with anticalmodulin drugs required for activation of one enzyme studied but not the other. Thus, calmodulin contains at least two drug interacting domains and different domains are required for activation of different enzymes.

Calmodulin exerts a tight coupling of Ca^{2+} and cAMP regulation of cellular processes by controlling cAMP levels and cAMP dependent phosphorylation. It interacts with the regulatory subunit of cAMP dependent protein kinase and activates a Ca^{2+} regulated protein phosphatase, calcineurin. The amino acid sequence of the Ca^{2+} binding subunit of this enzyme has been determined in collaboration with Dr. P. Cohen.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:

A. Manalan	Special Medical Staff Fellow	LB NCI
D. Newton	Research Chemist	LB NCI
M. Krinks	Chemist	LB NCI
R. Miller	Technician	LB NCI
W.C. Ni	Visiting Fellow	LB NCI

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 will be on cyclic nucleotide phosphodiesterase, and the Ca^{2+}-dependent phosphoprotein phosphatase, calcineurin. These studies are undertaken to elucidate the roles of the two second messengers, Ca^{2+} and cAMP in the regulation of cell function.

Methods Employed:

The project involves the study of properties of proteins, purified and analyzed by the usual techniques of protein isolation, including many types of chromatography and electrophoresis. The enzymes are studied by examination of their optical, hydrodynamic, kinetic, and immunochemical properties as well as by suitable chemical measurements and modifications. The roles of these proteins in cellular regulation are studied in cultured cell lines.

Calmodulin Regulation of Cellular Activity:

The Ca^{2+}-dependent regulation of several cellular processes is mediated by the ubiquitous, intracellular Ca^{2+}-binding protein, calmodulin. Interactions of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase and calcineurin with calmodulin are studied as model systems to elucidate the mechanism of action of calmodulin. Methods developed for these systems are applied to the study of interaction of calmodulin with other enzymes here and in collaboration with other investigators.

A. Interaction of Calmodulin with Ca^{2+}

The stimulus-response coupling mediated by Ca^{2+} involves several successive steps: (1) transitory increase in intracellular Ca^{2+} from 10^{-7} to 10^{-6} - 10^{-5}M, (2) interaction of Ca^{2+} with the Ca^{2+} receptor, calmodulin (3) interaction of the activated calmodulin·Ca^{2+} complex with the various target proteins and (4) coordinated activation of several enzymatic reactions. We previously reported that Ca^{2+} binding to calmodulin occurs in a stepwise fashion. The Ca^{2+}-binding properties of large calmodulin fragments obtained by limited proteolysis with trypsin (fragments 1-77, 1-90, 1-106, 78-148 and 107-148) have revealed that the two Ca^{2+} binding sites in the amino terminal

half of calmodulin (fragment 1-77, 1-90, 1-106) have a weaker affinity for Ca^{2+} than the two Ca^{2+} sites located in the carboxyl terminal half of the molecule (fragment 78-148). Fragment 78-148 undergoes a structural transition, upon binding two Ca^{2+} per mol, which affects the environment of the two tyrosyl residues and the phenylalanyl residues. This conformational change, followed by UV difference spectroscopy, is analogous to that observed with the native protein upon binding two Ca^{2+} per mol. Fragment 1-77, also undergoes a Ca^{2+}-dependent structural change which affects the environment of phenylalanyl residues. The calculated sum of the absorbance changes exhibited by the two fragments is very similar to the absorbance changes observed with the native protein upon binding four Ca^{2+} per mol, suggesting that the conformation of the isolated fragments is similar to their conformations in the native protein. These data therefore indicate that the stepwise binding of Ca^{2+} to calmodulin occurs first at sites III and IV, followed by occupancy of sites I and II.

Upon further proteolytic cleavage into individual Ca^{2+} sites such as that resulting in the formation of fragment 107-148, the affinity for Ca^{2+} decreases and the structural properties of the peptide are significantly different from those of the native protein. It seems, therefore, that the cooperative binding of Ca^{2+} to the two high affinity sites of calmodulin, requires the presence of the two Ca^{2+}-binding domains III and IV, but not of the two Ca^{2+} sites in the amino terminal half of the protein. If Ca^{2+} and Tb^{3+} have similar relative affinities for the four Ca^{2+} sites, the large increase in Tb^{3+} luminescence observed upon binding the last two Tb^{3+} (as described in last year's report) may reflect an overall conformational change of the protein following occupancy of sites I and II. These results are in good agreement with ^{113}Cd and ^{1}H NMR studies of Forsen et al. on calmodulin fragments prepared in our laboratory using our purification procedure (D. Newton).

B. Interaction of Calmodulin with its Target Proteins and Anticalmodulin Drugs

A unique characteristic of calmodulin is its ability to interact with and activate so many different enzymes. Evidence now suggests that whereas each calmodulin-regulated enzyme contains a regulatory, calmodulin-binding domain (see below), these domains may not be identical since interaction and activation of different enzymes by calmodulin required the integrity of different amino acid residues or peptides of calmodulin. We have prepared the large tryptic fragments of calmodulin: 1-77, 78-148, 1-106, 1-90 and 107-148 in highly purified states, and have tested their ability to either activate or interact with calmodulin-dependent cAMP phosphodiesterase or calcineurin. The use of HPLC to purify these peptides allows almost complete removal of calmodulin.

1. Regulation of cAMP phosphodiesterase. With the exception of fragment 78-148, none of the peptides can activate or inhibit the calmodulin-stimulation of cyclic nucleotide phosphodiesterase at concentrations up to 10^{-5}M. The small non-saturable stimulation (approximately 10%) observed at 10^{-5}M peptide can be attributed to contamination with native calmodulin. On the other hand, peptide 78-148 can reversibly activate phosphodiesterase but with a very low intrinsic activity (10-20% that of calmodulin) and can thereby act as a competitive inhibitor of calmodulin stimulation of the enzyme. The Km and Ki values for these

activation and inhibition were both between 10^{-7} and 10^{-6}M. Full calmodulin activity could not be restored by mixing complementary fragments. Furthermore, calmodulin lost its ability to activate phosphodiesterase following a single peptide cleavage at Lys 77 without prior separation of the two fragments. These results are taken as evidence that COOH terminal half of calmodulin, fragment 78-148, contains a phosphodiesterase interacting domain. The activation of the enzyme, however, requires the presence of the NH_2-terminal half which may also contain an interacting domain not detected by these methods and whose interaction with the enzyme is needed to promote strong affinity and activation. Alternatively, the presence of the NH_2 terminal fragment may exert an allosteric control on the COOH terminal fragment enhancing its binding constant and its ability to stimulate the enzyme (D. Newton, J. Schiloach, M.H. Krinks).

C. Regulation of Calcineurin

Calcineurin is a major calmodulin-binding protein present in large amounts in brain. This protein is a heterodimer composed of a 19,000 dalton Ca^{2+}-binding subunit calcineurin B and a 61,000 dalton calmodulin-binding subunit (Calcineurin A). The amino acid sequence of calcineurin B has been determined in collaboration with Drs. A. Aitken and P. Cohen and has confirmed the existence of four "EF hand" calcium binding domains analogous to those of calmodulin. These studies have also revealed that the NH_2 terminal blocking group of calcineurin B is myristic acid. This hydrophobic substituant which has also been identified as the NH_2 terminus of the catalytic unit of cAMP-dependent protein kinase could serve to anchor the protein in the membrane or to provide interaction with other proteins. Recently, a Ca^{2+}- and calmodulin-regulated phosphoprotein phosphatase activity was found to be associated with calcineurin. The calmodulin-stimulated phosphatase activity copurifies with calcineurin in soluble fractions from brain after affinity chromatography on calmodulin-Sepharose, which removed calmodulin-independent phosphatases. The large subunit, calcineurin A, was measured by [^{125}I]calmodulin gel overlay, and the small subunit subunit, calcineurin B, was monitored with anti-calcineurin antibody following SDS-gel electrophoresis and transfer to nitrocellulose filters. Using these methods, phosphatase activity, calcineurin A and calcineurin B were found to copurify through all purifiaction steps, including DE-23 chromatography, calmodulin-Sepharose affinity chromatography, gel filtration on G-200, and chromatofocusing. Calcineurin was shown to bind 1 mole of calmodulin per mole. Likewise, full activation of the phosphatase was achieved at a ratio of 1 mole of calmodulin per mole of calcineurin. The characterization of calcineurin as a phosphoprotein phosphatase has been carried out by M. Krinks, A. Manalan and J. Schiloach in collaboration with P. Cohen.

The effect of limited proteolysis of calcineurin on the Ca^{2+}-calmodulin stimulated phosphatase has further strengthened the identification of calcineurin as a protein phosphatase and clarified the role of subunit interactions in the regulation of the enzyme. Like other calmodulin-regulated enzymes, calcineurin can be activated and rendered calmodulin and Ca^{2+}-independent by limited proteolysis. Limited proteolysis of calcineurin with trypsin specifically alters the large subunit, calcineurin A. Digestion in the absence of calmodulin results in rapid cleavage, removing one fourth of the large subunit. Loss of this domain was accompanied by loss of ability of the remaining 44,000 dalton fragment to bind

calmodulin, assessed by glycerol gradient centrifugation and [^{125}I]calmodulin gel overlay. In contrast, the Ca^{2+}-independent interaction of this fragment with calcineurin B was preserved. As a result of limited proteolysis, the phosphatase was activated, and not further stimulated by the addition of Ca^{2+} or calmodulin. Thus, the loss of calmodulin binding to calcineurin resulting from proteolysis was accompanied by loss of calmodulin-stimulation of phosphatase activity. Inclusion of calmodulin in the digest mixture made calcineurin more resistant to proteolysis, and altered the pattern of digestion such that the calmodulin-binding capability of the 46,000-57,000 dalton fragments was preserved. In glycerol gradients, phosphatase activity cosedimented with calcineurin in the presence of EGTA ($s_{20,w}$ = 4.5S), in the presence of Ca^{2+} and calmodulin ($s_{20,w}$ = 5.0S), and after limited digestion ($s_{20,w}$ = 4.3S either in the presence of EGTA or in the presence of Ca^{2+} and calmodulin). Results of limited proteolysis demonstrate the existence of at least two domains within calcineurin A. One domain contains the catalytic site as well as the site for interaction with calcineurin B. This site is relatively resistant to proteolysis, and further resolution of potentially distinct catalytic and calcineurin B-binding domains was not observed. The other domain, which is highly susceptible to proteolysis, is essential for interaction with calmodulin. By analogy with other calmodulin-binding proteins, these results are consistent with the presence of a calmodulin-binding domain, whose inhibitory effect on enzyme activity is removed either by proteolysis or as a result of conformational changes upon calmodulin binding (A. Manalan).

Significance to Biomedical Research and the Program of the Institute:

The proteins being studied are important enzymes in the control of cell metabolism which are regulated by protein-protein interactions. cAMP phosphodiesterase is one of the two enzymes responsible for the control of cAMP levels. Calcineurin, as a Ca^{2+} regulated phosphoprotein phosphatase can modulate the function of many enzymes and other proteins regulated by phosphorylation. cAMP and Ca^{2+} mediated phosphorylations are critical for the regulation of cell growth and differentiation. Calmodulin, as a common modulator of these, and many other enzymes, provides a link between cyclic nucleotide levels and Ca^{2+} regulation of cellular processes. The ability of calmodulin to regulate a large number of biological processes represents a novel mechanism with great potential physiological importance.

Future Course of Research:

Calmodulin plays a unique role in the regulation of cellular processes mediated by cytosolic Ca^{2+}: that of a universal modulator of Ca^{2+} acting as a second messenger. It may also function as a coupling factor in the dual regulation of cellular processes by Ca^{2+} and cAMP. We will continue to study the structure of calmodulin in solution to characterize the Ca^{2+} conformers responsible for multiple functions, and to identify the calmodulin interacting site(s) with its target proteins. We will attempt to isolate and characterize the interacting sites of the target proteins cyclic nucleotide phosphodiesterase and calcineurin. We will try to identify the link between the two second messengers, Ca^{2+} and cAMP, by studying the effects of calmodulin and Ca^{2+} on cAMP-dependent protein kinase and protein phosphatase. These studies will be carried out at the molecular level with purified and characterized proteins as well as at the cellular

level with cells in tissue culture to correlate the "in vitro" observations with physiological events. Among the model systems in which the role of calmodulin and other Ca^{2+} binding proteins will be investigated are: The differentiation of neuroblastoma glioma hybrid NG-108-15 and in vitro transformation of cells.

Publications:

Manalan, A.S. and Klee, C.B.: Interaction of calmodulin with its target proteins. Chemica Scripta 21: 139-144, 1983.

Aitken, A., Cohen, P., Santikarn, S., Williams, D.H., Calder, A.G., Smith, A. and Klee, C.B.: Identification of the NH_2 terminal blocking group of calcineurin B as myristic acid. FEBS Lett. 150: 314-318, 1982.

Willingham, M.C., Wehland, J., Klee, C.B., Richert, N.D., Rutherford, A.V. and Pastan, I.H.: Ultrastructural immunocytochemical localization of calmodulin in cultured cells. J. Histochem. Cytochem. 31: 344-346, 1983.

Manalan, A.S. and Klee, C.B.: High performance liquid chromatography of calcium-binding proteins and their proteolytic fragments. In: Handbook of the Use of HPLC for the Separation of Amino Acids, Peptides and Proteins, in press, 1983.

Krinks, M.H., Haiech, J., Rhoads, A. and Klee, C.B.: Reversible and irreversible activation of cyclic nucleotide phosphodiesterase: Separation of the regulatory and catalytic domains by limited proteolysis. Adv. Cyclic Nucleotide Res., in press, 1983.

Klee, C.B., Krinks, M.H., Manalan, A.S., Cohen, P. and Stewart, A.A.: Isolation and characterization of bovine brain calcineurin: A calmodulin-stimulated protein phosphatase. Methods in Enzymology 102: 227-244, 1983.

Aitken, A., Klee, C.B., Stewart, A.A., Tonks, N.K. and Cohen, P.: The structure of calcineurin B. Proc. Symposium on Calcium Binding Proteins in Health and Disease, Carafoli et al., (Eds.) Elsevier/North Holland, in press, 1983.

Klee, C.B., Krinks, M.H. and Manalan, A.S.: Regulatory domains and protein phosphatase activity of calcineurin. Proc. Symposium on Calcium Binding Proteins in Health and Disease, Carafoli et al., (Eds.) Elsevier/North Holland, in press, 1983.

Klee, C.B. and Newton, D.L.: Calmodulin modulated protein phosphorylation. Proc. of Oklahoma Medical Research Foundation International Conference on Post-translational Covalent Modification of Proteins for Function. Oklahoma City, Nov. 7-10, 1982, Academic Press, in press.

Manalan, A.S. and Klee, C.B.: Activation of calcineurin by limited proteolysis. Proc. Natl. Acad. Sci. 80: in press, 1983.

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

PROJECT NUMBER
Z01CB05234-09 LB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Interrelations between the Genomes of SV40 and African Green Monkeys

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
M. F. Singer Chief, Nucleic Acid Enzymology Section, LB, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.3	0.3

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

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

A cloned segment of the African green monkey (Cercopithecus aethiops) genome that contains DNA sequences homologous to the control region of simian virus 40 is being studied. This sequence, 450 base pairs in length, is embedded in a genomic DNA region that is especially rich in interspersed repeated sequences. The segment homologous to SV40 is flanked by two members of the Alu family, one of which represents a novel Alu subfamily whose members contain a potential Z-DNA forming segment. The SV40-like region, which is hypersensitive to DNase I in monkey chromatin, serves as a transcriptional start site for cellular RNA synthesis. Also, the sequence provides information for initiation of transcription from vectors constructed by molecular cloning as measured by expression of an E. coli gene after transfection of the vector into mammalian cells. Expression was measured both by the percent of cells transformed by the E. coli gene and by analysis of messenger RNA transcribed from the vector.

OTHER INVESTIGATOR:

J. Saffer Staff Fellow LB NCI

Project Description:

Objectives:

A range of interactions occur between permissive cells and infecting viruses. Using simian virus 40 (SV40) as a model, we are studying one aspect of viral-host interaction, namely, the use of common regulatory components. When SV40 enters a permissive monkey cell it uses the host machinery for the initiation of early viral transcription and the synthesis of early viral proteins. Subsequently, interaction of an early viral protein (T-antigen) with the viral genome is required for viral DNA replication and the synthesis of late proteins. Replication too involves the use of a number of host enzymes. In addition to its effect on viral functions, T-antigen stimulates an increase in the level of the host enzymes required for DNA replication and host DNA replication itself. These events suggest that the viral and host DNAs might share similar sequences that are recognized by a variety of enzymes and regulatory proteins. Earlier we searched for, found and characterized DNA sequences in the monkey genome that are homologous to the control region of the SV40 genome. These regions, about 80 per genome and a few hundred base pairs in length, include sequences like those around the SV40 origin of DNA replication. We are now investigating the ability of one of the homologous monkey DNA segments to function in analogous ways.

Methods Employed:

Standard tissue culture procedures are used. Other methods include radioisotope tracer techniques, preparative ultracentrifugation, DNA-DNA and DNA-RNA hybridization both in solution and with DNA fixed to nitrocellulose filters, column chromatography. Extensive use is made of restriction endonucleases and both preparative and analytical gel electrophoresis for the analysis and preparation of DNA fragments. Specific enzymatic procedures are used for modification or isotopic labeling of DNA fragments. Primary nucleotide sequence determination of DNA fragments is carried out by direct DNA sequencing techniques introduced by Maxam and Gilbert. DNA fragments are purified and prepared in µg quantities by recombinant DNA techniques using E. coli K12 cloning systems. The ability of cloned monkey segments to function as replication origins or sites for initiation of transcription is studied with special recombinant vectors designed for use in animal cells. The prototypical vector is pSV2, designed by Mulligan and Berg; this shuttle vector includes E. coli plasmid sequences, an E. coli gene (xanthine-guaninine phosphoribosyl transferase, XGPRTase) and an SV40 control region. Molecular cloning allows replacing the SV40 control region with other DNA segments. In addition, DNA sequences can readily be introduced at various positions in the molecule. Desired constructions are made in vitro and then amplified in E. coli before transfection into mammalian cells. All recombinant DNA experiments are carried out under conditions required by the NIH Guidelines for Recombinant DNA Research as approved by the NIH Biosafety Committee. All our work has been facilitated by extensive use of the NIH computer for storage and

analysis of nucleotide sequence data. The mapping of RNA transcripts on DNA templates (including the constructed vectors) is done by the S1 nuclease technique and primer extension.

Major Findings:

In the earlier work a recombinant "library" of the DNA of the African green monkey (Cercopithecus aethiops) in a bacteriophage lambda vector was constructed. Using purified DNA fragments as probes, segments of monkey DNA homologous to the control region around the origin of replication of simian virus 40 (SV40) were isolated. Three distinctly different monkey segments homologous to the control region of SV40 were detected and characterized by subcloning and primary nucleotide sequence determination. Each segment is only a few hundred base pairs long and contains multiple and disconnected sequences homologous to the control region of SV40. The number and arrangement of the homologous sequences in each of the three segments differs and is distinct from the arrangement in the virus. The segments include homology to SV40 DNA regions known to be involved in the initiation of viral DNA replication and the start of early transcription. Specifically, the segments have the following features in common: (1) each contains multiple copies of the sequence GGGCGGPuPu, which also appears six times near the origin of SV40, (2) each contains several imperfect homologies to the central dyad symmetry of SV40, (3) each contains a long internal repeat, as does the origin region of SV40. Within the SV40 genome the GGGCGGPuPu repeats are known to be required for early transcription and to bind T-antigen. Recently, others have suggested that this region is a binding site for RNA polymerase II. The central dyad symmetry region also binds T-antigen, is equivalent to the origin of replication, and a portion of it is the start site for major early transcripts. The actual site of early transcription initiation appears to be determined by the sequence TATAAT, 20-30 bp upstream from the start site. In the SV40 genome the TATAAT sequence is between the G-rich repeats and the start site. None of the 3 monkey sequences contains a TATAAT box. The specific segment we are studying in detail (clone 7) was previously shown to contain a site that is hypersensitive to DNase I within monkey chromatin. In addition, it was found that cellular RNA hybridized to this region. These data suggested the SV40-like segment is indeed transcribed.

During the past year we have continued work on the sequences contained within clone 7. This has included further characterization of the genomic segments that immediately surround the SV40-like region. Earlier, we described a typical member of the Alu family of short repeated sequences that starts within 100 base pairs of the ori-like segment. We have now characterized another Alu family member within a few hundred base pairs of the opposite end of the ori-like segment.

Alu is the most abundant family of SINEs in primates. The typical structure, deduced from the study of cloned family member, is dimeric - composed of directly repeating units about 130 bp long each terminating with an A-rich region and with a 31 bp insert in the second unit. The newly characterized Alu hybridizes only weakly to typical Alu family probes. The segment was subcloned and sequenced and proved to be a divergent form of the consensus Alu. The divergence

is most marked at the 3'-end of each of the two monomeric units. At the 3'-end of the first monomer 24 nucleotides including 18 bp of alternating A and C replace the 16 nucleotides of the Alu consensus, including the A-rich stretch. At the 3'-end of the second monomer a 21 nucleotide long G-C rich stretch replaces a characteristic 9 nucleotide segment of the consensus. Hybridization of the aberrant Alu to monkey and human genomic libraries suggests that multiple copies of the variant Alu exist. Comparison of our sequence with published sequences indicated that one member of this Alu subfamily had been unwittingly isolated from human DNA by others. One of the very interesting features of this Alu subfamily is the potential for forming a Z-DNA structure at the site of alternating A-Cs.

The ability of the ori-like region to promote the initiation of transcription has now been studied extensively. This work involved two approaches. The first was to study cellular transcripts that initiate from the genome itself. The second was to study the initiation of transcription from recombinant vectors after transfection into cells in tissue culture. In both instances, we investigated transcription going in both directions from within the ori-like region since in the viral genome transcripts do originate in two directions.

Cellular transcripts homologous to the genomic ori-like segment and surrounding sequences were detected and their start sites mapped by S1-nuclease experiments. The starts are within the ori-like region and include a major site and several minor sites. These transcripts were in both directions, in analogy to SV40 transcription from the ori region.

More detailed studies were carried out with recombinant vectors in which the ori-like region was used, in both directions, to promote transcription of the bacterial xanthine-guanine-phosphoribosyl transferase gene (XGPRTase). The vector is based on the original construction by Mulligan and Berg; we modified it to inhibit the formation of functional transcripts starting within the pBR322 sequences by addition of a polyadenylation site. In experiments that measure transient expression from the vector we have found that the SV40-like segment, regardless of orientation, promotes expression of the bacterial XGPRTase after transfection into monkey cells. This is consistent with the bidirectional nature of RNA polymerase II interaction with the sequence GGGCGGPuPu as proposed by others. Efficient expression, however, is dependent on the presence within the vector molecule of a 72 bp long segment, the "enhancer", from the SV40 control region; enhancer is also required for efficient expression of SV40 genes. By itself, the 72 bp segment does not permit transcription of XGPRTase. Using S1 nuclease mapping techniques, the sites of initiation were determined. Transcription under the control of the cloned segment in both orientations is characterized by multiple initiation sites within the segment. In the orientation analogous to "late" SV40 transcription, the SV40-like region promotes transcription approximately 4-fold better when genomic sequences up to 350 bp upstream of the SV40-Like region in clone 7 are included in the vector. In this enhanced construction, the relative efficiencies of the multiple start sites is changed. These findings were confirmed by long-term experiments that measured the relative efficiency of the several constructions to transform cells. Transformation was estimated by the ability of cells to overcome inhibition by mycophenolic acid by virtue of the expression of the bacterial XGPRTase.

The cellular promoter we are studying is notable for the lack of a TATAAA box which is normally present 25 to 30 bp 5' to the site of transcription initiation by RNA polymerase II. Our current interpretation of this finding is that the promoter is a member of a rare class of polII promoters that may play special regulatory roles in cellular metabolism.

Significance to Cancer Research:

Our studies deal with the interactions between the viral genome and the genome of a permissive host. These studies are pertinent to the nature and mechanism of viral-host DNA interaction in permissive and transforming (oncogenic) infections. Further, it is now widely recognized that the genomes of tumor viruses contain sequences homologous to normal host DNA sequences. In the case of RNA tumor viruses these are oncogenes. Our experiments indicate that with some DNA tumor viruses such as SV40, regulatory sequences rather than coding sequences are shared by virus and host.

Proposed Course of the Research:

In the coming year, we will complete the mapping of transcripts initiated in vivo from the SV40-like region in chromatin. These transcripts will be characterized in detail as to length, potential splicing sites, and eventually gene product. This latter objective is important in determining what type(s) of cellular proteins are regulated in a manner similar to SV40. Further study will be done on the role of the sequences surrounding the SV40-like region. In particular, the localization of the upstream elements that enhance expression will be studied using expression vectors with appropriate deletions and changes. In addition, the role of the surrounding Alu sequences can be examined in an analogous manner. The function of this repetitive family is as yet unknown, but we have a potent system for the study of their effect on transcription of neighboring sequences. The transcription of the Alu sequences themselves will be studied in vitro in Xenopus oocytes in the presence and absence of the SV40-like promoter (in collaboration with S. Adeniyi-Jones and M. Zasloff). We are now also in a position to determine whether cellular transcription of the cloned segment is affected by SV40 infection. Determination of differences between expression of this sequence in normal and SV40-transformed cells will also serve to establish a possible role of the cellular gene in transformation. In vitro transcriptional studies using SV40 specific factors from Hela cell extracts will be carried out with W. Dynan and R. Tjian to determine whether specific proteins other than T-antigen are responsible for regulation of SV40-like promoters.

Publication:

Saffer, J. and Lerman, M.I.: Unusual Alu sequence from African green monkey containing a potential Z-DNA sequence. Mol. Cell. Biol. 3: 960-964, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB05244-06 LB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Organization of Repeated DNA Sequences in African Green Monkeys

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
M. F. Singer Chief, Nucleic Acid Enzymology Section, LB, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Nucleic Acid Enzymology Section

INSTITUTE AND LOCATION
National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	5.7	0.3

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

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

Two types of highly repeated DNA segments in the genome of the African green monkey (Cercopithecus aethiops) are being studied. (1) Satellite DNAs are characterized by tandem repetitions and are localized to centromeres. We investigated the inheritance of the polymorphic forms of deca-satellite that are found in individual monkeys of the species. Although the fluid organization of the monkey deca-satellite was confirmed, the analysis did not afford any direct evidence for major satellite reorganization in a single generation. However in tissue culture cells, in situ hybridization of deca-satellite probes to endoreduplicated chromosomes indicated extensive unequal crossing-over of deca-satellite sequences among sister chromatids. (2) The members of the primate KpnI family of long interspersed repeated sequences were shown to be a) polymorphic in length and internal sequence arrangement, b) transcribed in human and monkey cells, and c) homologous to a similarly abundant rodent LINE family (about 10^4 copies per genome). A cloned member of the R family of murine short interspersed repeated sequences contains a promoter that supports gene expression in mammalian cells.

OTHER INVESTIGATORS:

T. Lee	Research Chemist	LB	NCI
R. Thayer	Chemist	LB	NCI
G. Grimaldi	Fogarty Visiting Fellow	LB	NCI
A. Maresca	Fogarty Visiting Fellow	LB	NCI
C. Contente	Staff Fellow	LB	NCI
M. Lerman	Visiting Scientist	LB	NCI
S. Segal	Expert	LB	NCI

Project Description:

Objectives:

The detailed structure, organization and function of the highly repeated DNA sequences in the African green monkey (Cercopithecus aethiops) are being studied. These sequences fall into three groups: satellites are long tandemly repeated segments generally concentrated at centromeres, SINES are short repeated segments dispersed throughout the genome, and LINES are long repeated segments also dispersed throughout the genome. We are interested in characterizing the various families of such sequences, establishing their frequencies within the genome and their functions. Recently it has become evident that many of these sequences are changeable in position within DNA. They also undergo reactions which bring the sequences of the various family members into conformity within the genome of particular species (concerted evolution). We are interested in studying the mechanisms, the time course and frequency of such changes in order to define the fluidity of primate genomes.

Methods Employed:

DNA is isolated from fresh frozen tissue and from cells grown in tissue culture and purified by standard procedures. DNA structural analysis includes the use of a variety of enzymes, including restriction endonucleases, direct DNA sequencing methods (chemical procedure of Maxam and Gilbert), radioisotope tracers, gel electrophoresis, and centrifugation. A variety of nucleic acid hybridization techniques, both in solution and on nitrocellulose are used. DNA fragments are purified and amplified by molecular cloning in E. coli K12 host-vector systems. Genomic organization is studied by blotting and hybridization. Functional aspects focus first on transcription of these sequences both in vivo and in vitro. For this work RNA is isolated from cells and cloned recombinant cDNA libraries are used. Electron microscope analysis of heteroduplex molecules is made. Finally, recombinant DNA vectors are constructed and transfected into animal cells to study expression. All recombinant DNA experiments are carried out under conditions required by the NIH Guidelines for Recombinant DNA Research as approved by the NIH Biosafety Committee. Somatic cell hybrids are constructed by standard techniques.

Major Findings:

KpnI-LINE family. As shown previously by ourselves and others, the KpnI-LINE family includes approximately 10^4 related DNA segments that are dispersed

in primate genomes. Some family members are over 6 kilobase pairs in length but others are a good deal shorter. We have now characterized further the length polymorphisms that can occur within the family. A group of recombinant phage containing African green monkey KpnI family members were studied. Some of these had been studied previously and others were newly isolated for this work. Some of the cloned KpnI family members are embedded in or joined to α-satellite DNA sequences; others are from genomic regions that include unique DNA sequences. The length of the KpnI family segments in each was determined by construction of restriction endonuclease maps and hybridization of subsegments to short regions of KpnI family sequence that were purified by subcloning in pBR322. In this way, the extent of homology to sequences associated with the KpnI family was determined within each of the segments in the phage clones. More recently, we have begun to determine the length of KpnI family segments by analysis of heteroduplex molecules between different clones, in the electron microscope. The conclusions from this work are as follows. Some family members may be almost 7 kbp long. Others are considerably shorter. One cloned segment that is bordered on both sides by unrelated DNA sequences is no longer than 2.4 kbp. Another, whose borders are precisely known from primary nucleotide sequence determination, is 829 base pairs long. No cloned segment that we have studied contains all of the DNA segments that have been associated with the KpnI family. This suggests that a hypothetical "full length" member may be rare or even not exist.

Consistent with the data described so far, we found that different regions from within a KpnI family member may not be repeated an equal number of times in the genome and that these subregions can occur independently of one another. For these experiments, duplicate filters containing random portions of a monkey genomic library (in λCharon4A) were screened with cloned DNA segments representing the two ends and the center of a long KpnI family member. About 11% of the phage hybridized with the center and right hand regions of the long KpnI segment; about 7% hybridized with the left hand probe. The duplicate filters indicated that the left hand regions almost always occur in conjunction with the center but that the center segment only occurs in conjunction with the right hand segment 58 percent of the time. Thus, in addition to divergence within shared sequences, different family members contain different portions of the total possible length.

The polymorphism of family members extends to the arrangement of the shared sequences within family members. An approximately 2 kbp long family member (KpnI-LS1) was cloned, sequenced and compared to other family members which are over 6 kbp (KpnI-α7) and 829 bp (KpnI-RET) long. Both KpnI-LS1 and KpnI-RET lack sequences found at the ends of the longer family member. KpnI-LS1 sequences are colinear with part of the long family member, KpnI-α7. However while all sequences in KpnI-RET are represented in KpnI-LS1, the two are not colinear; KpnI-RET is missing 731 bp found in KpnILS1 and one segment flanking the deletion is inverted. The results demonstrate that KpnI family members are not only of different lengths, but may also contain scrambled arrangements of common sequences. At this time the significance of this observation is not known (see below).

The detailed structure of KpnI-RET provided evidence that at least some KpnI family members may be moveable elements. The 829 base pairs of KpnI-RET

are embedded in about 10 kilobase pairs of α-satellite sequence in the isolated clone. The entire nucleotide sequence of KpnI-RET was determined, along with the sequence across the junctions with the satellite. Since the satellite sequence had been determined some years ago, we could recognize precisely the borders of the KpnI element. We found that KpnI-RET is flanked by direct repeats of a 14 base pair long satellite segment that occurs only once in the satellite itself. This duplication of the target site of insertion is typical of moveable genetic elements in bacteria and in eukaryotes and is strong evidence that KpnI-RET was inserted into the satellite. Furthermore, since the satellite sequence is a "young" sequence from an evolutionary standpoint (it is species specific) and the KpnI family is well conserved among primates, it appears that the KpnI-RET segment moved into the satellite in relatively recent evolutionary time. We do not yet know whether KpnI family members are contemporaneously moveable.

KpnI-RET is similar in structure to a class of eukaryote moveable elements that differs markedly from the classical transposable elements of bacteria. The distinctive features of this class are 1) the absence of terminal repeats, 2) the presence of a 3'-terminal polyA stretch on one strand and 3) a variable size target site duplication. The class includes the Drosophila F element, Alu sequences of mammals and processed genes. KpnI-RET has the first two features but the third is undetermined as yet. It has been proposed that this class of elements transposes in a manner fundamentally different from the mechanisms associated with bacterial transposons. At present, the most favored proposals involve the transcription of the element, followed by reverse transcription to form a DNA copy of the RNA and finally insertion of the new DNA into random places in the genome, accompanied by duplication of the target site. Thus it seems reasonable to assume that KpnI-RET is the product of a transposition involving reverse transcription. However, when we determined the structure of KpnI-RET, there was no evidence that KpnI family sequences are transcribed and there were reports of negative findings in the literature. For this reason, and because of the more general importance of the question of KpnI family function, we have studied the transcription of the family.

Transcripts homologous to KpnI family members are readily detected both in CV-1 monkey kidney cells and in Hela cells (human). Cellular RNA from both nucleus and cytoplasm hybridizes to KpnI family DNA; hybridization was also found when the RNA was first selected on polydT-columns to concentrate the polyadenylated RNA. The probes used in these experiments were from KpnI-LS1 and therefore cover only a portion of the right hand half of a long KpnI family member. When the RNA is separated by gel electrophoresis and transferred to a nitrocellulose filter prior to hybridization with cloned KpnI family segments, RNA molecules between about 400 and 9000 nucleotides long hybridize. To study more directly the structure of transcripts, we have begun to isolate clones from a human cDNA library (kindly supplied by Okayama and Berg); clones are selected by their ability to hybridize with KpnI family sequences. Thus far, two clones have been isolated and are being characterized. Although this work is at a preliminary stage, we can conclude that there is a reasonably high level of transcription from at least some KpnI family members.

We have compared the overall genomic characteristics of KpnI family members in the monkey and the human genome. Cross-hybridization between the two species is very strong, indicating only a small degree of sequence divergence among most family members. Nevertheless, most of the family members in human DNA differ from most of the family members in the monkey genome by distinctive restriction endonuclease sites. This feature of repeated interspersed sequences, namely the variable abundance of different subfamilies among closely related species has been observed in a number of other species (e.g., sea urchins, Drosophila, mice). This unusual evolutionary behavior has been termed concerted evolution or homogenization. The explanatory mechanism is unknown.

One question of interest concerning LINEs is the possible relation between LINE families in distantly related organisms such as rodents and primates. In the past, conflicting results were obtained regarding the ability of primate LINEs to hybridize to mouse DNA or the reverse. Up until recently there was very little nucleotide sequence data available for direct comparison. Within the last few months, sequence data for the mouse LINE family called BamHl (or M1F-1) has become available. Also, we completed the sequence determination of the almost 1800 base pairs of KpnI-LS1. We compared all these sequences with the use of relevant computer programs. The comparison shows that KpnI-LS1 is colinear and between 60 and 70 percent homologous to the mouse segments over at least 1400 base pairs. Therefore we conclude that the major LINE families in rodents and primates had a common ancestor and although they vary extensively have been conserved in evolution. This observation lends some support to the view that LINE families, or at least some family members, have important functional roles in cellular processes.

Deca-satellite. We have continued the analysis of deca-satellite whose discovery was described last year. Deca-satellite makes up about 1 percent of the monkey genome and has a 10 bp repeat unit: consensus, 5'-AAACCGGNTC. We had originally discovered deca-satellite in genomic clones (in λCharon4A) that were selected because they hybridized with the very abundant α-satellite of the monkey; restriction endonuclease maps and direct sequence analysis indicated that the two satellites were joined directly to one another in the isolated clones. One of the more remarkable properties of deca-satellite is its highly polymorphic arrangement in individual monkeys; none of 10 individual monkeys yielded the same exact set of long deca-containing fragments upon restriction endonuclease digestion with, for example, endonuclease EcoRI (see last year's report). The polymorphisms related both to the size of discrete fragments in the sets found within different individuals and to the relative abundance of fragments of particular sizes. These results showed that deca-satellite organization is not fixed in the population.

We have now investigated whether junctions between α- and deca-satellites are frequent in the monkey genome. A monkey genomic library constructed from randomly generated fragments was constructed and random plaques hybridizing with deca-satellite were screened for hybridization with α-satellite. About 12 percent of the phage that hybridized to deca-satellite also hybridized to α-satellite indicating that direct linkage of the two is a common feature.

Our earlier studies of deca-satellite indicated that the degree of divergence of individual units from the 10 bp long consensus sequence varies from one base pair to another (from 50 to 0 percent) with the tetranucleotide core, 5'-CCGG particularly well conserved. We wondered whether the sequenced regions are representative of the whole deca-satellite family; since they were derived from regions close to junctions with α-satellite they might be more divergent than repeats close to the middle of long deca-satellite arrays. In addition, the dinucleotide 5'-C-G is the site of methylation in eukaryotic cells. If the tetranucleotide core is very conserved then deca-satellite might be highly methylated. To address these questions, the DNA of one monkey was digested with endonuclease EcoRI and then with endonuclease HpaII or MspI. The latter two restriction endonucleases recognize the sequence 5'-C-C-G-G, but HpaII will not cleave if the cytosine in the dinucleotide 5'-C-G is methylated. We could then ask a) what is the percentage of deca-satellite that has an invariant core sequence, b) what percentage of this sequence is methylated, and c) is the methylation correlated with some of the discrete polymorphic EcoRI fragments. The results indicate that the 5'-CCGG site is very well conserved and that only a very low percent of deca-satellite is methylated. The methylation seems to be randomly distributed in that it does not correlate with domains of deca-satellite that are defined by EcoRI cleavage sites.

We have used two approaches to estimate the overall frequency with which the organization of deca-satellite may be changing. In the first, deca-satellite organization was investigated within monkey families. The inheritance of the large polymorphic genomic restriction endonuclease fragments was studied by analyzing the EcoRI and BamHl digestion patterns of genomic DNAs from four monkeys and their offspring. In one cross the parental digestion patterns were more similar than those of any other pair of monkeys we have studied. Nevertheless, there were distinctive EcoRI and BamHl bands. Patterns of the two male offspring were practically identical, suggesting that the deca-satellite arrangement in each of the parents was close to being homozygose. Some bands, however, were reduced in intensity compared with the analogous parental bands. We suggest that these bands might reflect either amplification of the deca-satellite sequence within the time of this single generation or a heterozygosity in the amount of the band in the parental genomes. The parental pairs in the two other crosses examined had very different patterns of deca-satellite. No new bands appeared in patterns produced from the DNA of the offspring of these two crosses. The offspring patterns each contained only bands that were also present in their respective patents however neither offspring showed all the bands present in the two parents. These results indicate that at least one parent in each of these crosses was heterozygous with regard to deca-satellite organization. However, the analysis did not afford any direct indication that deca-satellite was being reorganized in the course of a single generation.

In the second approach to the frequency with which deca-satellite rearranges, we made use of the phenomenon of endoreduplication. On rare occasions, duplicated sister chromatids fail to segregate prior to the start of a second replication cycle. Spreads of nuclei in which the chromosomes are endoreduplicated are readily identified by the extra-wide chromosomes or by the joined double pair of sister chromatids. The percent of cells undergoing endoreduplication is enhanced in the presence of colcemid. We carried out in situ hybridization to

African green monkey chromosome spreads using a cloned deca-satellite probe. Most of the endoreduplicated spreads (approximately 15 out of 19 examined) contained at least one chromosome on which the hybridization was asymmetric and confined to only one of the two pairs of joined sister chromatids. We interpret this observation to indicate that in every somatic cell replication, about one out of the approximately 27 AGM chromosomes that contain deca-satellite (of the total diploid number of 60) undergoes unequal crossing-over resulting in a major reorganization of deca-satellite.

Mouse repeated sequences. As indicated above, we have established that the major rodent and primate LINE families are related. It has been known for some time that the major SINE families, Alu and B1 in primates and rodents, respectively, are also closely related. Other SINE families have also been identified in rodent genomes, one of the more abundant being the R-family. Experiments carried out over the past year indicate that at least some R family members contain promoter signals that permit transcription and expression of genes in eukaryote cells. An 850 bp long cloned mouse segment containing an R family member and some flanking sequences was used in these experiments. To study the possibility of this fragment being transcriptionally active in eukaryotic cells it was recombined with an expression vector for E. coli xanthine-guanine phosphoribosyl transferase (XGPRT) and introduced into cultured monkey CV-1 cells under selective conditions. Cells transformed by the E. coli enzyme arose with a frequency of 10^{-4} indicating the presence of a promoter for polymerase II within the mouse insert. Individual transformed CV-1 cells were cloned and the copy number of transfecting DNA plasmid per haploid genome was determined. All XGPRT transformed cells analyzed contained between one and two copies of plasmid sequences associated with high molecular weight chromosomal DNA. In order to identify the promoter region and the 5'-end of the mRNA for XGPRT, polyadenylated cytoplasmic RNA was extracted from one of the transformed cell lines and mapped using the SI nuclease method as well as the primer extension technique. Both methods showed that the transcription initiation site mapped to nucleotide 522 in the R sequence. When the R sequence was compared to the published sequences of five other cloned R-elements one major difference was observed; in position one of the putative "TATA" box (promoter) the five R sequences have an A but the one studied here has a T. This nucleotide change may be important in promoting transcription of an adjacent gene (in this case XGPRT) suggesting that some but not all R elements can affect expression of a downstream gene.

Significance to Cancer Research:

Cancer research has suffered from a lack of basic knowledge about the eukaryote genome at the molecular level. Methods developed during the last decade have already demonstrated their power to deal with this vast and important unknown. The work we are doing is part of the broad effort to apply new approaches to the elucidation of complex genomes. In particular, we are concentrating on the surprisingly large amount of mammalian DNA included in highly repeated sequences of as yet unknown function but with a capability for mobility and rearrangement in the genome. Work in many laboratories has now shown that genomic rearrangements are often found in tumor cells. It is therefore important to have a more complete picture of recombination in general and, more particularly, of mobile elements in primates. On a more fundamental level, LINE families present several major puzzles. What is the function of these segments

that comprise several percent of the genome? How do they effect gene expression? What is the effect of their mobility on contemporary genetic processes and what role did they play in the evolution of genomes?

Future Course of the Research:

A. We will concentrate, during the coming year, on the KpnI-LINE family with two aims in mind. In this work we will put more emphasis on human systems than we have in the past.

1. To elucidate the function of KpnI-LINEs

The RNA transcripts homologous to KpnI-LINE sequences will be characterized. Both cellular RNAs and cloned cDNAs will be studied. The position of transcriptional start and stop sites will be determined to investigate whether KpnI-LINEs include transcription units or whether transcripts stop and start in flanking sequences. We will determine which RNA polymerase is responsible for transcription. The potential of the RNAs to be translated will be investigated.

2. To understand the mechanism by which KpnI-LINE family members are dispersed within primate genomes.

For this purpose we will determine the nucleotide sequence at the ends of several KpnI family members including the junctions with other genomic segments.

B. The investigation of deca-satellite will be continued with emphasis on the following question. How do pericentromeric satellite sequences join the chromosome arms? Little is known about the relation between DNA sequences found at centromeres and the structural features of the centromeres. One approach to this question is to try to identify sequences that are joined at the centromere/noncentromere border. For this purpose we will isolate and study cloned genomic segments in which deca-satellite is linked to low copy genomic segments.

Publications:

Lee, T.N.H. and Singer, M.F.: Structural organization of α-satellite DNA in a single monkey chromosome. J. Mol. Biol. 161: 323-342, 1982.

Grimaldi, G. and Singer, M.F.: Members of the KpnI family of long interspersed repeated sequences join and interrupt α-satellite in the monkey genome. Nucleic Acids Res. 11: 321-338, 1983.

Maresca, A. and Singer, M.F.: Deca-satellite: A highly and polymorphic satellite that joins α-satellite in the African green monkey genome. J. Mol. Biol. 164: 493-511, 1983.

Thayer, R.E. and Singer, M.F.: Interruption of an α-satellite array by a short member of the KpnI family of interspersed highly repeated monkey DNA sequences. Mol. Cell. Biol. 3: 967-973, 1983.

Lerman, M.I., Thayer, R.E. and Singer, M.F.: The KpnI family of long interspersed repeated DNA sequences in primates: Polymorphism of family members and evidence for transcription. Proc. Natl. Acad. Sci. USA, in press, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05258-04 LB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

B. M. Paterson Research Chemist, LB, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	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.)*

Using cloned cDNA probes we have isolated the genomic sequences (from the chicken) for the following proteins: alpha skeletal muscle actin, alpha cardiac actin, beta cytoplasmic actin, myosin light chains 1 and 3, vimentin (a major intermediate filament protein), pyruvate kinase, and GAPDH (glyceraldehyde phosphate dehydrogenase). These have been defined by DNA sequence analysis. The various actin genes have been subcloned into the eukaryotic expression vector, pRSV-CAT, in order to study the developmental regulation of these genes after transfection (calcium phosphate precipitation) into the C2 mouse muscle cell line. Probes specific for the various chicken actin genes have been used to monitor expression during differentiation in vitro. In vivo transcription of the vimentin gene, a single copy gene, yields two distinct mRNA transcripts by Northern analysis. The coding potential and formation of these transcripts has been determined. The myosin light chains 1 and 3 are encoded by a single gene. The structure and regulation of this expression is under study. The mouse histone H4 gene is expressed in a cell-cycle dependent fashion. The structure of the gene has been altered to determine, in transfection studies with L-cells and C2 cells, which regions of the gene confer this regulation. Pyruvate kinase undergoes an isoform shift during myogenesis. The structure and regulation of the pyruvate kinase gene is under investigation. The three Acantha amoeba myosin polypeptides have been synthesized in vitro in preparation for the isolation of the three genes. The genes will be used in structural comparison of the myosins.

PHS 6040 (Rev. 1/83) GPO 895-100

OTHER INVESTIGATORS:

J. Hammer	Guest Researcher	LB	NCI
E. Korn	Guest Researcher	LB	NCI
J. Eldridge	Biochemist	LB	NCI
A. Seiler-Tuyns	Forgarty Visiting Fellow	LB	NCI
B. Billeter	Forgarty Visiting Fellow	LB	NCI
R. Horlick	Graduate Student	LB	NCI

Project Description:

(1) To prepare ds cDNA probes for the proteins of interest, and to utilize these probes to isolate the genomic sequences for structural studies. These studies primarily involve electron microscopic analysis and sequence analysis of all or part of the transcriptional unit for a given gene.
(2) To define the transcriptional start sites, splice junctions and termination signals for the genes of interest. This information will be used in a comparative study of differentiation specific and "house keeping" sequences.
(3) Some of the ds cDNA probes are to be used in chromosomal localization studies on mouse chromosomes. Several of the structural genes under investigation are highly conserved across species. It is of interest to know if isozymes are proximal or distal on the same chromosome, or on different chromosomes.
(4) When the promotor regions for various genes have been identified, it is intended to analyze promotor function in one of the eukaryotic vector systems now available. We intend to: 1) see if a promotor for a differentiation specific gene, such as α actin, can function when placed in an undifferentiated cell or if differentiation is required for function; 2) define the essential sequence elements for promotor function; 3) determine the role of the intron arrangement in the regulation of gene expression; 4) examine the role of polyadenylation in the regulation of gene expression.
(5) Dr. Seiler-Tuyns who has just joined the laboratory, will examine the optimal parameters for the utilization of the eukaryotic vector systems now available. This will be done with various constructs of the histone H4 gene from mouse which she has characterized in her doctoral studies.

Methods Employed:

Specific ds cDNA probes are being prepared from A^+ mRNA fractionated on preparative methylmercury gels. Fractions enriched for a particular mRNA, as judged by analysis in a cell-free protein synthesizing system, are cloned using standard methods. Clones are identified by positive selection.

The probes are used to screen the charon 4A library of the chicken genome with standard procedures, or with the supressor miniplasmid method of Maniatis. The distribution of the coding information and its polarity within a given isolate is determined by hybridization with kinased RNA and short cDNA. The 5' proximal and 3' proximal fragments in the appropriate restriction digest are sequenced to clearly determine the end points of the transcriptional unit for each gene. Electron microscopic studies give the information on the intron-exon

pattern and the relatedness of the various isolates for a given gene. Restriction fragments containing the promoter regions will be subcloned into one of the eukaryotic vector systems for further analysis.

Major Findings:

Actin. We have characterized the alpha skeletal, alpha cardiac and beta cytoplasmic actin genes in the chicken by restriction enzyme analysis and nucleotide sequence of the five prime end of the structural portion of the gene (the amino terminus is unique for each actin). Our nucleotide sequence studies reveal that the alpha skeletal actin and alpha cardiac actin polypeptides are initially translated in vivo with Met-Cys residues in the amino terminal position. These two amino acids are apparently cleaved off and the third residue (an Asp in both cases) is acetylated to give the mature amino terminus, and acetylated Asp, as determined by protein sequence analysis. The Met-Cys is lacking in the beta actin gene and only the Met is apparently cleaved prior to acetylation to give the acetylated Asp characteristic of the mature protein. The reason for this difference is under study. We have developed an assay to directly monitor processing. Each gene is single copy in the chicken.

Each actin gene has been subcloned into the pRSV-CAT vector in order to study its regulation on a plasmid during myogenesis in the mouse muscle C2 cell line. Transient expression is monitored with chicken specific probes prepared from the 3' noncoding portion of each actin gene. Time course studies with the parent vector indicate that CAT activity (chloroamphenicol acetyltransferase) increases in a linear fashion for at least 96 hours through the complete differentiation cycle, thus actin expression can be normalized to CAT transcription and activity. Both single cells and fused myotubes express CAT activity as judged by immunofluorescent studies with CAT antibody. If the genes do not regulate on the RSV-CAT plasmid, we are also preparing stable transformants with the sibling vector, pSV2-GPT. Vimentin. Vimentin is a single copy gene in the chicken which is transcribed in vivo to yield two distinct size classes of transcripts. Both transcripts are functional in vitro and direct the synthesis of the same polypeptide as judged by two dimensional gel analysis. Sequence analysis of the three prime end of the gene and of various cDNA clones which overlap this region indicates there are four polyadenylation signals clustered in pairs approximately 300 nucleotides apart. Analysis of either total or polyA RNA extracted from muscle or other tissues demonstrate the same transcripts are present in all tissues expressing vimentin. S1 analysis of either total or polyA RNA suggests three of the four adenylation signals are utilized in vivo. The function of the different size classes of vimentin mRNA is under investigation.

Pyruvate kinase. We have utilized a new approach for the synthesis of enriched cDNA libraries to isolate a 3200 base pair cDNA clone for pyruvate kinase. The clone has been identified by its ability to select an mRNA which directs the in vitro synthesis of a polypeptide immunoprecipitable with antibody to PK; it has the same molecular weight and pI as authentic PK. Complete nucleotide sequence of the cDNA clone reveals a 1700 base pair nontranslated 3' region. Comparison of the coding sequence with known sequence from the adult isoform indicates no homology, suggesting we have cloned a different isoform. Northern analysis reveals a 3400 base pair mRNA which increases over 50 fold in chicken muscle

post hatch, as expected for the PK enzyme. Common restriction sites between the cDNA and genomic clones are colinear.

Myosin light chains 1 and 3. The two light chain polypeptides appear to be encoded in a single gene since we have isolated a cDNA clone with no open reading frame which selects the mRNA encoding light chains 1 and 3, as judged by two dimensional gel analysis of the cell-free products. Amino acid analysis has previously shown each light chain has a common carboxy terminus but a different amino terminal half. We have isolated the gene for the light chain 1-3 polypeptides and preliminary analysis indicates the coding information is distributed over 15 kilobases even though the polypeptide molecular weight range is 17,000 to 25,000 daltons. New enriched cDNA libraries have been prepared in an effort to obtain complete cDNA clones for the light chains. These are to be used to analyze the organization of the gene.

Histone H4 gene. The mouse histone H4 gene is expressed in cell cycle dependent fashion. Regulation is thought to occur at two levels: transcriptional rate and message stability changes with position in the cell cycle. In order to investigate this in more detail we have created H4 variants with defined structural alterations. Initially, linkers were introduced into the 5' noncoding, the coding, and in both regions to provide a set of linker scanner mutants as well as markers to distinguish the variant from the endogenous gene when transfected into L cells. Some of the stable transformed L cell lines prepared for study were analyzed at various stages of the cell cycle. The endogenous gene is cell-cycle regulated but the one mutant studied to date, containing a linker both in the coding and noncoding region, is not regulated. Other isolated are being analyzed. Major structural variants of the H4 gene have also been prepared and include: addition of the vimentin adenylation cluster to either side of the 3'-stem-loop terminator in the histone gene, insertion of the human beta globin major intron within the body of the coding portion of the H4 gene, and replacement of the histone promotor with the mouse beta globin promoter. These will be put into L-cells and mouse C2 muscle cells and analyzed for cell-cycle regulated expression. The muscle cells will provide a perminant post mitotic state in which to study expression.

Acantha amoeba myosins. This protozoan synthesizes three myosin polypeptides of different molecular weight, as determined by the analysis of proteins extracted from the cells. Preliminary studies suggest they are different polypeptides. John Hammer from De. Ed Korn's lab has undertaken to clone these myosin genes inorder to study their structure. His preliminary work has shown the myosins are the products of different genes as each myosin polypeptide can be synthesized in vitro and immunoprecipitated by specific antibody. He has prepared high molecular weight DNA and is going to construct a genomic library in the Karn phage 2001. This library will be screened with cDNA clones and fragments of the myosin gene from C. elegans.

Projected Course of Research:

We intend to analyze the organization, structure, and regulation of constitutive and differentiation specific genes.

Publications:

Zehner, Z.E. and Paterson, B.M.: Characterization of the chicken vimentin gene: Single copy gene producing multiple mRNAs. Proc. Natl. Acad. Sci. USA 80: 911-915, 1983.

Musti, A.M., Zehner, Z., Bostian, K.A., Paterson, B.M. and Kramer, R.A.: Transcriptional mapping of two yeast genes coding for glyceraldehyde 3-phosphate dehydrogenase isolated by sequence homology with the chicken gene. Gene, in press, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05261-04 LB

PERIOD COVERED

September 30, 1982 to October 1, 1983

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

Measuring Malignancy of Human Cancer

PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.)
(Name, title, laboratory, and institute affiliation)

Cecil Fox Senior Scientist LB NCI

COOPERATING UNITS (If any) Institute for Histology, Karolinska Institute, Stockholm Sweden: Department of Otolaryngology, Walter Reed Army Medical Center: Department of Gynecological and Breast Pathology, AFIP, Department of Internal Medicine, Georgetown University, Department of Chemical Pathology, AFIP, Lab. of Pathology, NCI

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Office of the Chief

INSTITUTE AND LOCATION

DCBD, NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	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 standar unreduced type. Do not exceed the space provided.)

This program develops objective measurements of the malignancy of human cancers. It has the following goals: 1) To develop new grading systems for human cancer based on retrospective studies of histopathological material. 2) To find standardized methods for preparation of histopathological specimens for morphometry, stereometry, and image analysis. 3) To establish objective methods to discriminate between cell populations from hyperplasias, carcinomas in situ, and invasive carcinomas. 4) To study the mechanics of attachment of malignant cells to substrates and the influence of purified tissue and basement membrane components on attachment and cell spreading. 5) Understanding the mechanisms of cytoskeletal organization occurring in cell attachment and metastasis. 6) To identify indicators of malignancy such as monoclonal antibody detection of oncogene protein products or other tumor associated antigens and by using retrospective studies, establish their relevance to cancer management.

PHS 6040 (Rev. 1/83) GPO 895-106

Project Description:

Objective: Estimating the malignant potential of human cancers of epithelial and mesothelial tissues has traditionally been entirely subjective. This project intends to use objective measurements of tissue and cellular tumor components to add to the armamentarium of signs employed by pathologists in estimating malignancy. The project is a broad one and involves both morphometric and analytical components. The ultimate objective then is to improve the data base available to clinicians in treating human disease.

Methods Employed: The methods employed will be described under each study heading.

Study 1: To develop grading systems for human cancers using a retrospective human tissue repository collection.

The Armed Forces Institute of Pathology is a tissue repository of nearly two million specimens. This study uses the tissue retrieval system to collect demonstrative material and case histories for retrospective study. Tissues are being analyzed for characteristics known to be subjective markers of malignancy and results will be compared to outcome of the clinical disease. Three major types of tissues are being studied: malignant and premalignant lesions of human breast, malignant tissues from head and neck tumors, and normal and abnormal tissues of endometrium. Parameters now being used are nuclear morphometry, quantitative nuclear DNA, cytoplasmic volume and cell density. Measurements are performed by image digitization, scanning and point photometry and calculation of cell density. The head and neck tumor study includes tissues before and after chemotherapy and radiotherapy to estimate effectiveness of therapy in altering cellular populations.

Study 2: For nearly one hundred years pathological preparations have been killed and fixed in 4% aqueous solutions of formaldehyde, yet the kinetics and morphological effects of this process are poorly understood. This study describes the mechanism of formaldehyde action on tissues with regard to rates of penetration, covalent bond formation, and morphological effects. Specific morphological effects being studied are texture patterns of nuclear preservation, cytoplasmic volume, shrinkage and the marked disruption of cytological mitochondrial membranes by 1.33M formaldehyde as studied by a new method of transmitted light scanning interferrometry. The physical chemistry of aqueous formaldehyde in buffers and varying amounts of formaldehyde are being studied with nuclear magnetic resonance as is spontaneous oxidation of formaldehyde to formic acid. Tissue-formaldehyde kinetics using labeled formaldehyde are used to determine temperature and concentration effects on covalent bond formation. Ionic and osmotic pressure of various fixative buffer combinations have been studied to establish optimal formulations. Other processing effects such as temperature of paraffin embedding and interlaboratory variations in tissue processing have also been analyzed.

Study 3: The specific problem of differentiating between endometrial hyperplasia by quantitative morphometry has been tested and multivariate discriminate analysis has been found to be effective in discriminating between endometrial hyperplasia and carcinomas of the endometrium have been obtained in

about 80% of the cases analyzed. This technique is being expanded to include additional parameters such as DNA per unit of nuclear area. These parameters are also being applied to carcinoma in situ and dyplasias of other lesions such as Bowenoid hyperplasia.

Study 4: Cell attachment and cytoskeletal organization are being studied in relation to the presence of basement membrane purified proteins, by reflection contrast microscopy, transmitted scanning interferrometry and scanning electron microscopy. Proteins being studied are purified collagens I-IV, high density substrate which has been absorbed on specially prepared glass substrates. The following features are identified as they occur: formation of close contacts, formation of focal contacts, organization of microfilaments, retraction of the nucleus from the 200nm focal plane of the microscope, extension of lamellar cytoplasm and reduction of the cellular profile. The different protein substrates produce different sequences of events which are being recorded by low light video time lapse recording and on time lapse films. Low and high level metastatic cell lines are being compared with normal cells as are events in attachment of human epidermal cells.

Study 5: This is an extension of Study 4 and explores the organization of the cytoskeleton in cell attachment and movement as revealed by the techniques outlined above. Cytoskeletal extensions after organization of attachments are being disrupted by cytochalasins and vincristine to establish sequences of cellular organization and to determine the reversibility of differentiation of cellular organization.

Study 6: This consists of exploratory studies of other markers and mechanisms in oncogenesis. Methods have been developed to discriminate between unadorned asbestos fibers and ferrugenous bodies in human lung. A technique has been developed for rendering cellular and tissue components invisible through manipulation of the refractive index of the mounting medium of tissue sections while retaining visualization of the fibers by dark-field and phase microscopy. Using a programmable motor driven microscope stage, a predetermined area of known volume may be scanned and the numbers of adorned and naked fibers may be determined. This gives a total number of carcinogenic sized fibers per cubic volume of lung. These results will allow comparison of lung tissue to determine if there is a relationship between ferruginous body formation in lung and probability of carcinogenesis. An attempt is being made to identify gene product of oncogenes in formalin fixed tumor tissue. While monoclonal antibodies are preferable for these studies, affinity purified antibodies may also be effective. Fluorescence of gene product will be detected by low light video techniques. Other tumor associated antigens may also be studied by these techniques.

Significance: Epithelial cancer in humans seems to be part of the human estate since it is an age related disease that has afflicted humans throughout the course of our history. If this is true, the most important task in cancer research for the immediate future is to improve diagnosis so that cancer may be detected at a stage more amendable to treatment and to provide the clinician with information that will give specific information as to the biological portents of individual tumors. More appropriate therapy may then be instituted. It is to the second of these points that this research is directed: toward

generating information about what may be expected of cancer in individual patients. These modest goals seem to be a pragmatic approach to the immediacy of the cancer problem.

Proposed Course of Research: Because of the diversity of projects under way, the research must of necessity be adventitious. The Department of Defense has been generous in providing help in the form of DOD Summer Interns for Studies 1, 2, and 3 and as have other departments of the AFIP. Currently, all of the projects listed are proceeding at about the same rate, or at roughtly one-fifth full-time per project.

Publications:

Colgan, T.J., Norris, H.J., Foster, W., Kurman, R.J., and Fox, C.H.: Predicting the outcome of endometrial hyperplasia by quantitative analysis of nuclear features using a linear discriminate function. Int. J. Gynec. Path., 1: 347-352, 1983

Fox, C.H.: Caveat Emptor. (Guest Editorial). J. Histotechnology, in press

Green, J., Abler, T., Shotkin, S., Whiting, J.D., Hughes, R.O., Calvert, R.C., Johnson, F.B., and Fox, C.H.: Variation in formaldehyde fixation in routine histopathology laboratory. J. Histotech., in press

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05262-03 LB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Eukaryotic Gene Regulation: The Metallothionein System

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Dean H. Hamer, Senior Staff Fellow, LB, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Cellular Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.0	6.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The heavy metal induction of metallothionein synthesis is a useful model system for studying eukaryotic gene regulation. Metallothionein genes from mice and humans have been cloned and characterized. The DNA sequences involved in heavy metal induction have been analyzed by in vitro mutagenesis and gene transfer techniques. Gene dosage experiments suggest that induction requires a positive transcriptional activator, and approaches to isolating this factor are being explored. Metallothionein gene sequences have also been used to develop an efficient, versatile set of mammalian cell expression vectors.

PHS 6040 (Rev. 1/83) GPO 895-100

OTHER INVESTIGATORS:

G. N. Pavlakis	Fogarty Fellow	LB NCI
A. D. Carter	Guest Worker	American Cancer Society
M. F. Jubier	Guest Worker	CNRS
B. Felber	Fogarty Fellow	LB NCI
A. Leone	Fogarty Associate	LB NCI
C. S. Schmidt	Graduate Student	FAES
M. J. Walling	Chemist	LB NCI

PROJECT DESCRIPTION:

Objectives:

We wish to understand how eukaryotic genes are activated or repressed under different cellular conditions.

Methods Employed:

Our general strategy is to use recombinant DNA, biochemical and genetic techniques to identify the regulatory DNA sequences and the cellular factors with which they interact.

Major Findings:

1) Regulation of Metallothionein Gene Expression

Metallothionein (MT) synthesis is an especially useful system for studying eukaryotic gene regulation. These small, cysteine-rich proteins have been found in all eukaryotes examined, ranging from yeast to man, and are expressed in many different organs and cell types. MT gene expression is inducible by the same heavy metals to which the MT proteins bind. This homeostatic regulatory mechanism plays a critical role in detoxifying toxic ions, such as cadmium and mercury, and may also play a role in the metabolism of essential metals such as copper and zinc. We are using recombinant DNA, biochemical and genetic techniques to analyze the mechanism of this response in normal and diseased cells.

A) Structure and Chromosomal Distribution of Primate MT Genes

We previously isolated and sequenced two MT cDNA clones from cadmium-resistant monkey kidney cells. One of these represents simian MT-II whereas the second corresponds to MT-I or a related variant protein. These clones were used as probes to isolate 9 different phage λ clones carrying human genomic MT sequences. One of these corresponds to the functional human MT-II gene, while three other clones appear to contain MT-I-like genes or pseudogenes. Gel transfer hybridization experiments show that the human genome contains at least 12 different fragments that cross-hybridize to MT probes.

In collaboration with W. McBride, we have studied the distribution of these fragments in a series of human-hamster and human-mouse somatic cell hybrids. The initial results indicate that these sequences are dispersed on several different chromosomes.

B) Regulatory Sequences of the Mouse MT-I Gene

The presumptive promoter and control sequences of the mouse MT-I gene were fused to the E. coli galactokinase gene and inserted into an SV40 vector. Cultured cells transfected with this recombinant synthesize bacterial galactokinase and the enzyme level is induced 5-fold to 20-fold by cadmium. Analysis of a large series of deletion and "linker-scanning" mutants shows that two distinct regions of DNA are involved in the transcription of this gene: an upstream activator region that determines the efficiency of expression and a more distal control region required for heavy metal inducibility. The control region contains evolutionarily conserved primary and potential secondary structural features. Point mutants have been constructed to directly test the role of these sequences.

C) MT Regulation in Menkes' Cells

Menkes' kinky hair syndrome is an x-linked human genetic disease leading to abnormal hair coloration and structure, mental retardation and early death. Other investigators have shown that the underlying cause of this syndrome is a deficience in circulating copper, probably due to overproduction of MT in the kidney and cells lining the gut. It has also been demonstrated that cultured fibroblasts from Menkes' patients exhibit elevated basal levels of MT. We have obtained several such cultured lines and have shown, by gel transfer hybridization, that their MT genes are indistinguishable from those of normal cells. We also know, from our examination of somatic cell hybrids, that none of the MT genes are localized on the X chromosome. Therefore, it appears likely that Menkes' results from a mutation that effects MT synthesis in trans. We are examining the possible mechanism of this disorder by gene transfer experiments.

D) Regulatory Factors

Nothing is known about the presumptive cellular factors that interact with the MT control sequences and increase transcription in the presence of heavy metals. Although we have not yet succeeded in developing an in vitro assay for such factors, we have been able to perform two indirect experiments that speak to their nature. First, we have introduced the MT-galactokinase fusion gene into a series of cultured hamster cell lines (obtained from C. Hildebrand) that synthesize various levels of MT mRNA and protein. No consistent variations in the expression of the fusion gene were observed, indicating that MT gene transcription is not autoregulated. Second, we have performed "transfection titration" experiments in which we observe the effect of adding excess MT regulatory sequences on the transcription of the MT-galactokinase fusion gene. We find that excess regulatory sequences cause a

specific decrease in the induced level of expression. This suggests that MT regulation is controlled, at least in part, by a positively acting transcription factor.

E) MT in Yeast

The yeast Saccharomyces cerevisiae synthesizes a small, cysteine-rich protein that binds copper ions. The synthesis of this protein, which is encoded by the Cup1 locus, is dramatically increased when copper is added to the culture medium. Because of the obvious parallels with the mammalian MTs, and because of the tremendous technical and genetic advantages of yeast as an experimental organism, we have initiated an analysis of the Cup1 locus. In collaboration with T. Butt and J. Gorman, we have fused the Cup1 promoter to the E. coli galactokinase gene and have introduced the recombinant molecule into a yeast transformation vector. We will try to use this construct to select for mutants that fail to appropriately regulate Cup1 transcription. If successful, this will allow as to clone the regulatory genes, identify their products and, ultimately, study their interaction with the control sequences.

F) Bovine Papilloma Virus - MT Recombinants

MT synthesis is inducible by glusocorticoids as well as heavy metals. We constructed bovine papilloma virus recombinants carrying a hybrid gene in which human growth hormone structural sequences are fused to the promoter and presumptive control region of the mouse metallothionein-I gene. Mouse cells transformed with the recombinants synthesize metallothionein-growth hormone hybrid mRNA with the same 5' end as metallothionein mRNA. Hybrid mRNA is inducible by cadmium but not by dexamethasone, whereas the chromosomal metallothionein genes in the same cells are inducible by both agents. This indicates that heavy metals and glucocorticoids regulate the mouse metallothionein-I gene by independent mechanisms.

We also found that the transformed cells secrete exceptionally high levels of appropriately processed growth hormone polypeptide. This suggested that bovine papilloma virus - MT vectors might be useful for overproducing other eukaryotic proteins that must be post-translationally modified to become biologically active. Accordingly, we constructed a series of vectors that allow essentially any coding sequence to be placed under the control of the MT promoter. One such vector has been used, in collaboration with N. Hsiung and R. Fitts, to produce hepatitis B surface antigen particles. We have also constructed vectors containing the intact mouse MT-I gene and have shown that they confer cadmium resistance to transformed cells. We are attempting to use this dominant selection scheme to broaden the host range of bovine papilloma virus vectors.

2) Expression of an α-fetoprotein "minigene" in an SV40 vector (in collaboration with P. Young, R. Scott and S. Tilghman

A mouse α-fetoprotein "minigene", lacking 12 internal exons, was intro-

duced into cultured monkey cells on an SV40 vector. The minigene was transcribed from the normal initiation site and the transcripts were appropriately spliced and polyadenylated. In addition, a 220 nucleotide transcript was detected and mapped to a mouse Alu-like or B1 repeat on the opposite strand to that encoding the α-fetoprotein gene in the first intron.

3) Protein Production

See Section F above.

4) RNA Splicing

This project has been completed.

5) tRNA Processing

This project has been completed.

Publications:

Hamer, D.H. and Walling, M.J.: Regulation in vivo of a cloned mammalian gene: Cadmium induces the transcription of a mouse metallothionein gene in SV40 vectors. J. Mol. Appl. Genet. 1, 273-288, 1982.

Felber, B.K., Orkin, S.H. and Hamer, D.H.: Abnormal RNA splicing causes one form of α-thalassemia. Cell 29, 895-902, 1982.

Hizuka, N., Hedricks, C., Pavlakis, G.N., Hamer, D.H. and Gorden, P.: Properties of hGH polypeptides: purified from pituitary extracts and synthesized in monkey kidney cells and bacteria. J. Clin. Endrocin. Metab. 55, 545-550, 1982.

Young, P.R., Scott, R.W., Hamer, D.H. and Tilghman, S.M.: Construction and expression in vivo of an internally deleted α-fetoprotein gene: presence of a transcribed Alu-like repeat within the first intervening sequence. Nucl. Acids Res. 10, 3099-3116, 1982.

Pavlakis, G.N. and Hamer, D.H.: Regulation of a metallothionein-growth hormone hybrid gene in bovine papilloma virus. Proc. Natl. Acad. Sci. USA 80, 397-401, 1983.

Pavlakis, G.N. and Hamer, D.H.: Expression of cloned growth hormone and metallothionein genes in heterologous cells. In Recent Progress in Hormone Research, in press, 1983.

Schmidt, C.J. and Hamer, D.H.: Cloning and Sequence analysis of two monkey metallothionein cDNAs. Gene, in press, 1983.

Hamer, D.H.: Production of post-translationally modified proteins in the SV40 Monkey Cell System. In Experimental Manipulation of Gene Expression, in press, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05263-02 LB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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 on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

C. Wu Visiting Associate, LB, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

National Cancer Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	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.)*

The sequential arrangement of nucleosomes along the chromatin fiber is punctuated by highly nuclease-sensitive sites. We previously mapped such sites to the 5' terminus of several heat shock genes in Drosophila by a novel indirect end-labeling technique. Such preferentially accessible sites in chromatin may function as points of entry to the DNA for RNA polymerase and control proteins. In order to locate specific sequences to which these proteins might be bound, we have recently used indirect end-labeling in a new way to identify short, nuclease-resistant DNA sequences, and have found such "footprints" within hypersensitive sites in chromatin. To determine the functional relationship of 5' terminal hypersensitive sites in chromatin to gene activity, we have made a promising start in the development of an in vitro transcription system from Drosophila nuclei.

PHS 6040 (Rev. 1/83) GPO 895-100

OTHER INVESTIGATORS:

T. Paisley	Biologist	LB	NCI
Z. Krawczyk	Exchange Scientist	LB	NCI
B. Wood	Lab. Worker	LB	NCI

Project Description:

Objectives:

A knowledge of gene regulation is fundamental to an understanding of eukaryotic development and differentiation. We study gene regulation by probing the structure of DNA associated with proteins in chromatin. We have shown that the 5'-terminal and flanking sequences of Drosophila heat shock genes is uniquely accessible in chromatin to a nucleolytic probe, DNase I. We aim to analyze further the structure and function of nuclease hypersensitive sites in chromation.

Major Employed and Major Findings:

We are concentrating on studying the chromatin structure of the heat-inducible genes in Drosophila which encode the 70K, 83K, 68K, 27K, 26K, 23K and 22K heat shock proteins. Cloned DNA representing these genes have been generously provided by members of Dr. M. Meselson's laboratory, and by Dr. E. Craig. The fine structure of the DNase I hypersensitive sites in the chromatin around these genes are being mapped by the indirect end-labeling method which we developed previously. We are now separating the partially cleaved DNA segments on long agarose gels, which can give a length determination precise to 5-10 bp in the 1 to 2 kilobase pair range.

For biological material we have established a vigorous Drosophila mass population of 10^5 flies (Oregon R/P2 strain) which is being maintained continuously. The population yields about 150 g of Drosophila embryos per two week cycle. We also make use of Drosophila tissue culture cell lines grown in spinner culture.

Using data obtained from the fine structure map of the DNase I hypersensitive sites, we are developing a new footprinting technique which seeks to identify short sequences within the nuclease hypersensitive region to which putative eukaryotic regulatory proteins may bind when they activate or repress gene transcription. This technique stems from the fact that when procaryotic activators or repressors bind to promoter or operator sequences, they shield them from nuclease cleavage. Thus we are using the indirect end-labeling method to identify short nuclease-protected DNA sequences in chromatin. This complements our earlier use of indirect-end labeling for the identification of nuclease-sensitive sites in chromatin.

Our preliminary findings with this method indicate the presence of short (20-60 bp) DNA sequences flanking the 5' end of the hsp 70 and hsp 83 genes,

which are protected from cleavage by a variety of nucleases, including restriction enzymes. The appearance of some of these protected sequences correlates with the onset of gene activation, suggesting that these "footprints" could be due to the binding of putative activator proteins.

As an initial step in determining the functional importance of nuclease hypersensitive sites in chromatin we are developing an in vitro transcription system from Drosophila nuclei. We use γ-S-ribonucleotides as precursors for transcription and we selectively purify newly initiated transcripts on Hg-agarose columns. So far we have found that the nuclear transcription system does generate RNA molecules that are selectively bound to the Hg agarose column in a reproducible manner. We have shown by dot blot analysis of this Hg-bound RNA that hap 70 RNA is initiated and elongated when nuclei from heat induced cells were incubated in the transcription reaction.

Significance to Cancer Research:

Our work contributes to a broad effort in the study of gene regulation during normal development and differentiation, and will serve as a basis for the study of aberrant cellular functions which result in neoplasia.

Proposed Course of Research:

We plan on refining the nuclease protection technique and extending it to the rest of the heat shock genes, and to other Drosophila genes. We plan to refine the nuclear transcription system further and perform critical controls concerning the fidelity of the heat shock transcripts. We then plan to see how perturbation of the nuclease hypersensitive sites in chromatin will affect transcriptional efficiency in chromatin.

Publications:

Wu, C.: An exposed chromatin structure at the 5' end of eukaryotic genes. In: O'Malley, B. and Fox, C.F. (eds): ICN-UCLA Symposium on Molecular and Cellular Biology, Vol. XXVI, New York, Academic Press, pp. 147-156, 1982.

Wu, C.: Chromatin structure of Drosophila heat shock genes. In: Ashburner, A., Tissieres, A. and Schlesinger, M, (eds): Heat Shock Induction of Proteins. Cold Spring Harbor Publication, New York, pp. 91-97, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB05264-02 LB

PERIOD COVERED: October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Characterization of a Mouse Repetitive Gene Family

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

PI: K.K. Lueders Chemist LB NCI

COOPERATING UNITS *(if any)*: S. Segal, Nucleic Acid Enzymology Section, LB/DCBD
B. Paterson, Developmental Biochemistry Section, LB/DCBD

LAB/BRANCH: Laboratory of Biochemistry

SECTION: Biosynthesis Section

INSTITUTE AND LOCATION: DCBD, NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

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

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

We have used restriction analysis, heteroduplex formation, and thermal stability studies to identify and characterize a family of interspersed repetitive sequences from the mouse genome. The repeats are about 400 base pairs long and represent 1-2% of mouse genomic DNA. The sequences are found at variable distances on the 5' as well as 3' sides of genes. Related sequences are present also in the rat, Syrian hamster, and monkey genomes. Comparison of the sequence of one element with other known repetitive elements has revealed that it is a member of the R-sequence family. Computer analysis of the sequence has revealed an RNA polymerase II promoter, polyadenylation signal, and homology with the enhancer/activator region of the SV40 72 bp repeat. Experiments are in progress to determine whether the R-sequence family members function as promoters of transcription, and whether they can enhance the transcription of sequences under control of other promoters. To look at these questions, we have made constructs containing R-sequence and a vector carrying the bacterial gene chloramphenicol acetyl transferase which upon transfection into mammalian cells can be used in a transient expression assay to measure promoter and enhancer functions.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Investigators:

E.L. Kuff Chief, Biosynthesis Section LB NCI

Objective: We woud like to determine whether members of the R-sequence family have any functional role related to RNA transcription in mammalian cells.

Methods and Results: We have used restriction analysis, heteroduplex formation, and thermal stability studies to identify and charaterize a family of interspersed repetitive sequences from the mouse genome. The repeats are about 400 base pairs long and represent 1-2% of mouse genomic DNA. The sequences are found at variable distances on the 5' as well as 3' sides of genes. Related sequences are present in the rat, Syrian hamster, and monkey genomes. Structural and evoltionary studies have been completed.

Sequencing (by Dr. Bruce Paterson) of one element selected by virtue of its ability to promote transcription of a bacterial gene, has been completed. Comparison of the sequence with other known repetitive elements has shown that it is a member of the R-sequence family. The sequenced element has been tested by Dr. Shoshana Segal using a permanent transformation assay for promoter activity in mammalian cells. Computer analysis of the sequence for transcriptional control regions has revealed an RNA polymerase II promoter, polyadenylation signal, and homology with the enhancer/a ctivator region of the SV40 72 bp repeat.

Proposed Course: We would like to determine whether the ability to function as promoter of RNA transcription in mammalian cells is a general property of the R-sequence family, and whether R-sequences can enhance the transcription of genes under control of other promoters. To look at these questions, we have obtained vectors containing the bacterial gene chloramphemicol acetyl transferase, which upon transfection into mammalian cells can be used in a transient expression assay to measure promoter and enhancer activity. We have used these vectors to prepare constructs containing an R-sequence in positions which will permit us to assay for both functions. The main focus of future experiments will be on defining the functional role of R-sequence elements with these assays.

Significance: Certain sequences such as the long terminal repeats of retroviruses have been shown to be capable of modifying expression of cellular genes by their promoter and/or enhancer functions. It would be of interest to determine whether repetitive classes of sequences such as the R-family, which contain comparable signal regions, have similar functions.

Publications: Lueders, K.K. and Paterson, B.M. (1982) Nuc. Acids Res. 10: 7715-7729.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05265-01 LB

PERIOD COVERED

January 1, 1983 to September 30, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

P. Wagner, Guest Researcher, LB, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Macromolecular Interactions Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.35	0.75	0.60

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Cytoskeletal proteins and their regulation by Ca^{2+} are being studied to understand the role of these proteins in cell motility. The interactions of actin with smooth muscle and nonmuscle myosins are regulated by phosphorylation of one of the myosin subunits by specific Ca^{2+} calmodulin dependent kinases. Turbidity changes, ultracentrifugation, and electron microscopy were used to determine the effect of this phosphorylation on myosin filament formation. While phosphorylation does effect the degree of filament formation, the type of anion used to adjust the ionic strength is equally important, and it is unlikely that this is the principle mechanism by which phosphorylation regulates the participation of these myosins in force development. The effects of this phosphorylation on myosin subunit interactions is also being examined. Brain spectrin, fodrin, appears to link the cytoskeleton to the cell membrane. The affinity of fodrin for calmodulin has been estimated using fluorescently labeled calmodulin. The binding of actin to fodrin and its effect on calmodulin binding are being investigated. The binding of fodrin to actin is inhibited by myosin, and fodrin modulates the actomyosin ATPase.

PHS 6040 (Rev. 1/83) GPO 895-100

OTHER INVESTIGATOR:

B. Keene Microbiologist LB NCI

PROJECT DESCRIPTION:

Objectives:

To understand the role of cellular actins and myosins, their interactions with each other and with the cell membrane, the regulation of these interactions, and how they relate to the various motile activities of cells.

Methods Employed:

Standard protein isolation techniques are used to prepare actin, myosin and other cytoskeletal proteins from various types of muscles and from thymus and brain. Characterizations of their interactions are done using enzymatic assays, fluorescence spectroscopy, ultracentrifugation, and electron microscopy.

Major Findings:

Currently two aspects are being pursued, regulation of smooth muscle and nonmuscle myosins by phosphorylation of their 20,000 molecular weight subunits, P-light chains, and characterization of a spectrin like protein isolated from brain which may link the cytoskeleton to the cell membrane.

The P-light chains of smooth muscle and nonmuscle myosins are phosphorylated by specific Ca^{2+} calmodulin dependent kinases and dephosphorylated by Ca^{2+} insensitive phosphatases. In vitro this phosphorylation has been shown to effect filament formation; in 0.15 M KCl and MgATP, nonphosphorylated myosins are monomeric while the phosphorylated myosins are filamentous. If myosin filaments are required for force development, this could be a mechanism by which Ca^{2+} release controls the contraction of smooth muscles and the motile activity of other cells. However, electron micrographs have shown that relaxed smooth muscles contain many myosin filaments even though the myosins were not phosphorylated. We have reconciled this discrepancy by showing that the inability of nonphosphorylated myosins to form filaments in vitro is due to the use of KCl. When potassium propionate was used to adjust the ionic strength, most (80%) of the nonphosphorylated myosins and nearly all the phosphorylated myosins were in filaments. The equilibrium between monomeric and filamentous myosins is effected by both phosphorylation and specific anions. The chloride concentration inside cells is low, and in smooth muscle conditions must be such that even the nonphosphorylated myosins are mostly polymerized. Experiments are being performed to determine if a similar equilibrium exists for nonmuscle myosins. Myosin isolated from calf thymus is being used.

Fodrin or brain spectrin is a large asymmetric protein which binds both to actin and to the cell membrane. Proteins immunologically related to fodrin are found in many cell types. We have found that fodrin can both stimulate and inhibit the actin activated ATPase of myosin and that the binding of myosin to actin inhibits fodrin binding. Gel overlays and affinity chromatography have shown Ca^{2+} dependent binding of calmodulin to fodrin. The binding of fodrin to calmodulin affinity columns is weaker than that of other calmodulin binding proteins. Dansyl labeled calmodulin has been used to determine the affinity of calmodulin for fodrin. The fluoresence maxima of dansyl labeled calmodulin is shifted 10 nm by the addition of fodrin and there is a 25-40% increase in fluoresence intensity. An association constant of $3x10^6$ M^{-1} has been calculated.

Significance to Biomedical Research and the Program of the Institute:

Cell movement, endocytosis, capping of cell surface receptors, cell division and a variety of other cell activities are thought to rely on forces generated by the interactions of cytoplasmic actins and myosins. These interactions are in part regulated by Ca^{2+}. The experiments reported here are designed to help in the understanding of the roles of cytoskeletal proteins in these motile activities.

Proposed Course:

The regulation of smooth muscle and nonmuscle myosins by phosphorylation will be further characterized in vitro. The extent of phosphorylation of both the myosin P-light chains and heavy chains in vivo and whether they relate to the level of motility will be examined using cells grown in culture. An attempt will be made to determine whether the Ca^{2+} dependent binding of calmodulin to fodrin effects either its binding to actin or to the cell membrane. Fodrin appears to be phosphorylated by a Ca^{2+} dependent kinase. The effect of this phosphorylation will be examined and the level in vivo determined using neuronal cells grown in culture.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05266-01 LB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Regulation of the Immunoglobulin Gene Family

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Cary Queen, Senior Staff Fellow, LB, NCI

COOPERATING UNITS *(if any)*

Dr. David Baltimore, MIT

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Macromolecular Interactions Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We are studying the regulation of expression of the immunoglobulin gene family by attempting to answer two questions: (1) why do only cells of the B-lymphoid lineage synthesize immunoglobulins, (2) how do these cells transcribe only one or a few immunoglobulin genes, while leaving hundreds of other, similar immunoglobulin genes inactive? Our approach to these questions is to insert a cloned, rearranged kappa light chain gene into a plasmid in various configurations, to transfect the plasmid into various types of cells, and to determine whether the transfected gene is transcribed. We have shown that the complete kappa gene is transcribed after transfection into antibody-producing myeloma cells but not in non-lymphoid 3T3 or L cells. Hence the different cell types are able to appropriately regulate the kappa gene even when not in its usual chromosomal environment. By deleting different parts of the cloned gene, we have shown that certain sequence elements actually downstream of the promoter are necessary for its transcription in myeloma cells. Investigations are under way to further characterize these sequence elements and their mode of action.

PHS 6040 (Rev. 1/83) GPO 895-100

OTHER INVESTIGATORS:

S. Segal	Expert	LB	NCI
J. Stafford	Microbiology Technician	LB	NCI

PROJECT DESCRIPTION:

Objectives:

To study the regulation of the immunoglobulin gene family. In particular, to learn about the DNA sequences and protein factors that activate expression of immunoglobulin genes.

Methods Employed:

The project involves in vitro modifications of a cloned immunoglobulin gene. The modified genes are transfected into cultured cells of various types by the DEAE-dextran method. RNA is extracted from the cells and assayed by the S1 nuclease method to determine whether the immunoglobulin gene is transcribed.

Major Findings:

1) Regulation of the immunoglobulin gene family

Each mammalian cell contains several hundred immunoglobulin genes, which encode the proteins that constitute antibodies. Only cells of the B-lymphocyte class (B-lymphoid cells) express these genes, that is, transcribe them into RNA and translate the RNA into immunoglobulin proteins. Moreover, out of the large repetoire of immunoglobulin genes, each antibody-producing cell generally expresses only one light chain gene and one heavy chain gene. It is of fundamental importance to the study of the immune system, and to the study of gene regulation during development, to learn why only certain cells express immunoglobulin genes, and how these cells are able to transcribe only two of them while leaving many similar genes inactive.

Our approach to this problem is to focus on one particular immunoglobulin gene, cloned from the mouse, that synthesizes a kappa light chain. This gene has been inserted on a plasmid that also contains a large part of the animal virus polyoma, allowing it to replicate in mouse cells. For one set of experiments, a second gene was inserted on the same plasmid. This gene was unrelated to the immunoglobulin system and, therefore, should be expressed at equal levels in B-lymphoid and non-lymphoid cells. The final plasmid, designated pLX31, was transfected into two standard non-lymphoid lines of mouse cells, 3T3 and L, and one antibody-secreting lymphoid line, MPC 11. As determined by an S1 nuclease assay of extracted RNA, the second gene was transcribed at approximately equal levels in all three lines of cells, as expected. This

proves that the plasmid pLX31 was able to penetrate all of the cell lines in a transcribable state. However, the kappa immunoglobulin gene on the plasmid was only transcribed in the lymphoid MPC 11 cells, with no detectable transcription in the non-lymphoid 3T3 and L cells.

Two conclusions relevant to gene regulation during development can be inferred from this cell-type specific expression of a transfected kappa gene. First, since the DNA template is in the form of an unintegrated plasmid, the level of kappa gene activity is not controlled by chromosomal location or large-scale chromosome structure. Second, since the kappa gene was transfected into terminally differentiated cells, activation or inactivation of this gene does not depend on modifications made to it during the process of cell development. Rather, the already differentiated cells contain all the information needed to appropriately regulate the expression of new copies of the kappa gene.

The experiments described above begin to address the problem of why only certain cell types express immunoglobulin genes. To address the complementary problem of why given cells express only certain immunoglobulin genes, we have conducted experiments to locate the DNA sequences in our cloned kappa chain gene that activate its transcription. In collaboration with D. Baltimore, we removed the 3' half of the gene, coding for the carboxyl part of the protein. The truncated gene, contained in a plasmid, was no longer transcribed after transfection into lymphoid cells. This suggests that the 3' half of the gene contains information required to initiate transcription of the gene.

More recently, by deleting different amounts of the gene, we have sought to more precisely localize the required sequences. We have now localized the sequences to within 200 base pairs, inside the gene's second intron. The required region is notable because it is the only part of the intron that is conserved between the mouse, rabbit and human genes. Hence, it appears that the required sequences have been preserved during evolution.

Significance to Biomedical Research and the Program of the Institute:

The immunoglobulin gene family produces the proteins that constitute antibodies, a crucial aspect of the body's defense against infectious diseases and probably cancer. Understanding how these genes are regulated, especially how they are activated, is therefore of great potential utility in controlling disease processes.

Future Course of Research:

We will attempt to locate more precisely, at the nucleotide level, the DNA sequences involved in regulating expression of immunoglobulin genes. We will also use the differential expression of transfected immunoglobulin genes in lymphoid and non-lymphoid cells to develop an assay for the protein factors that activate transcription of immunoglobulin genes.

Publications:

Queen, C. and Baltimore, D.: An immunoglobulin gene is activated by downstream sequences. Cell, in press, 1983.

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

PROJECT NUMBER
Z01CB08701-09 LB

PERIOD COVERED
October 1, 1982 to September 30, 1983

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

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
M. Rosenberg, Research Chemist, LB, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Cellular Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

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

B

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

We have been investigating nucleic acid structure involved in the control of transcription initiation and termination as well as in translational expression of various RNAs. Using recombinant DNA techniques we have been able to examine the relationship between regulatory function and DNA sequence in a variety of cellular and viral operon control regions. The characterization of mutants which affect both transcriptional and translational expression in these regions has afforded information on precise molecular events which lead to control element function. We have used a variety of defined prokaryotic genes to examine the requirements for translation in both eukaryotic cells and cell-free systems. Recombinant DNA techniques have been used to develop plasmid, phage and bacterial vector systems which allow the isolation, characterization, and comparison of prokaryotic transcriptional regulatory signals. This system has been adapted to allow efficient expression of a variety of prokaryotic and eukaryotic gene products in E. coli. Efficient expression of the E. coli galactokinase gene attached to a mammalian virus vector has been obtained within mammalian cells. This vector has allowed us to study transcriptional and translational regulatory elements which function in eukaryotic cells.

OTHER INVESTIGATORS:

M. Reff	Sr. Staff Fellow	LB	NCI
A. Shatzman	IPA	Georgetown University	
D. Sobieski	Chemist	LB	NCI
H. Johanson	Visiting Fellow	LB	NCI
G. Ramos	Lab Worker	LB	NCI
A. Chepelinksy	Visiting Scientist	LB	NCI
B. Ferguson	Staff Fellow	LB	NCI

PROJECT DESCRIPTION:

Objectives:

1. To examine the relationship between function and DNA structure involved in the control of transcription initiation and termination of RNA synthesis, as well as in the translational expression of these mRNAs in both prokaryotic and eukaryotic systems.

2. To study the role of transcription termination and RNA processing in the control of phage and bacterial gene expression.

3. To develop the ability to express prokaryotic and eukaryotic genes of interest at high efficiency in bacterial cells.

4. To develop new techniques which can be applied to the above studies and to the general examination of nucleic acid structure in other biological systems.

Methods Employed:

Purification of specific RNA transcripts synthesized either in vivo or in vitro; nucleic acid sequence analysis of both RNA and DNA; primed extension of RNA transcription products with deoxyribonucleotides; isolation of protein factors involved either in the processes of transcriptional termination or post-transcriptional modification of RNA; isolation of purified DNA restriction enzymes and their employment in obtaining specific purified fragments of both viral and cellular DNAs; recombinant DNA techniques.

Major Findings:

1. The nucleic acid structure of the 3'-terminal regions of several RNA transcripts synthesized in vitro and in vivo from both phage and bacterial systems are being examined. These RNAs represent both independently terminated transcription products as well as transcripts requiring the protein factor, Rho, for their termination. Sequence analysis at the 3'-ends of some of these products indicated:

a) that all of the transcripts examined possess similar degrees of 3'-terminal sequence heterogeneity which consisted predominantly of the addition of 1 to 5 adenylate residues to the 3'-terminus of the transcript and
b) that rho factor enhanced termination results in a definite structural change in the nucleotide sequence with which an RNA molecule can terminate.

In addition, we have developed a new methodology for examining the nucleic acid structure in the untranscribed portion of the DNA immediately adjacent to the 3'-terminus of an RNA transcript. This technique has been applied to the determination of sequence information throughout the entire termination region of these transcription products. Little structural homology is apparent in the template DNA beyond the 3'-ends of these transcripts. The lack of homology suggests that this region might not be important to the termination process. Thus, the majority of the encoded termination signal is transcribed into RNA.

We determined the DNA sequence surrounding and examined termination at a totally rho dependent site (t_{R1}) in bacteriophage λ. The DNA composition at t_{R1} is 75% AT base pairs and termination occurs heterogeneously over four adjacent base pairs. No GC rich or U rich sequence is found at the 3'OH end of the RNA. This sequence is quite different from other transcripts that terminate independently of Rho. However, there is a stable base-paired stem and loop structure near the end of this RNA.

In the absence of Rho factor with normal transcription conditions (i.e. 37° and high triphosphate levels), RNA polymerase undergoes a substantial pause at this site. A mutation, cnc, that interferes with base pairing in the stem and loop, has two effects on transcription: RNA polymerase does not pause in the absence of Rho and transcription does not terminate in the presence of Rho. This suggests that the stem and loop induces RNA polymerase to pause, and that a paused polymerase is important for Rho action.

The entire intercistronic region in which this termination site is positioned was more extensively characterized. A number of related transcriptional and translational regulatory elements have been defined in this region. More mutations affecting terminator function have been characterized. In addition, we have defined a site (NutR) located immediately preceding the terminator which is apparently involved in anti-termination function. We have shown that the viral function, N, acts in some way to prevent termination (i.e. anti-terminate) at the t_{R1} site thereby allowing for the quantitative and temporal control of transcription through this intercistronic region (i.e. transcription attenuation). Our data indicate that the NutR site comprises an ~17 nucleotide sequence which displays a hyphenated two-fold rotational symmetry. This site is in some way involved in N protein recognition prior to the action of N at the various downstream termination sites.

We have also initiated detailed studies on another phage λ termination signal t_I. This site is involved in the regulation of the phage λ gene int.

Lambda has evolved a rather remarkable regulatory mechanism which utilizes overlapping and alternative signals for transcription termination and RNA processing to control int expression from the two different mRNAs. We have characterized the biochemical steps involved in this regulatory phenomenon. Presently, we are introducing mutation into this region in an effort to discern the importance of individual nucleotides to the regulatory mechanism operating to control int expression.

2. The galactose operon of E. coli is subject to both negative control by gal repressor and positive control by cAMP and its receptor protein (CRP). We have sought to define the nucleotide sequence of the promoter-operator sites responsible for gal regulation. Analysis of transcripts of restriction fragments combined with direct DNA sequencing enabled us to derive the sequence for the gal regulatory region. Cleavage by restriction endonuclease Hinf abolishes cAMP-CRP dependent, but not CRP independent, transcription. Formation of a cAMP-CRP dependent preinitiation complex prevents cleavage by Hinf. Thus the region upstream of the Hinf site is important for cAMP-CRP stimulation of transcription. This region contains sequence similarities with the CRP recognition site of lac. One gal operator constitutive mutant is resistant to Hinf cleavage suggesting the operator may include at least part of the Hinf site. Further analyses are required to confirm this possibility.

More recently we have demonstrated in vitro the existence on the plasmid pBR322 of a promoter signal that is strictly dependent on cAMP and its receptor protein CRP. Transcription initiates with pppG at nucleotide 2270 and proceeds counterclockwise on the standard pBR322 map. DNase protection studies show that CRP selectively binds to the -35 region of the promoter. This region exhibits strong structural homologies to the binding sites of other CRP-dependent promoters.

In addition, we have been using an RNA-dependent E. coli S30 translation system to examine the translational efficiencies of in vitro synthesized mRNAs. Because the products of both the transcription and translation reactions can be separately quantitated, the amount of protein produced per mole of a specific mRNA can be determined. Using this method, we have compared the relative translational efficiencies of two different mRNA transcripts of the E. coli galactose operon: the CRP-cAMP dependent mRNA (P1) and the CRP-cAMP independent mRNA (P2). Our results show that the P2 mRNA translates epimerase, the 5' proximal gene product of the gal operon, 4x more efficiently than does the P1 transcript, while the 5' distal gene product, kinase, is translated with equal efficiency. Since the P2 transcript differs from the P1 transcript only by the addition of 5 nucleotides at the 5' terminus, and these nucleotides are outside of the ribosome binding region for epimerase, the selective difference in the translational efficiency of epimerase may be mediated by RNA conformation. It is known that in cells deficient in cAMP, the ratio of epimerase to kinase is about 4x higher than in cells containing cAMP - a phenomenon called discoordinate expression. Moreover, cells deficient in cAMP are thought to produce only gal P2 mRNA, whereas cells with cAMP produce only P1 mRNA. Thus, discoordinate expression is explained by our observation that the P2 transcript produces 4x

more epimerase, but a similar amount of kinase, then does the P1 transcript.

These studies have been extended to examine the apparent coupled expression of the two distal genes of the gal operon, transferase (galT) and kinase (galK). Using recombinant DNA techniques we have introduced a number of defined frameshift and nonsense mutations upstream of the galT/galK intercistronic boundary. This has allowed us to manipulate precisely the sites at which upstream translation terminates with respect to the galK initiation codon. The results obtained with these constructed mutations confirm that galT and galK translation are naturally coupled and allow us to assess the consequences of upstream translation on the expression of an adjacent gene.

We are presently examining the effects of coupled expression on a variety of other operons. We are synthetically coupling the expression of one gene to that of another placed upstream. We wish to determine if coupling is a general mechanism which can be applied to obtaining increased expression of gene products.

3. Earlier work utilizing defined bacterial and phage mRNAs transcribed in vitro from a variety of lambdoid phages, investigated the relationship between the modification of 5' triphosphate end of these transcripts with a 7-methylguanosine moiety (i.e. "cap" structure) and the translational efficiency of these prokaryotic mRNAs in wheat germ cell-free extracts. Our results demonstrated an almost absolute requirement for the "cap" structure to obtain efficient translation of the prokaryotic transcripts. All the structural information necessary for proper and efficient recognition and translation of prokaryotic mRNAs using eukaryotic components is encoded in the prokaryotic transcript except for the presence of the 5' 7-methyl-guanosine modification. This implies strong evolutionary constraints on the RNA structure which is used for ribosome recognition and translation initiation, as well as similar constraints on those parts of the ribosome which must interact with this RNA structure.

We have now been able to achieve efficient expression of the E. coli galactokinase gene directly within mammalian cells. The Escherichia coli galactokinase gene (galK) was inserted into a modified early region transcription unit of simian virus 40 (SV40) contained on a bacterial plasmid. Introduction of this pSVK vector into monkey, mouse, and hamster cell lines by transfection resulted in efficient expression of the bacterial galK gene. This expression was shown to be dependent upon fusion of the galK gene to the early promoter of SV40 and did not appear to require SV40 splice signals. Moreover, expression in these cells could be obtained either transiently, 24-72 hr after transfection, or continuously, after stable transformation. In particular, pSVK-dependent galK expression was obtained in a hamster cell line genetically deficient in galactokinase activity. Expression of the bacterial enzyme was shown to complement the galactosemic defect of these cells, thereby allowing their selective survival and growth on galactose as the only carbon source. The ability to readily assay, select for, and potentially select against galK expression from pSVK and its derivatives

should prove extremely useful in studying eukaryotic gene regulatory signals.

These studies have now been extended by constructing a vector system carrying two independent gene transcription units which function in a variety of eukaryotic cell systems. Each transcription unit controls the expression of a different assayable, genetically selectable gene function. One is the E. coli galactokinase gene (galK) and the other is the E. coli xanthine-(guanine)-phosphoribosyltransferase gene (xgprt). The modular design of the vector system allows each of the regulatory elements controlling galK expression to be removed selectively and replaced by alternative DNA segments. Any vector changes which result in corresponding changes in levels of galK expression are measured accurately using xgprt expression as an internal standard. Measurements can be made transiently, 24-48 hours after DNA transfection, or upon stable introduction of the vector DNA into the host genome using the appropriate selective conditions. Both galK and xgprt activity are assayed from the same cell lysate using either a starch gel separation procedure or a more rapid filter assay technique. We have used the system to study and characterize a variety of gene regulatory elements including promoters, polyadenylation signals, splice signals, and translational control elements. We report here the use of this vector system to study the effects on gene expression which result from changes introduced into the 5' noncoding leader region of the galK transcription unit. We varied the length of this leader region and found no effect on galK expression, provided that the galK initiation codon remained the first ATG in the transcription unit. Using synthetic linkers, we then inserted specific sequences containing a single ATG codon at defined positions within the leader region. Our results indicate that the various ATG inserts had widely different effects on galK synthesis and that the sequences surrounding the newly inserted ATG codons determined the magnitude of these effects. Assuming that the observed interference with galK expression correlates with the relative ability of these different ATG sequences to be recognized by the translation machinery of the cell, then our assay is discerning between those ATG containing sequences which are well-recognized (i.e. highly interfering with galK expression) and those which are not (i.e. little effect on galK expression). In addition, the upstream inserted ATG condons were placed in each of the three possible translation reading frames positioned before the galK gene. Translation occurring in each of these frames terminates prior to, within, or downstream of the galK initiation codon, respectively. Our data indicate that a poorly recognized ATG containing sequence has little effect on translation initiation at the galK initiation codon irrespective of its reading frame. In contrast, an upstream ATG containing sequence which dramatically interferes with galK translation (i.e. is well-recognized) exerts its greatest effect when it translates out-of-frame through the galK initiation codon into the galK gene.

4. The lambda cII gene product is known to be required in vivo for transcriptional activation of both the lambda repressor gene (cI) and the integrase function (int). This activation is essential for phage lysogenic development.

We have selectively cloned the lambda cII gene onto a pBR322 derivative such that cII expression is under the control of the lambda P_L promoter. Transformation of this plasmid into certain bacterial hosts results in high level expression and accumulation of the cII product (~1% of total cellular protein). This single polypeptide product was purified to > 95% homogeneity (in mg quantities).

Standard in vitro transcription reactions were then carried out in the presence and absence of the purified cII protein using as templates appropriate lambda DNA fragments which contain the promoter sites for repressor (P_{RE}) and integrase (P_I) synthesis. Polyacrylamide gel and fingerprint analyses were used to characterize the RNA transcription products and their 5' start-sites. The results indicate that cII protein alone allows RNA polymerase to bind selectively and initiate transcription from these two promoter sites. In the case of the P_{RE} promoter, cII dependent polymerase binding and transcription were eliminated by using DNA templates which contained various cY point mutations.

We have purified the phage lambda transcriptional activator protein cII. The procedure described allows cII to be obtained in both high purity and yield, and thus allows detailed physical and chemical analysis. We demonstrate that cII in solution is a tetrameric protein and that it undergoes specific processing at its N-terminal end. In addition the protein is characterized as to its molar extinction coefficient, molecular weight, amino acid composition, isoelectric point, α-helical content, and antigenic capability.

In vitro transcription experiments carried out with purified cII indicate that the purified protein selectively activates transcription from the two phage promoters, P_E and P_I. Apparently, cII was both necessary and sufficient to activate both promoters. Protection studies were used to demonstrate that cII was a DNA binding protein which interacted specifically with the -35 region of P_E and P_I promoter. Mutant analysis and methylation protection studies indicated that cII recognized a repeat sequence on one face of the DNA helix. It now seems likely that two identical dimer subunits of the cII tetramer recognize and bind the repeating DNA structure in the major groove on one face of the DNA helix, and that this interaction allows RNA polymerase to recognize the -35 region sequence of the promoter positioned between the repeat sequences.

5. We have developed a recombinant vector system which can be used to isolate, compare and characterize essentially any promoter or terminator signal recognized by E. coli RNA polymerase. The system utilizes plasmid, phage and bacterial vectors and provides extreme flexibility in that any construction made with the plasmid vector can be readily transferred to the phage vector or into the bacterial chromosome. Thus the regulatory signal of interest can be studied in both multiple and single copy. This system is being used to study several specific phage and bacterial promoter and terminator regulatory

signals with particular emphasis on the introduction of mutations into these sites and the functional characterization of the effects of these base alterations.

These same studies have also been applied to the development of several plasmid vectors which allow the efficient, regulated expression of certain prokaryotic and eukaryotic genes in E. coli.

Future Course:

All research projects will terminate.

Cancer Research:

Understanding the mechanism by which gene expression is regulated is central to an understanding of cancer.

Publications:

Zasloff, M., Santos, T., Romeo, P. and Rosenberg, M.: Transcription and precursor processing of normal and mutant human $tRNA_1^{met}$ genes in a homologous cell free system. J. Biol. Chem., 257: 7857-7863, 1982.

Ho, Y.S., Lewis, M. and Rosenberg, M.: Purification and properties of a transcriptional activator: the cII protein of phase λ. J. Biol. Chem., 257: 9128-9134, 1982.

Rosenberg, M., McKenney, K., Schümperli, D.: Use of the E. coli. galactokinase gene to study prokaryotic and eukaryotic gene control signals. In Promoters: Structure and Function, Praeger Scientific Publishing Co., M. Chamberlin and R. L. Rodriquez, eds., 387-406, 1982.

Bedouelle, H., Schmeissner, U., Hofnung, M. and Rosenberg, M.: Protomers of the malEFG and malK-lamB operons in Escherichia coli K12. J. Mol. Biol., 161: 519-531, 1982.

Rosenberg, M. and Schmeissner, U.: Regulation of gene expression by transcription termination and RNA processing. In Interaction of translational and transcriptional controls in the regulation of gene expression, Elsevier North Holland, NY, B. Safer and M. Grungerg-Manago, eds., p. 1-16, 1982.

Schümperli, D., McKenney, K., Sobieski, D.A. and Rosenberg, M.: Translational coupling at an intercistronic boundary of the Escherichia coli galactose operon. Cell, 30: 865-871, 1982.

Zasloff, M., Rosenberg, M. and Santos, T.: Imparied nuclear transport of a mutant human $tRNA_1^{met}$. Nature, 300: 81-84, 1982.

Rosenberg, M., Ho, Y.S. and Shatzman, A.R.: The use of pKC30 and its derivatives for controlled expression of genes. In Methods in Enzymology, Academic Press, R. Wu, ed., in press, 1983.

Wulff, D. and Rosenberg, M.: The lysogenic program of bacteriophase lambda; establishment of repression. In Bacteriophase Lambda II, Cold Spring Harbor Laboratory, R. Hendrix, J. Roberts, F. Stahl and R. Weissberg, eds., in press, 1983.

Shatzman, A., Ho, Y.S. and Rosenberg, M.: Using phage λ regulatory signals to obtain efficient expression of genes in E. coli. In Experimental Manipulation of Gene Expression, Academic Press, M. Inouye, ed., in press, 1983.

Young, J.F., Desselberger, U., Graves, P., Palese, P., Shatzman, A. and Rosenberg, M.: Closing and expression of influenza virus genes. In Origin of panademic viruses (W.G. Laver, ed.) Elsevier Science Publications, Inc., New York, in press, 1983.

Ho, Y.S. and Rosenberg, M.: Transcriptional activity (cII) binds a -35 region repeat sequence on one face of the DNA. Nature, in press, 1983.

LABORATORY OF PATHOPHYSIOLOGY
ANNUAL REPORT SUMMARY
October 1, 1982 to September 30, 1983

The research effort of the Laboratory of Pathophysiology is focused on the mammary gland and mammary carcinomas. In the past years several other investigators with diverse research interests, were administratively included in the laboratory. During the 1982-83 Fiscal Year the laboratory has been reorganized to include only the mammary group, that is involved in 7 projects:

1. Gene organization of secretory proteins in normal and neoplastic mammary epithelium (P. R. Qasba, H. L. Nakhasi and coworkers)

The major objective of this project is to establish whether neoplastic transformation of mammary epithelium alters the organization of genes involved in the production of secretory milk proteins.

Molecules similar to mammary gland α-lactalbumin but distinct in their ability to modify the specificity of galactosyltransferase have been found in the epididymal fluid. This activity differs from mammary gland α-lactalbumin in that it transfers glactose from UDP-galactose not only to glucose but also to myo-inositol with equal efficiency. In the male reproductive tract where lactose is absent and free glucose levels are very low, α-lactalbumin-like activity may modulate sperm surface glycoproteins and may be an important factor in cell-to-cell interaction.

α-lactalbumin bears a structural homology with lysozyme, an enzyme catalyzing the hydrolysis of a 1-4 glucosilic linkage in polysaccharides. The complete nucleotide sequence of the rat α-lactalbumin gene carrying the coding and intervening sequences, including its 5'-flanking region has been established. Comparison of this gene structure with chicken egg white lysozyme gene shows: (a) both genes contain 3 introns at similar positions, (b) the first 3 exons of the two genes show high nucleotide homologies and are of comparable length, and (c) the fourth exon of α-lactalbumin, which codes for the amino acids essential for its interaction with glactosyl-transferase, is markedly different from the corresponding exon of lysozyme.

Galactosyl-transferase has been purified from human and rat milk using two rapid chromatography steps. The human milk enzyme showed a single band of MW54K on SDS-polyacrilamide gel, the rat milk enzyme showed 3 polypeptide bands of MW59K, 54K and 27K. Antisera against the 54K form immunoprecipitated the other forms. The antisera against the rat enzyme did not cross-react with the human galactosyltransferase.

To understand the modulation of galactosyltransferase activity, essential for generating specific cell surface antigenic determinants, isolation and sequence analysis of the cDNA clones corresponding to galactosyl-transferase is being pursued. To date a library of cDNA clones from rat lactating mammary gland has been generated.

The characterization of whey-acidic protein is continuing. The protein has been isolated from rat milk and sequenced. The protein sequence show the following unique features: (a) High content of cysteine, glutamic acid, aspartic acid and serine, but lack of tyrosine. Half-cysteines appear in unique arrangements and are repeated in two domains of the protein. The cysteine arrangements show striking similarities with that of neurophysins and certain lectins such as Con A and wheat germ agglutinin. (b) The second domain has also similarities with the second domain of the protease inhibitor for red-sea turtle.

2. Cyclic nucleotides in growth regulation of mammary tumors (Y. S. Cho-Chung and coworkers)

The major objective of this project is to investigate the effects of factors such as cyclic nucleotides and hormones on growth and regression of mammary carcinomas.

In past work on this project it was found that protein kinase type II increases sharply during regression of hormone-dependent mammary carcinomas. Monospecific antibodies were obtained against the regulatory subunits of type I and II protein kinase derived from bovine skeletal and heart muscle. These antibodies cross-reacted with the regulatory subunits of MCF7 human mammary carcinoma line. In the nuclei of growing MCF7 tumors the antiserum precipitate proteins of 47,000, 44,000 and 35,000 dalton cAMP receptor proteins but in regressing tumors two new receptor proteins appeared (50,000 and 52,000) that were specifically precipitated by the anti-receptor II antibody. Comcomitant with the appearance of these two proteins the 35,000 dalton protein disappeared from the nuclei. Indirect immunofluorescence revealed that during regression the nuclei of MCF7 tumors become very rich in type II cAMP-dependent protein kinase.

In previous work we observed that treatment with DBcAMP (N^6,d^2 dibutyryl cyclic adenosine 3',5' monophosphate) prevented carcinogenesis by 7-12 DMBA. This action appears to be due to inhibition of the covalent binding of 7-12 DMBA to DNA of mammary epithelium. As a consequence, the in vitro translation products of poly(A)+RMAs from mammary gland showed a difference in 4 protein bands of the electrophoretic pattern.

Since the inappropriate expression of some transforming gene might be responsible for neoplastic transformation, efforts to evaluate the role of the ras gene expression is ongoing. The expression of the ras gene is studied in growing and regressing mammary tumors, in hormone-dependent versus hormone-independent mammary tumors, in the mammary gland with dysplastic-hyperplastic lesions or undergoing chemical carcinogemesis. Factors that affect phenotypic reversion of transformed cells are also being analyzed in cell line 433 of NIH 3T3 cells containing the transforming ras gene of Harvey sarcoma virus flanked by LTR of MMTV which acts as promoter and is influenced by glucocortocoids. The controlling effects of cyclic AMP on 433 phenotype and p 21 production is being investigated.

As an extension of the finding that cyclic AMP binding proteins are involved in the growth of rodent mammary tumors, a clinical application is being pursued at the diagnostic level. A study is ongoing in which the ratio of steroid receptor to cyclic AMP binding proteins is being used to discriminate between hormone dependent and independent tumors.

3. Growth and diffentiation of normal and neoplastic mammary cells (W. K. Kidwell and coworkers)

The major objective of this project is to evaluate the role of the microenvironment in the growth and neoplastic transformation of the mammary epithelium. Production of faulty basement membrane has been found to cause regression of mammary tumors. The production of basement membrane appears to be controlled by 3 different conditions: (a) modulation of collagen turnover which is suppressed by glucocortocoids, (b) increased synthesis as a consequence of cell contact with "foreign" substrate, and (c) stimulation by growth factors and hormones. One growth factor has been isolated and partially purified from rat, mouse and human milk. This factor is produced by the mammary cells and stimulates them to produce more collagen. The factors from human milk and human mammary tumors have the same PI and are probably identical. A similar factor is produced by MCF7, a human mammary carcinoma.

Azitidine carboxylate, thioproline and cis-hydroxyproline have been utilized to block proline incorporation into collagen and were found to substantially reduce cell growth both in vivo and in vitro.

It was not possible to show that cells plated on collagen substrate could be rescued from the killing effects of proline analogs, however, proline had a growth stimulatory effect on the tumor cells.

Experiments concerning the interaction of mammary epithelium and adipocytes revealed that mammary epithelium is dependent on essential fatty acids for proliferation and that prolactin stimulated epithelium, recruits the fatty acids from the adipocytes. Mast cells are involved in the signal via histamine release that triggers release of fatty acids from adipocytes. Some of these fatty acids are transformed into PGE_1 that is a potent growth stimulator of mammary epithelia.

The possibility of obtaining clinically useful compounds was pursued in the studies of compounds able to inhibit poly(ADP-ribose)synthetase, a chromatin bound enzyme that is involved in DNA repair. Six compounds were synthetized and the ability of 4 of them to block DNA repair was found to directly correlate to their inhibitory action on poly(ADP-ribose)synthetase.

4. Effects of prolactin on mammary and ovarian cells (R. A. Knazek and coworkers)

The major objective of this work is to evaluate the influence of cell membrane-associated receptors with consequent changes in the response of the target tissue.

In previous work it was shown that the number of detectable prolactin receptors is controlled in vivo by the level of circulating prolactin or growth hormone by a positive feedback mechanism. Inhibition of prostaglandin synthesis results in a loss of prolactin receptor and prevents their induction by prolactin. The data obtained showed that prolactin up-regulates its receptor by modifying target membrane fluidity via modification of prostaglandin sunthesis. In hormone dependent tumors, the membranes of regressing mammary carcinomas bind less prolactin because they are more viscous than membranes of growing tumors. An assay for prostaglandin receptors has been developed which has demonstrated that regressing tumors have an increased capacity to bind prostaglandin and that copper ions increase the binding capacity.

In line with the results obtained on membrane rigidity, it has been found that patients afflicted with adrenoleukodystropy or adrenomyeloneuropathy have the inborn propensity to accumulate long chain fatty acids in their cellular membranes. Consequently, the fluidity of the erythrocyte membrane was found to be altered and the same may occur in the adrenals and gonads determining adrenal and gonadal failure, common in these patients.

The role of prostaglandins in the ovarian function was also studied using the newly devised assay for prostaglandin. Suppression of endogenous prolactin secretion caused an increase in the number of prostaglandin receptors in ovarian membranes, an effect reversed by replacement of physiologic levels of prolactin. Supraphysiological concentrations of prolactin resulted in a marked decrease in the number of prostaglandin receptors. This suggests that prolactin exerts its effects through the prostaglandin cascade and explains, in part, the mechanism by which infertility occurs in hypoprolactinemic women.

The methodology of cell cultures in artificial capillaries has been further perfected with the objective of making the procedure useful for continuous production of hormones for both laboratory and clinical use. Modifications permit study of nutrients and product transport through the tissue formed in the intercapillary spaces, the effect of lymphatic-type drainage upon cell function and

the use of monoclonal antibodies directed against specific types of neoplastic cells.

5. Growth and development of the mammary gland (B. K. Vonderhaar and coworkers)

The major objective of this project is to understand the role of hormones and various growth factors on mammary gland development, differentiation and neoplastic transformation. Using explants of mid-pregnancy mammary gland in culture it was found that T3 increased α-lactalbumin synthesis but not casein. The activity of lactose synthetase was enhanced but not that of galactosyl-transferase. Lactose production was enhanced by T3 addition to the media.

For casein synthesis the optimal concentration of hydrocortisone in the media was found to be 10^{-6}M while for α-lactalbumin synthesis was between 10^{-8} and 10^{-7}. Higher concentrations inhibited synthesis. Addition of T3 did overcome the inhibitory effect of hydrocortisone on α-lactalbumin secretion but did not on casein secretion. In the presence of T3 the casein secreted by the gland in vitro was not modified but α-lactalbumin found in the gland showed 2 peaks while the secreted α-lactalbumin showed a single peak in SDS-PAGE. Both forms have equal activity in the lactose synthetase assay system; both forms were equally affected in their synthesis and glycosylation by tunicamycin. By the use of endoglycosidase D, endoglycosylase H and mRNA translation in the wheat germ translation system, it was concluded that the 2 forms of α-lactalbumin are not different in size but are different in charge.

Development and function of the mammary gland was studied in vitamin D deficient mice. No major morphological differences were found between the vitamin D deficient and controls, however, serum prolactin levels were decreased 30% in vitamin D deficiency. The glands of vitamin D deficient mice, in organ culture, synthesized 10-20% less α-lactalbumin and 50-60% less casein. The 44K casein was the one maximally affected.

In a comparison of the optimal effects of steroids for milk secretion in cultured mammary explants, it was found that aldosterone and corticosterone in the presence of T3 determine milk protein synthesis in vitro which is qualitatively and quantitatively similar to that obtained in vivo.

In another set of experiments mammary gland explants with aldosterone in the media were used to assess the role of epidermal growth factor and mammary tumor-derived growth factor of Drs. Kidwell and Zweibel on lobulo-alveolar development of the mammary gland. It was found that both factors are involved in the process but the mechanism of their action is not yet clear.

The work on the interaction between lactogenic hormones and their receptors was directed toward an analysis of the stability of the hormone receptor-complex, the nature of the cryptic sites and the effects of alterations in membrane lipids and membrane aggregation. In order to study the regulation of the lactogenic hormone receptor, the purification of the prolactin receptor for human placenta has been initiated using a procedure developed to isolate prolactin binding sites of mouse liver. The work on the characterization of the human prolactin receptor is ongoing.

6. Structure and function of cell membranes (P. Pinto da Silva and coworkers)

The major objective of this project is to define the localization of cell membrane components and to correlate their topology with physiological functions.

A group of projects was concerned with the partition of wheat germ agglutinin receptor sites over the protoplasmic and the exoplasmic faces of several cell types. In the recent past a method called "fracture-label" was developed in the laboratory for this purpose. The following results were obtained: (a) In cultured cells MOLT 4 (from acute lymphoblastic T leukemia) and HUT 78 (from

mycosis fungoides) wheat germ agglutinine (WGA) receptor sites were always localized on the exoplasmic face of the plasma membrane, never on the protoplasmic face. (b) In secretory and non-secretory type cells of rat tissues the distribution of WGA binding sites revealed two compartments; one characterized by absence of WGA receptor includes membrane of mitochondria, peroxisomes, endoplasmic reticular and nuclear envelope, the other, strongly labeled comprises the membrane of lysosomes, phagocytic vacuoles and secretory granules as well as plasma membrane. (c) The glycolipid binding Con A in Acanthamoeba castellanii was abundant over exoplasmic fracture-faces of plasma membranes but absent from protoplasmic fracture-faces. (d) Distribution of Con A and WGA were visualized on the acrosomal membranes of spermatozoa and stable surface domains formed by specific glycoconjugates are primarily conditioned by transmembrane glycoproteins. (e) Differences in Con A and WGA distribution in the membranes of normal and neoplastic cells were not found.

In a second set of experiments tight junctions strands have been induced in toad mammary bladder. Thus, it is possible to artifically induce a characteristic modification of the cell membrane.

In a third group of experiments a method was developed to study the existence and distribution of protein-free spaces in the cytoplasm by the exclusion of probes of known dimensions. The probe utilized is non-cationized ferritin (100 A^{o}). Variations in the cytoplasm permeability to ferritin have been observed in lymphocytes. Cytoplasmic compactness may change during differentiation, and is reduced in growing cells. To increase the possibilities of characterizing cell components, a variation of the freeze-fracture technique is being developed; i.e., a freeze-fracture radioautography.

7. Angiogenesis and neoplastic transformation (P. M. Gullino and coworkers)

The objective of this project is to establish the conditions necessary for a cell to acquire the ability to induce new formation of vessels from the surrounding tissue. The reason for this effort is based on the observation that cells normally not able to be angiogenic, are at much higher risk of neoplastic transformation when they acquire angiogenic capacity.

The evaluation of substances able to induce neovascularization has been completed with the documentation that several chemically defined effectors can be angiogenic and that copper ions are indispensable for angiogenesis to occur.

A group of experiments was performed that demonstrated the appearance of chemotactic factors for capillary endothelium in rabbit corneas treated with angiogenesis effectors.

Purification and characterization of the chemoattractant is underway. In another group of experiments it has been shown that capillary endothelium migrates preferentially on a fibronectin substrate and that antifibronectin serum can block endothelial cell migration and impair angiogenesis. The importance that this observation may have on tumor growth is being evaluated.

Dr. Shelby L. Berger has been assigned to another unit, however, her work has been done in the Laboratory of Pathophysiology for most of 1982-83 and the summary of this work is included:

The synthesis of single-stranded globin cDNA by the RNA-directed DNA polymerase activity of reverse transcriptase in the presence of oligothymidylate primers was investigated in order to determine the limitations to higher yields. The results indicated that the associated ribonuclease H activity, an integral part of reverse transcriptase, plays a large role in the synthesis of the first strand of cDNA and that the interplay of the two enzyme activities for any

specific set of conditions determines the yield of single-stranded products. In both the presence and absence of polymerization, the associated ribonuclease H catalyzed the deadenylation of mRNA, producing molecules that were somewhat shorter, highly homogeneous in size, and fully translatable into globin protein. They were also entirely lacking in the ability to serve as templates for cDNA synthesis. The reaction was completely dependent on oligothymidylate and completely independent of deoxyribonucleoside triphosphates. The initial rate of deadenylation was one-fourth the initial rate of initiation of polymerization when saturating levels of deoxyribonucleoside triphosphates were used in the polymerase reaction. In the presence of ribonuclease H activity, the DNA polymerase catalyzed the synthesis of an array of cDNAs including some that were full length. The initiation of polymerization was rate limiting: once synthesis had begun, it required 1-1.5 min to transcribe globin mRNA. However, most primers that were elongated were aborted prematurely. Maximum synthesis of full-length cDNA required stoichiometric levels of enzyme and high triphosphate levels, but regardless of conditions, the sum of completed cDNA and deadenylated mRNA accounted for only 50% of the input mRNA. The data fit a model in which synthesis of full-length cDNA molecules depends on the arrangement of primers and transcription initiation complexes on the poly(A) "tail" of mRNA. A mRNA library from human T-lymphocytes activated for differing periods with several mitogens has been prepared.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05211-11 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Poly(ADP-ribose) and Chromatin structure and Function.

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: W.R. Kidwell Chief, Cell Cycle Regulation Section, LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cell Cycle Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

Poly(ADP-ribose) synthetase is a chromatin bound enzyme that adds chains of ADP-ribose in tandem to nuclear proteins. This enzyme is activated by DNA damaging agents such as gamma, x-ray and u.v. irradiation and by DNA alkylating agents. We have synthesized and tested 6 compounds which are inhibitors of the synthetase and found that the ability of 4 of 6 of them to block DNA repair is directly correlated with the compound's potency as a synthetase inhibitor. The compounds ranked in order of their ability to block DNA repair are 3-acetl-aminobenzamide> 3-hydroxybenzamide= benzamide>>3-aminobenzamide. 3-nitroben-amide, was found to be much more inhibitory for the repair of DNA chain breaks than was expected based on its potency as a poly(ADP-ribose) synthetase inhibi-tor. This compound appears to block DNA repair indirectly via its potency as an RNA synthesis inhibitor. Indeed, all of the benzamides were found to block RNA synthesis to some extent but this characteristic was not related to the potency of the compound as a synthetase inhibitor. Activity of the benzamides to block RNA synthesis was linearly related to the electron withdrawing potency of the substituent group at the 3 position of the benzamide ring-i.e., to the Hammet's constant. 3-methoxybenzamide was found to be a potent synthetase inhibitor but was a relatively poor inhibitor of DNA repair. Differences in benzamide analog effects on DNA repair may be partly a consequence of differ-ences in analog uptake. They are probably not due to pertubation of nucleotide triphosphate pools in the cells since the most potent synthetase inhibitor, 3-acetylaminobenzamide at very high concentrations had no effect on the nucleotide triphosphate level in cells. Whether or not the benzamides block DNA repair via their inhibition of poly(ADP-ribose) synthetase is not clear. However, the com-pounds may be clinically useful since they potentiate the cell killing effects of chemotheraputic agents such as bleomycin, adriamycin, NMU and methylmethane sulfonate, agents that cause DNA damage.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: M. R. Purnell Visiting Fellow LPP, NCI

Project Description

Objectives:

A series of benzamide analogs which are variably capable of blocking poly(ADP-ribose) synthetase activity are being evaluated for their effects on cell growth, cell cycle arrest and for effects on DNA repair processes. These experiments are designed to elucidate the role of poly(ADP-ribose) in cell function.

Major Findings:

Poly(ADP-ribose) synthetase inhibitors block DNA repair. A variety of benzamides substituted in the 3'-position were synthesized and their potency as synthetase inhibitors tested. These were 3-acetylaminobenzamide, k_i = 0.43 μmolar; 3-methoxybenzamide, k_i = 0.61 μmolar; benzamide=3-hydroxybenzamide, k_i = 1.0 μmolar; 3-amino-benzamide, k_i = 2.8 μmolar; 3-nitrobenzamide, 9.8 μmolar. These compounds were tested for their ability to block the repair of γ-ray induced DNA chain breaks by measuring the effect of the compounds at various concentrations on the single strand break repair rate as determined from alkaline elution of DNA through polycarbonate filters. First order rate constants for the elution of DNA were determined and these were converted into RAD equivalents of DNA breaks. Plots of the reciprocal of the repair rate vs the concentration of the synthetase inhibitor normalized against its k_i were constructed. These results showed that at low inhibitor concentrations the ability of all the benzamides (except 3-nitrobenzamide and 3-methoxybenzamide) to block DNA repair was directly correlated with the potency of the compound as a synthetase inhibitor. Poly(ADP-ribose) synthetase inhibitors block cell division and reduce cell viability. Cytotoxicity of the benzamide analogs were assessed by treating cells for two cell doubling times with various concentrations of the compounds followed by cloning efficiency measurements in the absence of further analog exposure. Plots of the cloning efficiency vs inhibitor concentration normalized against its k_i were constructed. The results indicated that the benzamide analogs caused a loss of cell viability. At all inhibitor concentrations, the benzamides apparently produce these effects on cell viability by blocking poly(ADP-ribose) synthetase. An evaluation of the cytostatic potential of the compounds indicated that cell division could proceed in the absence of poly (ADP-ribose) synthesis but that cumulative damage to cells leading to cell death resulted after 2 cell doublings. One compound, 3-acetylaminobenzamide was studied in detail. It was found to produce a cell growth arrest in the G_1 and in G_2. This compound which is the most potent synthetase inhibitor synthesized to date may be very useful for assessing the role of poly(ADP -ribose) in cell cycle progression. Poly(ADP- ribose) synthetase inhibitors may block DNA repair indirectly rather than via inhibiting synthetase. ^{3}H-uridine uptake into RNA was found to be dramatically and rapidly affected by the synthetase inhibitors. The most potent inhibitor was 3-nitrobenzamide. Plots were made of the election withdrawing potency of the election withdrawing potency of the 3-'substitutents of the analogs (Hammet constant) and the

% inhibition of RNA synthesis and these demonstrated a linear relationship. A plot of the RNA synthesis rate vs inhibitor concentration normalized against k_I indicated that the effect of the benzamides in blocking RNA synthesis was probably not due to the effect of these compounds on poly(ADP-ribose) synthetase since the curves obtained with individual inhibitors were not superimposable. Poly(ADP-ribose) synthetase inhibitors synergistically act with DNA damaging agents to kill cells. Although their mechanism of action may be independent of effects on poly(ADPribose) synthetase, the benzamides potentiated the killing effects of NMU, bleomycin, methylmethane sulfonate and adriamycin. These effects were seen concentrations of benzamides which were by themselves not appreciably cytotoxic or cytostatic. The synergistic killing potential was inversely propartional to the k_I of the benzamides except for 3-nitrobenzamide and 3-methoxybenzamide, the former being more potent and the latter less potent than predicted from their k_I for synthetase.

Significance to Biomedical Research and the Program of the Institute:

Evidence has accumulated which suggests that poly(ADP-ribose) synthetase plays some important role in the maintenance of the fidelity of chromatin structure. The enzyme is activated by a variety of agents that cause DNA damage and becomes deactivated when the damage is repaired. This fact has suggested that chemotheraputic agent action may be potentiated by compounds that block poly(ADP-ribose) synthetase and prevent DNA repair and indeed, two recent reports from T. Sugrimura's Laboratory support this postulate. The purpose of our studies is to develop selective inhibitors of the synthetase which are innocuous by themselves but which enhance cell killing by chemotheraputic agents which cause DNA damage.

Proposed Course of Research:

A series of new inhibitors will be synthesized with sugar substituents at the 3'-position of the benzamide ring. The cytotoxicity, cytostatic action and synergistic killing potencies will be assessed in vitro and in experimental animals carrying transplantable tumors and treated ∓ bleomycin.

Publications:

Kidwell, W.R., Nolan, N. and Stone, P.R.: Variations of poly(ADP-ribose) and poly(ADP-ribose) synthetase in synchronously dividing cells. In Hayaishi, O and Ueda, K. (Eds.): ADP-ribosylation Reactions, Academic Press, Inc., New York, 1982. pp 374-387.

Kidwell, W.R. and Purnell, M.R.: Temperature sensitivity of poly(ADP-ribose) synthetase in whole cells. In Sugrimura, T. and Hayaishi, O. (Eds.), Poly(ADP-Ribose) Synthetase and DNA Repair. Proceedings of the 13th Princess Takamatsu Cancer Research Conference. In Press.

Kidwell, W.R. and Purnell, M.R.: Poly(ADP-ribose) glycohydrolase and phosphodiesterases in the analysis of poly(ADP-ribosyl)ated proteins. In Moldove, K. and Wold, F. (Eds): Posttranslation modifications, Methods in Enzymology. In Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05216-12 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cyclic AMP Binding Proteins in Breast Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
PI: Y.S. Cho-Chung Chief, Cellular Biochemistry Section LPP, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cellular Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	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.)*

Cyclic adenosine 3',5'-monophosphate (cAMP) and its binding proteins are involved in the regulation of the growth of mammary tumors in experimental animals (Cho-Chung, Cancer Res. 38: 4071, 1978). Whilst human breast cancers which possess estrogen receptor (ER) and progesterone receptor (PgR) activities are likely to be hormone responsive, many do not respond to endocrine therapy. In hormone-dependent rat mammary tumors the ratio of steroid receptors to cAMP binding proteins was found to better discriminate hormone dependent from independent tumors than steroid receptor alone.

In this study we will investigate the relationship between cAMP binding proteins, ER and PgR in human breast cancers and clinical parameters including prognosis. Several molecular species as well as proteolytic fragments of cAMP binding proteins have been found in normal and neoplastic tissues. Thus, molecular species of cAMP binding proteins will be determined by utilizing the photo-affinity ligand, 8-azido-[^{32}P]cAMP and immunoprecipitation using affinity purified antibodies to cAMP binding proteins. Utilizing immunocytochemical method intracellular distribution and nuclear compartmentalization of cAMP binding proteins will be also determined. Finally, in a cell-free system, the binding of cAMP binding proteins directly to DNA of normal vs cancer cells will be studied utilizing molecular biology techniques. The goal of this proposal is to provide us a fundamental growth regulatory mechanism of cAMP action in breast cancer.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	T. Clair	Chemist	LPP, NCI
	W. R. Miller	U. of Edinburgh;	Scotland
	S. O. Døskeland	U. of Bergen;	Norway
	M. E. Lippman	Chief, Med. Breast Cancer	M,NCI
	C. L. Kapoor	Lab. Visual Research	LVR, EI

Project Description

Methods Employed:

1. Tumors: Primary and metastatic tumors from patients with breast cancer and primary, 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary carcinoma and transplantable MTW9, DMBA #1, MT13762, mammary carcinomas in rats were used.

2. MCF-7 cells: The MCF-7 human breast cancer cells (Mason Research Institute) were grown in McCoy's 5A medium supplemented with bovine insulin, penicillin, streptomycin and fetal calf serum (10%) and ± additives.

3. cAMP assay: cAMP was measured by the competitive protein-binding method of Gilman using cAMP assay kit of Amersham.

4. Protein kinase assay: the activity was determined by measurement of the incorporation of ^{33}P from γ-labeled ATP into histone ± 10^{-6}M cAMP.

5. Estrogen-binding assay: Estrogen binding was measured by the modification of the charcoal adsorption assay described originally by Korenman.

6. cAMP-binding assay: cAMP-binding activity was measured by competion assay of Gilman at pH 6.5 at 23° for 3 hr (^{3}H cAMP-binding to proteins includes not only free sites but also endogenously bound sites by the exchange). The binding reaction was stopped by the addition of ice-cold 3.6 M ammonium-sulfate (pH 7.2) to precipitate protein-bound [^{3}H]cAMP. The precipitates were collected on membrane filter and the radioactivity measured by liquid scintillation.

7. Photo-affinity labeling of cAMP binding protein: The photo-affinity incorporation of 8-N_3-[^{32}P]cAMP was performed by the method of Pomerantz et al. (Biochemistry, 14: 3858, 1975), the binding proteins labeled with the 8-N_3-[^{32}P] cAMP were analyzed by SDS-PAGE.

8. Antibodies: cAMP binding proteins (R^{I} and R^{II}) were purified from the bovine skeletal muscle and bovine heart. Antibodies were raised in rabbits. The antibodies were affinity purified using a glutaraldehyde cross-linked immunoabsorbent technique. The monospecificity of antibody was confirmed. The cross-reactivity of R of MCF-7 cells with bovine anti-R^{I} and -R^{II} was demonstrated by radioimmunoassay (Kapoor and Cho-Chung, Cancer Res. 43: 295, 1983).

9. Immunocytochemistry: Unfixed cryostat sections (~4μ) of human tumors were used for the indirect immunofluorescence cytochemistry of cAMP binding proteins (R^{I} and R^{II}). The sections are examined with a Zeiss epifluorescence microscope.

Major Findings:

1. Prognostic value of cAMP binding proteins in human breast cancer: Cyclic AMP binding activity was measured in the cytosols from 75 human breast cancers. All tumors contained measurable binding proteins, levels varying from 0.81 to 15.05 pmol/mg cytosol protein (mean=5.34). No relationship was found between level of cAMP binding activity and menopausal status of the patient, clinical stage of the disease, presence or absence of nodal involvement or histological grade of the tumor.

A group of 11 patients who at the time of primary treatment had no evidence of metastatic disease but have developed recurrence within 36 months have been compared with a similar group (10 patients) who have had a minimum of 20 months follow-up and were disease-free. Levels of cAMP binding proteins were significantly higher in tumors from women having early recurrence than in those who were disease-free ($p<0.01$ by Wilcoxon and Rank Test). The range in disease-free patients was 1.57-7.21 pmol/mg cytosol protein; whereas that in 10 of 11 tumors which subsequently recurred early was 7.75-13.02. It is concluded that although levels of cAMP binding proteins are not associated with the clinical parameters described, it may be of independent prognostic significance.

2. Relationship of hormone sensitivity to estrogen receptor and cAMP binding capacity in human breast cancer: Estrogen receptor (ER) and cAMP binding capacity [cAMP receptor (CR)] were measured in cytosols from human breast tumors. Patients with advanced, evaluable breast cancer were biopsied before start of endocrine treatment, and ER and CR measurements performed. All patients included in this study were ER positive. Sixteen of 30 patients (53%) had an objective response to endocrine treatment. When ER and CR were expressed as a ratio and this ratio was related to treatment response, it was found that all objective responders had ratio values above 2.5×10^{-3}. Nine of 14 non-responders had ER/CR ratios below this value. In this study, a threshold limit of 2.5×10^{-3} (ER/CR) would have predicted a correct response to endocrine treatment in 25 of 30 patient (83%). The results show that measurement of cAMP binding proteins might strengthen the predictive value of steroid receptor measurement for hormone-dependency of human breast cancer.

3. DNA-Binding of cAMP binding protein R^{II}: Immunocytochemical localization of R^{II}-cAMP binding protein in the nucleoli of MCF-7 cells suggested the role of R^{II} cAMP binding protein in the nuclear event, especially in cell division (Kapoor and Cho-Chung; Cancer Res. 43: 295, 1983). DNA-binding of R^{II} was demonstrated using a cell-free system of DMBA-induced mammary tumor. The 8-azido [^{32}P] cAMP was incubated in dark with cAMP-dependent protein kinase type II of bovine heart at 23° for 1 hr then further incubated with isolated nuclei from DMBA tumor at 0° for 1 hr, and photolyzed the complex, and DNA was isolated using the phenol extraction, and the radioactivity bound DNA was localized by agarose-electrophoresis. It was found that the radioactivity band was localized on the DNA of 69×10^6 M.W. This radioactivity band was not detected on the control DNA obtained from the nuclei incubated with the 8-azido [^{32}P] cAMP only in the absence of the protein kinase. The results indicate covalent binding of R^{II} cAMP binding protein to DNA of mammary carcinoma.

Significance to Cancer Research and the Program of the Institute:

These studies contribute to the understanding of the fundamental growth regulatory mechanism of cAMP action. The measurement of cAMP-binding proteins may be an important predictive probe for hormonal sensitivity in human breast cancer. The regulatory role of cAMP-binding protein R^{II} in cell division may be of clinical significance. These results together with our previous studies suggest a therapeutic potential for cAMP analogs in human breast cancer. The use of cAMP analogs may substitute for, or synergize the effects of antiestrogens or some of the cytotoxic agents presently in use.

Proposed Course of Research:

To extend the investigation on the regulatory role of cAMP in breast cancer growth control, the following proposal is made: 1). Assess the relationship between the molecular species of cAMP-binding proteins in human breast cancer and clinical stage of the disease or prognosis. 2. Localize the intracellular distribution of cAMP binding proteins (R^{I} vs R^{II}) in human breast cancer by immunocytochemistry. 3. Localize the binding sites of cAMP binding protein (R^{II}) in DNA utilizing molecular biology techniques.

Publications:

Cho-Chung, Y.S.: Mode of cyclic AMP action in growth control. In Leung, B.S. (Ed.): Hormonal Regulation of Mammary Tumors. Vol. II, Eden Press Inc., Montrel, Canada, 1982, pp 155-177.

Cho-Chung: Y.S.: Intracellular mediators of cancer growth and metastasis. In Stoll, B.A. (Ed.): Prolonged Arrest of Cancer. John Wiley & Sons Ltd, England, 1982, pp 199-220.

Huang, F.L. and Cho-Chung, Y.S.: Dibutyryl cyclic AMP treatment mimics ovariectomy: New genomic regulation in mammary tummor regression. BBRC, 107: 411-415, 1982.

Cho-Chung, Y.S., Clair, T., Shepheard, C. and Berghoffer, B.: Arrest of hormone-dependent mammary cancer growth in vivo and in vitro by cholera toxin. Cancer Res. 43: 1473-1476, 1983.

Huang, F.L. and Cho-Chung, Y.S.: Alteration in gene expression at the onset of hormone-dependent mammary tumor regression. Cancer Res. 43: 2138-2142, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05219-12 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

In Vitro Simulation of Hormone-dependent Mammary Tumor Regression

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: R.A. Knazek Senior Investigator LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cell Cycle Regulation

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.35	0.6	0.75

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

Alterations of the hormone-receptors on or within cells will modify the response of target tissues to various hormones thus serving to control cellular growth or function. We have shown that the number of detectable prolactin receptors is controlled in vivo by the circulating levels of prolactin or growth hormone by a positive feedback mechanism. Inhibition of in vivo prostaglandin (PG) synthesis by either enzymatic blockade or precursor depletion results in a loss of existing PRL receptors and prevents their induction by PRL. Membrane fluidity increased in a variety of hormonal, dietary or developmental states, is invariably accompanied by an increase in the number of PRL receptors. It has been demonstrated that PRL alters PG synthesis in vivo. These data show that PRL up-regulates its own receptor by modifying target membrane fluidity and that this may occur through modification of prostaglandin synthesis. Extended to the DMBA rat mammary tumor, the regressing tumor membranes are more viscous and bind less PRL than those of the growing tumor. An assay for PG receptor has been developed, which has demonstrated both that these regressing tumors have an increased capacity to bind PG and that copper increases this binding capacity 8-fold. Copper may, thus augment the effect of prostaglandins in vivo and play a role in tumor growth. Patients afflicted with adrenoleukodystrophy or adrenomyeloneuropathy have an inborn propensity to accumulate long chain saturated fatty acids in their cellular membranes. We have demonstrated that this occurs within erythrocytes and thus alters the fluidity of these membranes. Such changes in membrane fluidity may reflect similar changes within the adrenal and gonads and account for the states of adrenal and gonadal failure observed in these patients. Our studies show quite clearly that membrane-associated receptors are modulated by alteration of membrane fluidity.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:			
	S.C. Liu	Chemist	LPP, NCI
	J.R. Dave	Visiting Fellow	LPP, NCI
	W.B. Rizzo	Clinical Associate	DP , NICHD
	J.D. Schulman	Senior Investigator	DP , NICHD

Project Description

Objectives:

Reproduce and study in vitro, the hormone-dependent regression process of mammary tumors in vivo.

Methods Employed:

A radioreceptor assay for prolactin and growth hormone receptors is used to determine the ability of cell membranes to bind these polypeptide hormones.

Assay techniques for prostaglandin E and $F_2\alpha$ have been devised consisting of extraction from incubation buffers by organic solvents, silicic acid double column chromatography, and specific radioimmunoassay. High pressure liquid chromatography is used to quantitate prostglandin intermediates synthesized by various tissues.

Cell membrane fluidity was determined by fluorescence polarization. This was accomplished by measuring the ease of rotation of the fluorescent lipid probe diphenylhexatrine (DPH), that had been inserted into the membranes in vitro. The polarization constant was then translated mathematically into viscosity.

A method for the determination of the number and affinity of prostaglandin receptors was developed as follows: Either DMBA - or NMU-induced mammary carcinoma was excised from female Sprague-Dawley or Buffalo rats, respectively. The tumors were minced and then homogenized in 250 mM Sucrose, 10 mM Tris, 0.1% Na azide, 1 mM dithiothreitol, and 1 mM $CaCl_2$. After a preliminary low speed spin, the supernatant was centrifuged at 15,000 x g, and the resultant membrane pellet was used for binding studies. The membrane fractions were incubated at 15°C for 30 minutes with graded concentrations of [^{3}H] PG in a total volume of 250 µl which included 100 µl of membrane suspension. After incubation was complete, a 125 µl aliquot was filtered rapidly through a Whatman GF/C glass microfiber filter disc and washed with ice cold Tris buffer. The amount of PG bound to the membranes was determined by counting the air-dried filter in a scintillation counter.

Major Findings:

PRL receptors can be induced in the rodent liver by in vivo injections of either purified bGH or bPRL. GH was shown to induce but not to bind to the receptor whereas PRL did both. These studies, plus the observation that induction occurred even if protein synthesis had been blocked, indicated that the induction was being achieved by exposing or altering receptor in such a way that its bind-

ing site was made available to PRL. Increases in the number of PRL binding sites could also be achieved by direct alteration of the phospholipid bilayer. This was accomplished both in vitro and in vivo. The PRL binding capacity increased by 60% when hepatocytes were incubated with either phospholipase A_2 or lysolecithin but decreased by 60% when C_3H mice were depleted of essential fatty acids by dietary means, a loss that could not be reversed by treatment with exogenous PRL. Blockade of prostaglandin synthesis by in vivo treatment with indomethacin also reduced existing hepatic-PRL receptors in a dose-responsive fashion that, again, could not be overcome by exogenous PRL injections. Modification of the hepatic prolactin receptor could, therefore, be achieved by changes in the character of the lipid bilayer, an effect related both to the ease with which pre-existing PRL receptors could assume an active configuration within the lipid bilayer: a change in the viscosity of the membranes could modify the activity of the receptor. Since the prolactin induces its own receptor in vivo, studies were undertaken to determine if this could, in fact, be a result of changes in membrane fluidity with concommitant exposure of pre-existing receptors. Injection of exogenous PRL in dosage schedules designed to achieve physiologic PRL levels in hypophysectomized rats did, indeed, serve to increase the phospholipid/cholesterol ratio by 24%, the membrane fluidity by 7%, and prolactin binding capacity 3-fold, whereas injection of supraphysiologic amounts of PRL reversed the effect. Measurement of the capacity of these hepatic membranes to synthesize PG revealed that physiologic replacement doses of PRL injected into either male or female rats or mice caused an increase in the synthesis of $PGF_2\alpha$ and PGE as well as membrane fluidity.

These data indicate that PRL modifies both the viscosity and PG synthesis by hepatic membranes in vivo and that these phenomena, together, might be responsible for the auto-regulation of detectable PRL receptors.

Additional studies demonstrated that incubation of hepatic membranes with PGI_2 in vitro resulted in an increase in both membrane fluidity and the number of prolactin receptor sites. Further experiments showed that in vitro incubations with phospholipase A_2, arachidonic acid, or bradykinin, all participants in the early portion of the PG cascade, served to increase membrane fluidity and prolactin binding. In fact, any treatment of the hepatic membranes that increased membrane fluidity also resulted in increased prolactin binding. This was also shown in developmental studies wherein PRL receptors appeared in murine hepatic membranes at 21 days of age, a time when the membranes were also shown to be the most fluid of any developmental stage. Similar observations hold in the pregnant mouse: both PRL binding and hepatic membrane fluidity are greatest at days 12-16 of gestation. Prolactin binding levels and fluidity were not significantly different between the non-pregnant, non-lactating and the lactating group. Again, membrane fluidity was proportional to the phospholipid/cholesterol ratio. Studies performed on aged mice (~460 days of age) showed a significant decrease in hepatic membrane fluidity compared to 70 day-old adults which were, in turn, less fluid than 21 day-old mice. Membranes obtained from male mice were more fluid than those of females at all ages studied.

The concept of a correlation between PRL binding and membrane fluidity was extended to those hormone-dependent rat mammary tumors induced by DMBA and NMU.

When the hosts bearing these tumors are oophorectomized, serum levels of PRL fall, the numbers of tumor-associated PRL receptors decrease, and the tumors regress.

As predicted by the studies of liver, the tumor membranes were significantly more viscous after oophorectomy and possessed only a small percentage of the original number of prolactin receptors. The rates of PGE and $F_{2\alpha}$ synthesis rose from 0.13 and 10.5 ng/mg protein in growing tumors to a maximum of 1.2 and 26.5 ng/mg protein by 5 days after oophorectomy. Thus, the apparent role of prostaglandins in the modulation of cell membrane fluidity with resultant changes in plasma membrane receptors, suggests that cellular response to prostaglandins might be controlled further by alterations in their ability to respond to various prostaglandins. To this end, a prostaglandin-receptor assay was devised wherein mitochondrial membranes were obtained from DMBA and NMU tumors, incubated with ^{3}H-PG (E_2 or $F_{2\alpha}$) ± excess unlabeled PG. The bound and free PG were separated using the filtration technique developed as above. Binding of both PGE_2 and $PGF_{2\alpha}$ to tumor membranes reached equilibrium after 30 minutes incubation. The binding was 44-58% reversible within 15 minutes of adding excess unlabeled PGE_2 or $F_{2\alpha}$, respectively. Specific binding of both types of PG increased 2-3 fold by ~ 5 days after oophorectomy. The K_d = 2.9 nM for $PGF_{2\alpha}$ binding to growing tumor membranes from intact hosts with a binding capacity of 25 fmole/mg protein. An increase in binding capacity was noted after oophorectomy while K_d remained unchanged. Only a very low affinity PGE_2 receptor was detectable in both growing and regressing tumor membranes.

In view of our previous studies on the hormonal control of mammalian follicular maturation and oogenesis (Z01CB08230-06 LPP), we suspected that prostaglandins might be playing a role in stimulating the formation of new blood vessels within the tumor. This was supported by the studies of Ziche and Gullino that showed PGE was angiogenic in the rabbit cornea. Furthermore, they demonstrated that copper was also angiogenic. This prompted us to study the effect of copper on PGE_1 binding to tumor membranes.

Addition of graded doses of $CuCl_2$ to the prostaglandin binding assay resulted in an 8-fold increase in PG binding, reflecting the appearance of a high affinity receptor. [^{3}H] Prostaglandin E_1 binding to the receptor was rapidly reversable by PGE_1 and inhibited by various prostaglandins in a concentration-dependent manner. The degree of potency was: $PGE_1 > PGE_2 > PGA_2 > PGF_{2\alpha} > PGB_2$. Neither $CaCl_2$ nor $MgCl_2$ were able to achieve the same effect. Similar increases in PG binding to liver membranes were induced by the addition of Cu^{++} to the incubation mixture. The data presented herein suggest that copper may play an obligatory role in neovascularization by increasing the number of binding sites for the angiogenic prostaglandins.

Significance to Biomedical Research and the Program of the Institute:

Demonstration that prolactin receptors are induced by growth hormone indicates that this hormone may play a role in the responsiveness of mammary carcinoma to hormonal stimuli. The fact that changes in membrane fluidity are induced by

prolactin may help to explain the mechanism by which prolactin induces its own receptor. The requirement for essential fatty acids and prostaglandins to modulate the prolactin receptors indicates that the lipid stores play an important role in maintaining the responsivity of target tissues to circulating hormones. These studies support and explain, in part, the demographic correlation of mammary carcinoma with high dietary fat intake throughout the world.

The importance of prostaglandins in the growth and metastasis of mammary tumors has been suggested by many investigators. Changes in their rates of production and ability to bind to tumor membranes as a function of hormonal status strengthens this hypothesis and suggests another mechanism by which tumor growth and metastases may be modified. The thesis of receptor modulation by changes in membrane fluidity can now be extrapolated to humans because of the observations made in patients with ALD and AMN. This suggests a linkage between physiological or pathological alterations in lipid status with modification of cellular responses to trophic agents in clinical settings.

Proposed Course of Research:

The role of prostaglandins in the induction of the prolactin receptor and prolactin action will continue to be studied both in vivo and in vitro. The involvement of prostaglandins in the development of the mammary tumors and mammary glands will also continue to be studied using radioimmunoassay, radioreceptor, and HPLC techniques.

Publications:

Liu, S.C. and Knazek, R.A.: PG synthesis and binding is increased in regressing NMU-mammary carcinomas. Prostaglandins and Medicine 8: 191-198, 1982.

Knazek, R.A., Rizzo, W.B., Schulman, J.D. and Dave, J.R.: Membrane microviscosity is increased in the erythrocytes of patients with adrenoleukodystrophy and adrenomyeloneuropathy. J. Clinical Investig (In press).

Dave, J.R. and Knazek, R.A.: Changes in the prolactin-binding capacity of mouse hepatic membranes with development and aging. Mechanisms Ageing and Development (In press).

Liu, S.C. and Knazek, R.A.: Prostaglandin synthesis and binding by growing and regressing mammary carcinoma. In: Proceedings of the International Conference on Prostaglandins and Cancer, A.R. Liss, Inc. 1982 pp. 705-711.

Dave, J.R., Brown, N.V. and Knazek, R.A.: Prolactin modifies the prostaglandin synthesis, prolactin binding, and fluidity of mouse liver membranes. Biochem. Biophys. Res. Comm. 108: 193-199, 1982.

Dave, J.R., Richardson, L.R. and Knazek, R.A.: Prolactin-binding capacity, prostaglandin synthesis, and fluidity of murine hepatic membranes are modified during pregnancy and lactation. J. Endocrinology (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08205-12 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Application of Artificial Capillary Culture Technique to Hormone Production

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: R. A. Knazek Senior Investigator LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cell Cycle Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

The artificial capillary culture technique provides a pericellular microenvironment that closely resembles the in vivo state. This may allow normal cells to maintain their differentiated functions for prolonged periods of time in vitro. Cells are maintained in a physiologic state without many of the artifactual constraints imposed by more standard methods of tissue culture. The technique is, therefore, especially well suited for the culture of cells both of endocrine origin and their target cells. Response of cells to various hormones and their culture over long periods have been achieved thus making the techniques useful for continuous production of hormones for both laboratory and clinical uses. Modifications have been made to the basic design. These permit study of nutrient and product transport through the tissues formed in the intercapillary spaces, the effect of lymphatic-type drainage upon cell function, the study of cell physiology by nuclear magnetic resonance, and use of monoclonal antibodies directed against specific types of neoplastic cells.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: P. M. Gullino Chief LPP, NCI
R. S. Balaban Staff Fellow LKE, NHLBI
A. S. Lichter Senior Investigator RO , NCI

Project Description

Objectives:

A method is being developed for studying hormonally responsive tissues in vitro. Mammary, ovarian, and renal tissues are being used to study the mechanisms of hormonal stimulation.

Methods Employed:

A bundle of tube-shaped semi-permeable membranes perfused with tissue culture medium, simulates the in vivo capillary bed. Isolated cells or tissue masses injected into the extracapillary space receive nutrients and have cell products removed by diffusion through the capillary walls. In this nearly physiologic environment, established cell lines form solid masses of tumor while primary tissue explants continue to function in a normal manner, in some cases, for several months. The culture unit has been inserted into an NMR probe and perfused with culture medium and thus maintained at near-physiologic conditions while using ^{31}P NMR to study intracellular metabolites. Monoclonal antibodies developed against the MCF-7 human breast cancer cell line were labeled with I^{125}. The antibodies were then injected into the extracapillary space of culture units in which solid masses of MCF-7 cells were growing. Autoradiographs of histologic cross sections of these cultures were then made.

Major Findings:

The standard artificial capillary culture unit, which was developed in this laboratory, has previously been shown to provide a nutrient matrix which permits established cells to grow and form solid tumor masses in vitro. Secretory cells have retained their function in such a system for several months. Modification of the recirculating perfusion medium into a single pass mode of operation has demonstrated its value in studying the response of cell masses to physiologic hormones and chemotherapeutic agents.

Two separate capillary bundles were woven together and placed in a plastic shell fitted with Y connectors at each end. This provided matrices that were simultaneously intimately intertwined but still distinctly separate. Operation of the same or different hydrostatic pressures in co-current or counter-current perfusion modes permitted study of transport of solutes by diffusion or bulk flow through tissues grown within this braided network. The culture unit has been modified by wrapping the fiber bundle with a porous polymeric sheet which permits cells and particulate cell products to migrate or diffuse away from the cell mass. This permits harvesting of these substances without gross disturbance of the cell culture.

NMR investigation of HTCBH cells and human lymphocytes have been initiated, studying both intracellular pH and energy metabolism. A6 cells have grown within the capillary culture device and preliminary ^{31}P NMR spectra have been collected.

MCF-7 cells grew well, forming three-dimensional duct-like and alvealar-like structures within the capillary culture unit. [^{125}I]-anti MCF-7 antibody was then injected into the extracapillary spae of these units, subsequent autoradiography revealed grains not only in cells on the periphery of the cell masses, but also deep within the masses. This indicates that rapid transport of these specific, large proteins can be achieved into all areas of the tumor via diffusion within the extracellular fluid space.

Significance to Biomedical Research and the Program of the Institute:

The characteristics of the capillary culture unit enable rapid responses to both stimuli and inhibitors of cell function to be studied in a nearly physiologic in vitro environment. This work has demonstrated that convection as well as diffusion of cell nutrients and products plays a role in the transport of various substances within tumors. This may be of assistance in pharmacokinetic modeling and in understanding and improving the transport of both physiologic and pharmacologic agents within solid tumors. The study of cell behavior using NMR techniques may now be possible and provide a unique opportunity to study tumor physiology by a non-invasive method. Artifical capillary culture systems can now be considered as an in vitro model in which anti-tumor antibodies can be studied.

Proposed Course of Research:

Ovarian, mammary tissues, and hormone-producing transformed cells will be studied within the artificial capillary culture unit in conjunction with our efforts to study the growth and regression of hormone-responsive tumors in vitro. NMR studies will be made on high density cultures, studying the effects of hormonal, nutrient, and drug manipulations upon the glycolytic pathway. Anti-tumor antibodies will be labeled with various isotopes. The subsequent cytotoxic effect of the radionucleides and antibodies effects upon the solid tumor masses grown within the artificial capillaries will then be studied.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08212-09 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Shelby L. Berger, Research Chemist, Laboratory of Pathophysiology, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pathophysiology, Office of the Chief

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	3.50	0.00

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 synthesis of single-stranded globin cDNA by the RNA-directed DNA polymerase activity of reverse transcriptase in the presence of oligothymidylate primers was investigated in order to determine the limitations to higher yields. The results indicated that the associated ribonuclease H activity, an integral part of reverse transcriptase, plays a large role in the synthesis of the first strand of cDNA and that the interplay of the two enzyme activities for any specific set of conditions determines the yield of single-stranded products. In both the presence and the absence of polymerization, the associated ribonuclease H catalyzed the deadenylation of mRNA, producing molecules that were somewhat shorter, highly homogeneous in size, and fully translatable into globin protein. They were also entirely lacking in the ability to serve as templates for cDNA synthesis. The reaction was completely dependent on oligothymidylate and completely independent of deoxyribonucleoside triphosphates. The initial rate of deadenylation was one-fourth the initial rate of initiation of polymerization when saturating levels of deoxyribonucleoside triphosphates were used in the polymerase reaction. In the presence of ribonuclease H activity, the DNA polymerase catalyzed the synthesis of an array of cDNAs including some that were full length. The initiation of polymerization was rate limiting: once synthesis had begun, it required 1-1.5 min to transcribe globin mRNA. However, most primers that were elongated were aborted prematurely. Maximum synthesis of full-length cDNA required stoichiometric levels of enzyme and high triphosphate levels, but regardless of conditions, the sum of completed cDNA and deadenylated mRNA accounted for only 50% of the input mRNA. The data fit a model in which synthesis of full-length cDNA molecules depends on the arrangement of primers and transcription initiation complexes on the poly(A) "tail" of mRNA.

A mRNA library from human T-lymphocytes activated for differing periods with several mitogens has been prepared.

Other Professional Personnel:

Robert S. Puskas	Senior Staff Fellow	LPP, NCI
William H. Eschenfeldt	Senior Staff Fellow	LPP, NCI
Marc Krug	Staff Fellow	OD, NCI

Project Description

Objectives: Reverse transcriptase from avian myeoloblastosis virus is a key ingredient in recombinant DNA technology. It is essential for the synthesis of single-stranded complementary DNA, the initial step in all cloning projects that depend on messenger RNA. Although many investigators have attempted to optimize this reaction, yields of full-length cDNA rarely exceed 40% regardless of the conditions advocated. Subsequent steps in cloning are therefore placed at a disadvantage particularly when the desired mRNA is rare. In the present study, part of an ongoing project aimed at understanding the relationships among the products of nonabundant mRNAs from T-lymphocytes, we set out to determine the limitations to higher yields by examining the mechanism of action of reverse transcriptase.

Methods Employed: New assays were developed for evaluating the two intrinsic activities of reverse transcriptase, polymerase activity and ribonuclease H (RNase H) activity. A labeled primer, (5'-^{32}P)oligo(dT), was synthesized with (γ-^{32}P)ATP and polynucleotide kinase. The molecule was not initially trichloroacetic acid-precipitable, but after elongation by the polymerase activity, using a suitable RNA template, the (^{32}P)oligo(dT) became acid-insoluble. The rate at which this molecule became acid-precipitable was used to measure the initial rate of the initiation of cDNA synthesis.

RNase H activity was measured in two ways in the absence of polymerization. Rabbit globin mRNA is a mixture of two species, the mRNAs for the α and β chains of hemoglobin. After polyacrylamide gel electrophoresis, the mixture appears as a diffuse band owing to variable lengths of the poly(A) "tails". We treated this material with reverse transcriptase and found that in the presence of oligo(dT) the mixture was converted into two very sharp, slightly smaller bands. Band sharpening could be used as a qualitative indication of RNase H activity. For quantitative work, the 3'-termini of rabbit globin mRNAs were labelled with (α-^{32}P)ATP and poly(A) polymerase. The rate of disappearance of acid-insoluble radioactivity when labeled globin mRNA and unlabeled primer were incubated together served as a measure of RNAse H activity suitable for kinetic studies.

Major Findings: Reverse transcriptase is a single molecule with two activities that are an integral part of the enzyme: an RNA-dependent DNA polymerase function and a ribonuclease H function. The polymerase activity is responsible for reverse transcription of RNAs. For the synthesis of cDNA *in vitro*, this is the only important reaction catalyzed by the enzyme. The associated RNase H is an exonuclease capable of cleaving the RNA strand of a DNA-RNA hybrid into 5'-phosphoryl oligomers. Although important *in vivo*, this reaction was considered to be irrelevant *in vitro*. According to dogma, RNase H remained inactive in the reaction *in vitro* until the polymerase activity generated a suitable substrate.

Then, RNase H became functional and degraded the RNA strand of the cDNA-RNA hybrid leaving the first cDNA strand available for subsequent second strand synthesis. Poly(A)-poly(dT) was frequently used as a synthetic substrate for studying the reaction but virtually no RNase H activity was detectable using poly(A)-oligo(dT)$_{12-18}$ hybrids unless TTP for polymerization was also supplied. Most investigators consequently assumed that the enzyme could degrade only mRNA that had already been reverse transcribed and, in attempting to understand the mechanism of transcription, eliminated the RNase H activity from their analyses of the synthesis of the first strand of cDNA. In complete contradiction, we have found that the associated RNase H activity plays a critical role in the synthesis of the first strand of cDNA in vitro and that the interplay of the polymerase activity and the RNase H activity determines the yield of full-length cDNA.

From the enzymological point of view, the synthesis of the first strand of cDNA was anomalous. Only a small portion of the input mRNA was converted to full length cDNA. Furthermore, we found that the yield of mature cDNA molecules at completion increased with an increase in either the reverse transcriptase concentration, or the deoxyribonucleoside triphosphate concentration, or both. In simple terms, the yield of full-length cDNA at completion appeared to be dependent on the initial rate of the polymerization reaction. If the synthesis of cDNA had been straightforward, all reaction mixtures would have produced the same amount of cDNA from a fixed amount of messenger RNA regardless of conditions. However, those reactions with greater amounts of enzyme or triphosphates would have synthesized the product in a shorter period of time. The deviation of the reverse transcription reaction from expected behavior strongly suggested that a competing reaction(s) had occurred.

In order to elucidate the nature of the putative interfering reaction, we asked why reverse transcriptase had stopped synthesizing full-length cDNA molecules. Under the conditions used, approximately 6 to 7% of the input globin mRNA was converted to mature cDNA when the reaction ceased spontaneously. Could more cDNA be made by adding one or more substrates or additional reverse transcriptase? We found that only one substance was capable of reactivating cDNA synthesis, globin mRNA, itself. Although very few of the mRNA molecules had been converted to cDNA, the reaction stopped because it was depleted of mRNA. In accompanying experiments, such trivial experiments as destruction of deoxyribonucleoside triphosphates by contaminating phosphatases or thermal inactivation of the enzyme were ruled out.

Incubation of globin mRNA with reverse transcriptase in the absence of the other components of the reaction mixture eliminated the possibility that contaminating ribonucleases had degraded the RNA. Such "preincubated" mRNAs subsequently gave rise to the same yield of full-length cDNA as control mRNAs preincubated without reverse transcriptase.

The source of the contaiminating nucleases was investigated further. We found that no single component of the reaction mixture when incubated with globin mRNA caused degradation, but that preincubation of mRNA with both reverse transcriptase and the oligo(dT) primer reduced subsequent yields of full-length cDNA by 50%. The experiments showed that the nuclease in the reverse

transcriptase preparation was dependent on oligo(dT) for its activity. The putative contaminant appeared to have substrate requirements consistent with those of a ribonuclease H.

The products of the suspected ribonuclease H activity were investigated. In the absence of the polymerization reaction, globin mRNA, was converted by reverse transcriptase in the presence of oligo(dT) to moieties that were highly homogeneous in size and slightly smaller than globin mRNA when assayed electrophoretically. The products of the suspected RNase H reaction in the presence of polymerization could not be analyzed meaningfully on gels. However, when the synthesis of cDNA came to a halt, the nucleic acids could be reisolated and translated in a cell-free protein synthesizing system to characterize surviving RNA. In such a system, hybrids of intact mRNA and cDNA are not translatable and mRNA degradation products give rise to easily recognizable small peptides. When the translation products of mRNA that had previously been reverse transcribed to completion were examined, a polypeptide the correct size for globin was obtained. Thus, reaction mixtures devoid of intact templates for globin cDNA synthesis nevertheless contained molecules capable of directing the synthesis of authentic globin protein in a cell-free system. Taken together, these data suggested that reverse transcriptase stopped synthesizing cDNA because the associated RNase H, an intrinsic part of the enzyme, had deadenylated the mRNA.

Deadenylation of mRNA was confirmed using 3'-end-labeled mRNA as detailed in Methods. The reaction was found to be independent of the triphosphate concentration.

The kinetics of the polymerase and the RNase H activities were examined in order to explain the experimentally observed relationships between the rate of polymerization and the yield of full-length cDNA. Using a modified Lineweaver-Burk approach, we showed that the initial rate of initiation of polymerization with the 5'-end-labeled oligo(dT) primer described in Methods was 4-fold higher than the rate of deadenylation of the mRNA, regardless of enzyme concentration. We also showed that most primers were aborted prematurely owing to obstruction by other oligo(dT) molecules located downstream (i.e. nearer to the 5'-end of the mRNA). When the rate of initiation of polymerization was corrected for abortive starts, the initial rate of productive initiation of transcription was 1.3-fold higher than the initial rate of deadenylation provided "optimized" polymerization conditions were used. A maximum velocity could not be discerned in either reaction.

The data were interpreted by means of a model. In the presence of stoichiometric levels of enzyme and very high levels of deoxyribonucleoside triphosphates, all oligo(dT) primers can be serviced simultaneously. Therefore, only the primer located furthest downstream need be considered; all others are blocked. Under these conditions, there is a 1.3-fold greater probability that reverse transcriptase will use the molecule under consideration to prime cDNA synthesis than to cleave the poly(A) tail. Hence, the maximum theoretical yield of full-length cDNA is 57%. Under suboptimal conditions for polymerase, that is, with catalytic quantities of enzyme and triphosphates at concentrations near the value of K_m, all primers cannot be handled simultaneously. Statistically,

upstream primers are favored because, of the many bound primers per poly(A) tail only one can be located furthest downstream. If reverse transcriptase chooses to copy the mRNA, the oligo(dT) primer is elongated. Nevertheless, completed cDNA is not produced because transcription is blocked by one or more primers located downstream. The result is a gradual increase in the size of oligo(dT) primers. If reverse transcriptase chooses, instead, to cleave the mRNA, a less favored alternative initially, the poly(A) tail becomes shorter. Eventually the length of the oligo(dT) primers will exceed the length of the surviving poly(A) tail. Then, perfect hybridization of the two is impossible. Those hybrids with the 3'-end of the oligo(dT) primer dangling free cannot prime synthesis of cDNA but can serve as substrates for RNase H. Under these conditions, cleavage of the poly(A) tail predominates. Hence, reaction mixtures with a low concentration of enzyme together with a low concentration of triphosphates for polymerization favor the deadenylation reaction. The model completely explains why yields of full-length cDNA greater than about 50% have never been achieved.

A messenger RNA library has been prepared from human lymphocytes activated for differing periods of time by a repertoire of mitogens. Preliminary experiments have been carried out in anticipation of creating a cloned full-length cDNA library.

Significance to Biomedical Research and the Program of the Institute: This project conforms to Objective #3, Approach #1 of the National Cancer Plan. It aims at developing methods that can be used to understand the biochemical control mechanisms by which normal cell growth and function are maintained. Disordered cell growth in neoplastic populations may then be better understood and rational attempts made to prevent or to modify it.

Proposed Course of the Project: Further studies will focus on manipulating the reverse transcriptase reaction in order to synthesize mature cDNA from all input mRNA molecules. These methods will be applied to the study of rare mRNAs coding for the secreted products of activated human T-lymphocytes.

Publications:

Puskas, R. S., Manley, N. R., Wallace, D. M., and Berger, S. L.: Effect of ribonucleoside-vanadyl complexes on enzyme-catalyzed reactions central to recombinant deoxyribonucleic acid technology. Biochemistry 21: 4602-4608, 1982.

Siegal, G. P., Thorgeirsson, U. P., Russo, R. G., Wallace, D. M., Liotta, L. A., and Berger, S. L.: Interferon enhancement of the invasive capacity of Ewing's sarcoma cells in vitro. Proc. Natl. Acad. Sci. USA 79: 4064-4068, 1982.

Berger, S. L., Wallace, D. M., Puskas, R. S., and Eschenfeldt, W. H.: Reverse transcriptase and its associated ribonuclease H: Interplay of two enzyme activities controls the yield of single-stranded cDNA. Biochemistry 22: 2365-2372, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08215-06 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Angiogenesis and tumor growth

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: K. Raju Staff Fellow LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews C

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

A group of copper carrying compounds and PGE1 were found to induce new formation of capillaries in the corneal assay. Neoplastic tissue as well as the chemically defined angiogenesis effectors stimulate in the cornea the production of a chemoattractant for the capillary endothelium. The chemoattractant was extracted, isolated by gel filtration chromatography, purified about 200X and shown to be identical regardless of the angiogenesis effector used to stimulate the cornea. The chemotactic activity appears to be exclusively directed toward the capillary endothelium and probably consists of two components that must be combined for optimal mobilization effect.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:			
	P.M. Gullino	Chief,	LPP, NCI
	G. Alessandri	Visiting Fellow	LPP, NCI
	S. Ungari	Visiting Fellow	LPP, NCI

Project Description

Objectives:

To elucidate the mechanism of angiogenesis.

Methods employed:

1. Chemotaxis activity on capillary endothelium was measured utilizing two approaches (A) Boyden chamber assay and B) gelatin-agarose assay developed in our laboratory.

2. The chemotaxis activity induced in the corneas by different angiogenic effectors was isolated by sepharose-6B gel filtration chromatography.

3. The chemotactic effect was further purified by gelatin-sepharose affinity chromatography.

Major Findings:

1. Several angiogenic effectors have been found able to induce in the rabbit cornea the mobilization of endothelium followed by new formation of capillaries.

2. The chemoattraction appears to be directed almost exclusively toward the endothelium of capillaries not the endothelium of large vessels.

3. The chemotactic activity was purified about 200X.

4. Preliminary data suggest that two components are probably necessary to obtain mobilization of endothelium. These components are now being characterized.

Significance to Biomedical Research and the Program of the Institute:

New formation of vessels is indispensable for tumor growth. Capillary endothelium mobilization is a key event in neovascularization. Purification of endothelium mobilizing activity will permit the production of antibodies to be tested clinically for two purposes: (1) to establish whether tissues normally deprived of angiogenic capacity are at higher risk of neoplastic transformation when they acquire angiogenic capacity (see previous results of this project) and (2) to ascertain whether an antiserum against the chemoattractant for endothelium may alter or arrest new formation of capillaries thus reducing tumor growth.

Proposed Course of Research:

To complete the project.

Publications:

Ziche, M. and Gullino, P.M. Angiogenesis and neoplastic progression in vitro. J. Natl. Cancer Inst. 69: 483-487, 1982.

Ziche, M., Jones, J., and Gullino, P.M. Role of prostaglandin E_1 and copper in angiogenesis. J. Natl. Cancer Inst. 69: 475-482, 1982.

Raju, K., Alessandri, G., Ziche, M. and Gullino, P.M. Ceruloplasmin, copper ions and angiogenesis. J. Natl. Cancer Inst. 69: 1183-1188, 1982.

Alessandri, G., Raju, K., and Gullino, P.M. Mobilization of capillary endothelium in vitro induced by effectors of angiogenesis in vivo. Cancer Res. 43: 1790-1797, 1983.

Raju, K.S. Isolation and characterization of copper binding sites of human ceruloplasmin. Mol. Cell. Biochem. (In press).

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

PROJECT NUMBER
Z01CB08219-06 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
cDNA sequence and gene structure of whey-acidic protein and k-protein

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
PI: P.K. Qasba Expert LPP, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0

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

C

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

A. Murine milk contains, besides caseins and α-lactalbumins another abundant class of protein, the whey-acidic proteins. We first isolated this protein from rat milk and the sequence of the first 20 amino-terminal residues were determined by direct protein sequence analysis. The full sequence of the protein was further derived by isolating a cDNA clone, corresponding to rat whey-acidic phosphoprotein, and sequencing its DNA. The protein sequence shows the following unique features: a) Whey-acidic phosphoproteins contain high content of cysteine, glutamic acid, aspartic acid and serine, but lack tyrosine. Half-cystines appear in unique arrangements and are repeated in two domains of the protein. The arrangement of half-cystines show striking similarities with the arrangement of cysteines in neurophysins and certain type of lectins like Con A and wheat germ agglutinin, b) the second domain has also striking similarities with the second domain of the red sea turtle protease inhibitor.

B. We have also isolated a cDNA clone encoding a protein which we have named k-protein. The cDNA derived protein sequence lacks in cysteine and has several potential phosphorylation sites.

C. We have isolated the genomic clones corresponding to whey-acidic and k-protein from a bacteriophage Charon 4A/rat partial EcoRi genomic library and established the genomic organization for these genes.

Other Professional Personnel: A. Dandekar Visiting Fellow LPP, NCI
E. Appella LCB, NCI

Project Description

Objectives:

The purpose of this work is: a) to obtain the information about the mRNA sequences for noncasein milk proteins--Wp, k-protein and α-LA--which is essential for understanding metabolism of these mRNAs and possible function of these proteins, b) to obtain the amino acid sequence of noncasein milk proteins from the corresponding cDNA sequences; c) show the relationship of DNA methylation with the expression of these genes.

Methods Employed:

1. NH_2-terminal sequence analysis of in vivo and in vitro synthesized Wp-proteins. RNA complementary to Wp-cDNA clone was hydbrid selected and translated in vitro in the presence of [^{35}S]methionine of [^{3}H]leucine. The radiolabeled protein or the protein isolated from milk was subjected to automated Edman degradation.

2. Pst I cDNA inserts were isolated from cDNA clones. The DNA restriction fragments labeled at 3′ or 5′ -ends were sequenced by the chemical method of Maxam and Gilbert.

3. For the studies on the methylation status of the gene sequences of α-LA, Wp and k-proteins in mammary gland and mammary tumors, the DNA was digested with appropriate restriction enzymes and then subjected to Southern Blot analysis.

Major Findings:

1. Sequence analysis of cDNA clones: a) The sequence analysis of rat α-LA cDNA clones has shown that rat α-LA is larger than any known α-lactalbumins as was predicted from our previous protein data. It has 17 extra residues beyond the COOH terminus of the α-lactalbumin isolated and sequenced to date from other species. The predicted COOH-terminal sequence is hydrophobic and proline rich and bears some resemblance to β-casein sequences. The results suggest an interesting possibility that all other α-LAs are synthesized with the extensions at the COOH-terminal end as in the rat and during the secretory process, the COOH-terminal extension is cleaved off in a manner similar to a presequence.

b) The sequence analysis of whey phosphoprotein cDNA clones have shown that whey phosphoproteins contain high content of cysteine, glutamic acid, aspartic acid, and serine, but lacks tyrosine. The cysteines appear in unique arrangements and are repeated in two domains of the protein. The second domain has striking similarities with the second domain of the red sea turtle protease inhibitor. The whey phosphoprotein mRNA is detected during mid pregnancy in lactation

in the rat mammary gland but is barely detected in mammary tumors in which other milk protein mRNAs are expressed.

c) The k-protein mRNA sequence encodes a protein which lacks cysteine. The encoded protein shows a homology to a part of SV40 T-antigen. The mRNA sequence also shows a region of homology with the DNA sequence found near the origin of replication of SV40 and Polyoma DNA.

2. Methylation status of α-LA, Wp and k-gene sequences in rat mammary gland and in certain tumors:

Msp and Hpa II restriction endonucleases which recognize the sequence C-C-G-G (Msp I cleaves both 5-C-C-G-G-3 and 5-C-^{m}C-G-G-3 sequences whereas Hpa II cleaves the sequences 5-C-^{m}C-G-G-3 and 5'-C-C-G-G-3' but not the sequence 5-C-^{m}C-G-G-3 were used to study the methylation state of these sequences in α-LA, Wp and k-genes in the rat mamamry gland at various stages of functional differentiation and in several rat mammary tumors. It was found that:

a) In the organs other than the mammary gland, C-C-G-G sites around these genes are highly methylated gene sequences of α-LA, Wp and k-proteins. However, there is a steady increase in the proportion of these unmethylated sequences in the mammary gland during gestation, initiating around 8 to 10 day of pregnancy. This increase in the proportion of undermethylated gene sequences follows closely i) the increase in the mRNA sequences corresponding to these genes and ii)increase in the epithelial cell population known to occur in the mammary gland. During lactation these gene sequences are completely demethylated which parallels with the maximum expression of these genes in the gland;

b) In MTW9 and MCCLX mammary tumors which synthesize α-LA protein, the α-LA gene sequences are demethylated. Whey phosphoprotein gene in these tumors is hypermethylated, correlating with the reduced expression of this gene;

c) MNU and 7-12-DMBA tumors show an altered methylation profile for these gene sequences which does not resemble to any stage of the mammary gland development. This pattern is also different from the patterns obtained from kidney and liver DNA.

Significance to Biomedical Research and the Program of the Institute:

Studies on the milk protein gene expression during normal differentiation of mammary cells will help us to understand underlying mechanism of de-differentiation of the mammary epithelial cells during malignant transformation.

Proposed Course of Research:

The experiments will be further carried out: 1) To identify the cell types present in the mammary gland which have these demethylated gene sequences

and 2) to determine if hormones are involved in demethylation of these gene sequences.

Publications:

Qasba, P.K., Dandekar, A.M., Sobiech, K.A., Nakhasi, H.L., Devinoy, E., Horn, T., Losonczy, I. & Siegel, M.: Milk protein gene expression in the rat mammary gland (1982). In Critical Reviews of Food Sciences & Nutrition (CRC press) 16(II), pp. 164-189.

Dandekar, A.M., Robinson, E.A., Appella, E. and Qasba, P.K.: Complete sequence analysis of cDNA clones encoding rat whey phosphoprotein: Homology to a protease inhibitor (1982) Proc. Natl. Acad. Sci. USA 79: 3987-3991.

Horn, T.M., Sodroski, J. and Qasba, P.K.: Protein-coding capacities of polyadenylated RNAs from normal and neoplastic Rat Mammary Tissues (1983) Cancer Res. 43: 1819-1826.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08220-02 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Structure & nucleotide sequence of rat α-LA gene: comparisons with lysozyme gene

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: P.K. Qasba Expert LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

α-Lactalbumin, is a modifier protein of galactosyltransferase thereby promoting the transfer of galactose to glucose, resulting in a -1--4 glycosidic linkage and synthesis of lactose. It bears a structural homology with lysozyme, an enzyme catalyzing the hydrolysis of a -1--4 glycosidic linkage in polysaccharides. We have isolated clones carrying rat α-LA genomic sequences from a bacteriophage Charon 4A/rat partial EcoRI genomic library. We have established the complete nucleotide sequence of the rat α-lactalbumin gene carrying the coding and intervening sequences including its 5'-flanking region. Comparison of this gene structure with chicken egg white lysozyme gene shows a) both genes contain 3 introns at similar positions; b) the first three exons of the two genes show high nucleotide homologies and are of comparable lengths and c) the fourth exon of α-lactalbumin, which codes for the amino acid residues essential for its interaction with galactosyltransferase, is markedly different from the corresponding exon of lysozyme. It is suggested that the 4th exon of α-LA, coding for a new functional unit, might have replaced the DNA region of a primordial lysozyme gene and led to a protein with a new function.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: S. Safaya Visiting Fellow LPP, NCI

Project Description

Objectives:

The purpose of this work is: a) to establish the genomic organization of α-lactalbumin gene and b) to determine the entire DNA sequence of the α-LA gene and its 5'-flanking region and c) to compare it with the chicken egg white lysozyme gene.

Methods Employed:

1. The amplified rat liver DNA library (in λ Charon 4A phage DNA) was screened using the in situ plaque hybridization techniques of Benton and Davis (Science 1977, 196, 180-187). Plaques containing α-lactalbumin structural gene sequences were purified as described by Maniatis et al. (Cell 1978, 15, 687-701).

2. For restriction enzyme mapping DNA is digested and fragments separated on agarose gels. After visualization of the DNA bands by ethidium bromide staining, DNA is analyzed by Southern blots. Nick-translated [^{32}P] labeled p-α-LA 18 is used as a hybridization probe to localize the structural gene sequences for α-LA. 5'-end labeled α-LA mRNA is used as a hybridization probe to localize the 5-end sequences.

3. DNA sequence analysis is carried out as follows: DNA restriction fragments are sequenced a) after labeling at 3' or 5'-ends by the chemical method of Maxam and Gilbert or b) by M13mp7 dideoxy sequencing technique of Messing et al. (Nucleic Acid Res., 1981, 9, 309-321).

Major Findings:

1. α-Lactalbumin mRNA sequence of about 720 bases spans on a cellular gene which is about 2.5 kb long.

2. There are three intervening sequences which are at similar positions within the coding regions as in chicken egg white lysozyme gene.

3. The intervening sequences in the two genes differ in lengths: in the rat α-LA gene the intervening sequences are 341, 429 and 1016 bp long and in the chicken egg white lysozyme gene 1270, 1810 and 79 bp long. All the introns in both genes only begin with GT dinucleotide and end with AG, sequences thought to be necessary for correct RNA splicing of various other eukaryotic genes, but also have additional common sequences at the exon/intron junctions.

4. Rat α-LA exons are 165, 159, 76 and 328 bp long compared to lysozyme exons which are 165, 162, 79 and 180 bp long, respectively. Three introns are located on the codons 26, 79 and 104 within the coding regions of rat α-LA, compared to the codons 28, 82 and 108 of the chicken egg white lysozyme.

5. Exons 1, 2, and 3 of the two genes show high nucleotide homologies. The 4th exons of α-LA and lysozyme show the least homology and also differ in length.

6. It is proposed that the 4th exon according to the split gene hypothesis might have replaced the 4th exon of a primordial lysozyme gene by a mechanism of rearrangement of the DNA region coding for new functional unit of the protein. The unique features in the intervening sequence preceding the 4th exon of α-LA gene tends to favor such a hypothesis.

7. First intervening sequence contains a trinucleotide sequence TCC repeated 23 times. In the third intervening sequence a dinucleotide TG is repeated 25 times and then after a long repeat of TAT sequence again repeated 22 times. The TG repeats are known to attain a Z conformation which is thought to be involved in gene rearrangement and recombination.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of the organization of the α-LA gene sequence and of its 5'-flanking region in normal and neoplastic mammary cells will help in understanding the altered gene expression in the transformed mammary cells.

Proposed Course of Research:

See Project Number Z01CB08286-01.

Publications:

Results to be published.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08225-08 LPP

PERIOD COVERED: October 1, 1982 to September 30, 1983

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

Membrane Structure and Dynamics of Normal and Neoplastic Mammary Gland

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: P. Pinto da Silva Chief, Membrane Biology Section LPP, NCI

COOPERATING UNITS *(if any)*

Dr. A. Peixoto de Menezes, Institute of Pathological Anatomy, School of Medicine, University of Lisbon, Lisbon, Portugal

LAB/BRANCH: Laboratory of Pathophysiology

SECTION: Membrane Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.72	0.61	0.11

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

We applied a method recently developed in this laboratory--"fracture-label"--to investigate the topology of lectin (concanavalin A and wheat germ agglutinin) binding sites with plasma membrane halves of cancer vs. normal cells. We used ovary-dependent breast carcinomas of the rat (DMBA and MTW9). Cells from growing and regressing tumors were studied and compared with epithelial cells from normal rat mammary gland. The aim of the project was to search for differences in the partition, on freeze-fracture, of membrane glyconjugates of mammary gland cells after malignant transformation, and during tumor regression. In all cases the lectin receptors were preferentially seen in association with the exoplasmic halves of the plasma membrane; significant differences in Con A and WGA labeling among normal, malignant, and tumor regressing cells were not disclosed.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	C. Parkison	Chemist	LPP, NCI
	A. P. Aguas	Visiting Fellow	LPP, NCI

Project Description

Objectives:

To search for differences in the topology of specific glycoconjugates on the plasma membrane between normal and neoplastic mammary cells.

Methods employed:

We have extended our results using the same methods that were described in detail in the previous report.

Major Findings:

We have not disclosed significant differences in the topology, partition, and density of Con A and WGA receptors among mammary epithelial cells from normal tissue and from breast carcinomas, either growing or undergoing regression.

Significance to Biomedical Research and the Program of the Institute:

Our results show that DMBA and MTW9 mammary carcinomas do not present changes in the distribution, and topology of Con A and WGA binding sites marked at the surfaces of this neoplastic cells, when compared with the same glycoconjugates from normal mammary gland cells. These findings stress the difficulty in detecting alterations in membrane architecture induced by neoplastic transformation, in particular in the topology of lectin binding sites in mammary cell carcinomas of the rat.

Proposed Course of Research:

This project has been terminated.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08226-07 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

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 & Tumorigenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

PI:	B.K. Vonderhaar	Research Chemist	LPP, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.95	1.2	0.75

CHECK APPROPRIATE BOX(ES)

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

C

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

This project is designed to understand the role of hormones and growth factors in normal mammary gland development and differentiation. We wish to understand how milk-protein production is controlled by various hormones. Studies include: 1) examination of the role of thyroid hormones, adrenal steroids and Vitamin D in synthesis and secretion of milk proteins in organ culture, 2) examination of the role of epidermal growth factor and mammary gland-derived growth factors in lobulo-alveolar development of the immature mouse mammary gland.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: M. Bhattacharjee Visiting Fellow LPP, NCI
E. Ginsburg Biologist LPP, NCI

Project Description

Objectives:

The purpose of these studies is to investigate the role of various hormones, vitamins and growth factors in mammary gland development, differentiation and tumorigenesis. The roles of prolactin, thyroid hormones, adrenal steroids and vitamin D in milk protein synthesis and secretion is emphasized. The role of epidermal growth factor (EGF) and mammary tumor-derived growth factor (MTF) in lobulo-alveolar development of immature mouse mammary glands in whole gland culture and in vivo was examined. The purpose was to define the conditions involved in normal and hyperplastic development of the gland and production of mammary-derived growth factors.

Methods Employed:

C3H/HeN, NIH Swiss and BalbC mice and Long-Evans rats were used. Whole gland and organ culture was performed using chemically defined serum-free medium supplemented with various hormones, growth factors, and metabolic inhihibitors. Production of α-lactalbumin and casein was determined by radioprecipitation or radioimmunoassays using mono-specific antibodies prepared against the milk-proteins. Total RNA was obtained from mammary tissue and liver using a standard phenol extraction method. The mRNAs were obtained by using a polyU-Sepharose column. Translation of the mRNAs utilized a standard wheat germ assay. SDS-polyacrylamide gels were run after the method of Laemmli. Vitamin D deficient ricketic rats and mice were obtained from Dr. Schlomo Weintroub, NIDR. Lobulo-alveolar development of mammary glands was assessed by hematoxalin staining of whole mounted glands. Estrogen/progesterone (E_2/Prog) priming of 4 week old BalbC mice was performed by inserting a pellet (cholesterol: progesterone: estradiol 1001:1000:1) under the skin of the animals for the indicated period of time. Mammary-derived growth factor (MF) was isolated from mammary tissue by homogenization in PBS containing 2% Triton X-100 followed by centrifugation at 20,000 xg for 20 min. EGF levels and MF concentration in the tissue extracts were determined by standard RIA techniques and competition for ^{125}I-EGF binding to hepatic microsomal membranes.

Analysis of EGF binding to glands of E_2/Prog primed mice was performed by the method of Scatchard. Mammary tumor-derived growth factor (MTF) was either obtained from Dr. Jamie Zwiebel, LPP or prepared from primary NMU-induced rat mammary tumors according to his published acid-alcohol extraction procedure.

Major Findings:

Mid-pregnancy mammary tissue was cultured in the presence of insulin (I), hydrocortisone (F) and prolactin (PRL) in the presence and absence of thyroid hormones (T_3). After 48 hr in culture with IFPRL, an increased synthesis of

both α-lac and casein was observed. T_3 had little or no effect on casein synthesis when cultured tissue was examined after 48 hr. A 2-3 fold increase in α-lactalbumin was found in the tissue at this time. The media was examined for effects of the hormones on secretion of milk proteins. A low level of casein was found in media both in the presence and absence of T_3, but thyroid hormone did not enhance secretion of these peptides. α-lactalbumin was found in the medium to a significant extent with T_3 enhancing secretion 3-4 fold. Since α-lac is a secretory milk protein important in production of the milk sugar lactose, we examined synthesis, secretion and activity of both components of the lactose synthetase system (galactosyl transferase and α-lac) as well as the product lactose. Lactose synthetase activity and α-lac itself were enhanced by the presence of T_3 in the media. No such increase was observed for galactosyl transferase. Using ^{14}C-glucose as a substrate for endogenous lactose synthetase in mid-pregnancy explants, we found a 2-fold increase in lactose content of the culture medium with addition of T_3 to the cultures.

We examined the effects of various concentrations of hydrocortisone (F) on synthesis and secretion of milk-proteins in organ culture. The optimal concentration for casein synthesis was 10^{-6} M while α-lac synthesis was optimal between 10^{-8} and 10^{-7} M. At higher concentrations of F, α-lac synthesis was inhibited. The presence of T_3 in the cultures did not alter the concentration curves for F. Secretion of newly synthesized caseins and α-lac into the media was inhibited up to 60% by all concentrations of F examined in the presence of IFPRL. Addition of T_3 to the media did not overcome the inhibition of casein secretion but did overcome the inhibition of α-lac secretion at all concentrations of F even as high as 10^{-6}M.

The products of synthesis of milk proteins in organ culture were then characterized by SDS-PAGE. The immunoprecipitated products in both tissue and media were analyzed on 12% SDS gels. No differences in casein patterns were seen in either tissue or media in the presence or absence of T_3. However, the α-lactalbumin produced in tissue in the presence of IFPRL showed a single peak, while in the presence of T_3 two distinct peaks (I and II) were seen. Only a single peak was secreted into the medium even in the presence of T_3. The second peak seen in the tissue in the presence of T_3 is neither the milk protein Kappa nor whey acidic protein (WAP) reported by others. Previously we purified mouse α-lactalbumin from lactating mammary glands and found it to exist in 2 distinct charged forms (pI 6.2 and 5.8). Both forms have equal activity in the lactose synthetase assay system.

We then determined which of these two forms synthesized in the presence of T_3 was the one secreted into the media. Using parallel cultures labelled with either ^{14}C or ^{3}H-amino acids we co-electrophoresed immunoprecipitated products found in tissue and media in IFPRL ± T_3. The peak designated I (slower moving on the gel) was the one synthesized and secreted in the presence of IFPRL and secreted in the presence of IFPRLT_3.

Thus for quantitative as well as qualitative α-lactalbumin synthesis and secretion by mouse mammary tissue in organ culture, the presence of T_3 in the medium is essential.

Next we examined whether the two forms of α-lac synthesized in the presence of T_3 represents different types or quantities of carbohydrates on a common polypeptide core, or two distinct peptides. The drug tunicamycin inhibits core oligosaccharide addition of N-linked carbohydrates. It also can inhibit protein synthesis. Tissue was cultured in the presence of IFPRL ± T_3 with and without added tunicamycin. Cultures were labeled 48 hr with both ^{35}S-methionine and ^{3}H-galactose and ^{3}H-mannose. Total protein synthesis in all cases was inhibited 30-35%. Carbohydrate addition to proteins was inhibited by 45% in IFPRL and by 65% in IFPRLT_3. Synthesis and glycosylation of α-lac reflected that seen for total proteins. Both peaks of α-lac in tissue cultured in IFPRLT_3 were affected to the same extent.

We then utilized two enzymes endoglycosidase D and endoglycosylase H. Endo-D cleaves unsubstituted α-mannosyl residues linked to the innermost β-mannosyl residue by 1-3 linkage. Endo-H hydrolyzes "high mannose" oligosaccharides. Using these 2 enzymes (37°, 3 hr) we were able to show complete loss of the 2nd peak of α-lac immunoprecipated from tissue cultured in IFPRLT_3. Thus we felt that the two peaks were the same (or similar) polypeptides with different glycosylation patterns. This latter conclusion was supported by in vitro translation of mRNAs isolated from mid-pregnancy mammary tissue cultured in IFPRL ± T_3. The total cellular RNA was unaffected by the presence of T_3 while the fraction of mRNA was slightly increased. In the wheat germ translation system, the mRNA fraction from tissue cultured in the presence of T_3 synthesized twice the amount of α-lac as the mRNA fraction from tissue cultured in the absence of T_3. The level of α-lac synthesized was very similar to that from the mRNA of lactating mammary gland. Characterization of the products made in the wheat germ system by SDS-PAGE, showed only one peptide size for the lactating mammary gland or tissue cultured in the presence of T_3. Thus it appears that the 2 forms of α-lac seen in tissue cultured in IFPRLT_3 are not different sized α-lacs, but different charged.

Vitamin D metabolites are present in milk. Vitamin D is essential for bone formation in the young. Recently receptors for vitamin D have been found in human mammary cancer cells. Vitamin D has been reported to affect the synthesis and secretion of prolactin by pituitary cells in culture. Since the normal mammary gland in the source of milk for the young and a highly prolactin responsive tissue, we examined milk-protein synthesis in vitamin D deficient (ricketic) mice and rats. The vitamin D deficient diet did not affect the growth of the animals as the body weights of virgins and lactators on the D- diet did not differ significantly from the body weights of the D+ control animals. By whole mount staining and histological sections no differences in the mammary glands of D+ and D- mice or rats was detected.

Serum prolactin levels however were decreased 30% in D- mice (65.8 ng/ml vs 94.6 ng/ml in D+). Hepatic prolactin binding activity was reduced by 40% (D- binds 2812 cpm/mg membrane protein; D+ binds 4533 cpm/mg protein). When placed in explant culture, mammary glands from D- mice incorporate ^{3}H-thymidine into DNA in response to hormones at a similar rate as tissue from D+ mice. In the presence of insulin, prolactin, aldosterone and corticosterone, a 13 to 15 fold increase in DNA synthesis was observed compared to the absence of hormones.

The addition of T_3 to the other 4 hormones resulted in a 35 to 38 fold increase in both D- and D+ mice. Total protein synthesis was also unaffected with a 375 to 430% increase in ^{3}H-amino acid incorporation into TCA precipitable counts in both D- and D+ animals. However in response to the hormones, mammary explants from D- mice synthesized 10-20% less α-lactalbumin and 50-60% less casein. This effect could not be reversed by adding 10^{-6}M 1,25-dihydroxyvitamin D to the culture medium. However placing D- mice on a D+ diet 10 days prior to explantation of the mammary gland restored full hormonal responsiveness to the tissue.

SDS-gel electrophoresis of the caseins synthesized by explants of glands from D+ and D- mice, showed that the 44K casein was inhibited by 25-30%, the 40K casein by about 15% while the 27K and 22K caseins were unaffected. RIA of extracts of of lactating mammary tissue and milk collected from D- mice showed a 30-40% decrease in casein content and a 10-20% decrease in α-lactalbumin content. Extracts of lactating mammary glands and milk from ricketic rats showed a similar 20-30% decrease in casein and 10-15% decrease in α-lac contents.

These data clearly indicate that vitamin D plays an important role in normal lactagenesis and primarily functions in mammary gland differentiation rather than proliferation. Whether the vitamins acts directly on the mammary tissue is still not clear.

We further examined the nature of the adrenal steroids used in the mammary gland organ culture. Since hydrocortisone has a different concentration optimum for casein vs α-lac synthesis, we examined the use of the naturally accuring steroids aldosterone and corticosterone. Both of these steroids rise during pregnancy and lactation and fall after the pups are weaned. We found that by using insulin, prolactin, aldosterone and corticosterone (IPRLAC) in the culture rather than IFPRL, there is synchronous synthesis of casein and α-lac and both classes of milk proteins have the same optimal concentration for these steroids. DNA synthesis in mid-pregnancy mammary gland explants is 25-30% greater in IPRLAC vs IFPRL and in virgin tissue the incorporation of ^{3}H-thymidine in the presence of IPRLAC is twice that in the presence of IFPRL.

These data suggest that use of I, PRL, A and C in the presence of T_3 will result in milk protein synthesis in vitro which is qualitatively and quantitatively similar to that which occurs in vivo.

Aldosterone is a necessary component of culture media used to assess lobulo-alveolar development in whole gland cultures of immature mouse mammary glands. This sytem was used to assess the impact of epidermal growth factor (EGF) and mammary tumor-derived growth factors (MTF) on lobulo-alveolar development of the glands in the presence of I, PRL, F and A.

4 week old BalbC mice were implanted with E_2/Prog pellets. At various times the #2 thoracic glands were removed and cultured in the presence of IFPRLA ± EGF or MTF. After as long as 9 days of priming with the pellet, no detectable differences in mammary gland morphology were detected between primed and unprimed glands. days in culture with IFPRLA. Glands from animals primed for 9 days or longer had extensive lobulo-alveolar development after culturing with IFPRLA. However, when animals were primed for 6 days, the glands could develop if 60 ng/ml of EGF was

added to the culture media. This requirement for EGF was equally met by addition of a MTF obtained from Dr. Jamie Zwiebel of LPP. However, the growth obtained with MTF on occasion looked hyperplastic. This could reflect the origin of the MTF which is an extract of NMU or DMBAinduced rat mammary tumors. Platelet derived growth factor and fibroblast growth factor do not substitute for EGF. MSA will partially substitute for EGF.

We next determined if the lack of requirement of 9 day primed tissue for EGF reflects endogenous EGF carried into the culture by the tissue. Extracts of mammary and submaxillary glands were examined for EGF by RIA at various times after implantation of the E_2/Prog pellet. E_2/Prog priming resulted in a significant and progressive increase in EGF content of the submaxillary gland. A 4-fold greater concentration in EGF in primed vs unprimed animals was observed as early as 3 days after pellet implantation. No EGF was detected in the mammary extracts at any time point.

We examined two possibilities: 1) the tissue is very sensitive to very low (not detectable by RIA) levels of EGF and 2) the mammary gland in primed animals elaborates a growth factor for which EGF substitutes in culture. The first possibility was examined in 2 ways: Glands from E_2/Prog primed animals were tested for their ability to bind ^{125}I-EGF. Glands from 6 day primed animals bound 15-20 times more EGF/mg tissue than glands of unprimed mice. EGF binding was specific to epithelial rich regions of the gland and undetectable in fat pad free of epithelium.

Scatchard analysis of the binding to the epithelial rich region of E_2/Prog primed glands showed 2 classes of receptors with Kas of 1.25×10^9 M^{-1} and 3.6×10^9 M^{-1}. These values are very similar to those reported by others for epithelial cells of mid-pregnancy and lactating mammary glands.

Secondly, animals were primed for 9 days using testosterone pellets to elevate the EGF levels maximally or Alzet pumps containing EGF. In neither case were glands from these animals able to respond to IFPRLA in culture suggesting that elevated EGF alone is not sufficient to prime the animals. Anti-EGF antibody given to the animals during the E_2/Prog priming did not prevent the glands from becoming competent to respond to the 4 hormones after 9 days. Anti-EGF added the culture medium could not block the responsiveness of glands from 10 days E_2/Prog primed mice again suggesting EGF may be necessary, but not sufficient for the tissue to respond in culture.

Since MTF substitutes for EGF in culture and also can bind to EGF receptors, the mammary extracts which were negative for EGF were examined for the presence of a MF which competes for EGF binding to liver microsomes. No such MF was detected in extracts of fat pad or unprimed tissue. However MF was detected in epithelial rich regions of mammary glands primed with E_2/Prog for as little as 3 days and continued to increase up to 9 days (the latest time point examined).

This MF (from E_2/Prog primed tissue) was added to cultures of 6 day primed in the presence of IFPRLA. Lobulo-alveolar development was seen in the glands at a concentration of 3 ng/ml of MF (based on EGF binding competition).

Thus, we tentatively concluded that E_2/Prog priming enhances the synthesis of a MF and its receptors on mammary epithelial cells of immature female mice. To determine whether this is a direct effect of E_2 and/or Prog or is mediated through EGF we attempted to promote lobulo-alveolar development in unprimed glands using IFPRLA and various combinations of MTF, EGF and E_2/Prog. Neither factor alone or together or with E_2/Prog promoted lobulo-alveolar development after 10 days in culture. However, preliminary results indicate that in the presence of IFPRLA, a combination of MTF and EGF results in development of unprimed glands after 3 weeks in culture. This is consistent with the observation that MTF and EGF act synergistically to promote development in 6 day primed glands in culture.

Thus we tentatively conclude that lobulo-alveolar development of immature mammary glands in vitro requires both EGF and a mammary derived growth factor.

This conclusion is supported by preliminary results using cholesterol: EGF or cholesterol: MTF pellets inserted directly into the glands of 5 week old mice and allowed to remain in vivo for 2 more weeks. Neither type of pellet alone resulted in glandular development in vivo. Only when an E_2/Prog pellet was simultaneoulsly placed subcutaneously in the interscapular regions did lobulo-alveolar development of the EGF or MTF containing glands occur. The E_2/Prog pellet alone had no effect on the glands' development.

Significance to Biomedical Research and the Program of the Institute:

Prolactin-thyroid interactions are important in growth and differentiation of mammary glands. Altered thyroid status may be implicated in risk of human breast cancer. It is not yet clear from the literature whether the effects of thyroid hormones are primary (i.e. directly on the mammary gland) or secondary (i.e. through alterations in other homones). Therefore, all aspects of thyroid hormone control of growth and differentiation of the normal gland as well as development of mammary tumors must be understood. The development of hyperplastic alveolar nodules (HAN) in mouse mammary glands has been correlated with subsequent mammary tumor development. Thus, we wish to examine those growth factors (both from normal and tumor tissue) which lead to lobulo-alveolar development and HAN formation in mammary glands in culture. The ability of unprimed tissue to respond to hormones and growth factors in defined culture medium affords us a unique opportunity to study mechanisms for controlling development and differentiation of the HAN.

Proposed Course of Research:

We will continue to study the effect of thyroid and adrenal hormones on milk protein synthesis and secretion. The nature of the MF involved in lobulo-alveolar development and HAN formation will be examined. MFs from normal (E_2/Prog primed) glands as well as mammary tumors will be characterized in collaboration with Dr. W. R. Kidwell's group. Development of HAN in culture and their neoplastic potential will be examined under controlled conditions of hormones and growth factors.

Publications:

Vonderhaar, B.K.: Effect of thyroid hormones on mammary tumor induction and growth. In B.S. Leung (Ed.): Hormonal Regulation of Experimental Mammary Tumors, Vol. II: Peptide and Other Hormones, Eden Press, Montreal, Canada, 1982, pp.138-154.

Vonderhaar, B.K. and Greco, E.A.: Effect of thyroid status on development of spontaneous mammary tumors in primiparous C3H mice. Cancer Res. 42: 4553-4561, 1982.

Bhattacharjee, M. and Vonderhaar, B.K.: Purification and characterization of mouse α-lactalbumin from lactating mammary glands. Biochim. Biophys. Acta., 755: 279-286, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08229-07 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of Dietary Lipids in Mammary Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
PI: W. R. Kidwell Chief, Cell Cycle Regulation Section LPP, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Branch

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

CHECK APPROPRIATE BOX(ES)

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

C

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

Growth regulation of the mammary epithelium of both normal and neoplastic states is a complex process involving the interaction between the epithelium and stromal cell populations, including the adipocytes. These interactions may be important in the process of preneoplastic to neoplastic conversion of mammary epithelium and the role of dietary lipids in this process. Experiments to date indicate that the epithelium is dependent on essential fatty acids for proliferation and that prolactin stimulated epithelium recruits these fatty acids from mammary adipocytes. Prolactin's role in this process appears to be mediated by signals from the epithelium directed at mast cells in the gland. The activated mast cells release histamine and this compound then triggers the release of fatty acids from the proximal adipocytes. The prolactin activated epithelial cells then selectively take up the unsaturated fatty acids. Part of these are inserted into membrane phospholipids with the consequent stimulation of cell growth. Some of the essential fatty acids are converted to prostaglandins. Of these, prostaglandin E_1 is a potent growth stimulator of the epithelium. The essential fatty acids thus appear to be important for mammary cell growth by serving as membrane structural components and as substrates for prostaglandin synthesis.

Other Professional Personnel: None

Project Description

Objectives:

Dietary lipids are known to have a significant impact on the development of breast cancer in humans and in experimental animals treated with carcinogens. Thus far this effect seems to be unrelated to circulating hormonal levels but may be due to a direct effect of the lipids on the glandular epithelium. These studies are designed to assess the interrelationship between the mammary fat cells and the epithelium in the growth response of the epithelium to hormonal stimulation.

Methods Employed:

Mammary ducts and alveoli were isolated from human, mouse or rat tissues or tumors by collagenase digestion and selective membrane filtration procedures. Cell cultures were grown in serum-free, lipid defined medium to establish growth responses to fatty acids. The uptake or release of free fatty acids by tissues or cells in culture was determined by fractionation of the growth media lipid fraction on thin layer plates followed by derivitization and analysis by high performance liquid chromatography or gas-liquid chromatography. Anti-oxidants were included in the extraction, fractionation and derivitization steps and the quantitites of fatty acids found were adjusted for losses utilizing recoveries of internal standards.

Major findings:

1. Prolactin stimulates the release of free fatty acids from mammary adipocytes. As little as 5×10^{-10} M prolactin effects the release of free fatty acids from explants of normal mammary tissue. The released fatty acids are almost certainly derived from the adipocytes of the explant since prolactin stimulates uptake rather than release of fatty acids by purified mammary epithelium in culture. Since the prolactin receptors of the mammary gland are confined to the epithelium the prolactin effect must be manifest through the epithelium.

2. Histamine may be an intercellular signal which functionally couples adipocytes and epithelial elements of the gland. The ability of prolactin to stimulate free fatty acid release from explants of mammary tissue containing both epithelium and adipocytes is blocked by Benadryl, an antihistamine which acts through H1 receptors on fat cells. Furthermore, histamine at physiological concentrations (2×10^{-8} M) is a very effective stimulator of free fatty acid release from mammary fat cells.

3. The histamine which triggers adipocytes to release fatty acids probably originates from mast cells in the mammary gland. Mast cells are found in abundance in the mammary gland and are especially abundant in hormone dependent mammary tumors. Some mast cells have been found in close association with the

glandular epithelium, separated from it only by a basement membrane. Purified preparations of epithelium contain about 1 mast cell per 1000 epithelial cells. When the mast cells are removed by serial passaging of the epithelium on collagen gels, prolactin does not stimulate histamine production. If, however, the mast cells are present, prolactin stimulates histamine release in amounts which are capable of stimulating free fatty acid release from mammary fat cells.

4. The assimilated essential fatty acids probably facilitate growth by serving both as structural components and as substrates for prostaglandin formation. Comparison of membrane phospholipid acyl groups of resting and proliferating

epithelium have shown that a 2 fold enrichment in the abundance of linoleic acid in phosphotidyl ethanolamine results following growth initiation. This compositional change may favor nutrient transport or hormonal receptor function. Additionally linoleic acid serves the well known function of substrate for prostaglandin synthesis. Prostaglandins E_1, E_2, $F_{2\alpha}$ and I_2 were screened for their growth effects on isolated epithelium. In concentrations from 1-10μg/ml culture medium only the E_1 was found to be appreciably active.

Significance to Biomedical Research and the Program of the Institute:

In attempts to understand the role of dietary lipids in the development of breast cancer we have begun to analyze the types, quantities and sources of fatty acids taken up by mammary epithelium. Our studies indicate a special role of unsaturated fatty acids in the physiology of the glandular epithelium. These fatty acids are apparently recruited from proximal mammary adipocytes which release their stores of fatty acids in response to an indirect signal from hormonally stimulated epithelium. The fat cells of the gland provide an effective buffer which normally restricts the availability of unsaturated fatty acids to the epithelium. Exceeding the buffering capacity such as may occur in individuals on high fat diets may sensitize mammary epithelium to basal levels of circulating hormones and thereby increase the proliferation rate of the epithelium and expand the population at risk to transformation.

Proposed Course of Research:

It has already been demonstrated that histamine levels in the mammary gland are directly correlated with the degree of mammary cell proliferation. Furthermore, it has been demonstrated that antihistamines block the proliferation of the mammary epithelium in experimental animals. We will attempt to determine whether the effects of Benadryl seen *in vivo* are the result of a direct action on the mammary gland. For this purpose we will assess the effects of Benadryl on the proliferation of the mammary epithelium in explant cultures in which both the epithelium and adipocytes are present as well as determining whether the antihistamine affects the proliferation of the isolated epithelium. Additionally we will examine the effects of antihistamines on the growth of hormone dependent and independent rodent mammary tumors.

Publications:

Kidwell, W.R.: Prolactin effects on fatty acid metabolism by isolated mammary epithelial cells. J. Amer. Fat & Oil Chemists Society 1983. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08230-07 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Hormonal Control of Mammalian Follicular Maturation and Oogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

PI: R.A. Knazek Senior Investigator LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.85	1.0	.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

Synthesis of prostaglandin (PG) $F_{2\alpha}$ by the ovary is known to be necessary for ovulation to proceed in an orderly fashion. Our studies have demonstrated that FSH stimulated the synthesis of prostaglandins (PG) E and $F_{2\alpha}$ by rat ovarian granulosa cells in a dose-related fashion augmented by exogenous hCG. PRL also exerted marked effects on PG synthesis. Physiologic amounts of PRL increased the rates of PGE and $PGF_{2\alpha}$ synthesis whereas injections of supraphysiologic amounts of PRL decreased the rates of synthesis. The stimulation by PRL over such a narrow concentration range is in agreement with *in vitro* studies on ovarian steroidogenesis. The role of PG in ovarian function was also studied using a newly devised assay for prostaglandin receptors which allowed further study of the role of PG in ovarian sterodogenesis and ovulation. Suppression of endogenous PRL secretion by bromoergocryptine or hypophysectomy caused an increase in the number of PG receptors in ovarian membranes, an effect reversed by replacement of PRL in physiologic amounts. Supraphysiologic concentrations of prolactin resulted in a marked decrease in the number of PG receptors. The observations suggest that prolactin exerts its effects through the PG cascade and explains, in part, the mechanism by which infertility occurs in hyperprolactinemic women.

Other Professional Personnel: S.C. Liu, Chemist, LPP, NCI
J.R. Dave, Visiting Fellow, LPP, NCI
J.W. Karanian, Staff Fellow, LPP, NCI

Project Description

Objectives:

Describe the effect of prolactin upon both prostaglandin receptors and prostaglandin synthesis in ovary as a mechanism by which this mammotrophic hormone modulates ovarian function.

Methods Employed:

Female Sprague-Dawley rats are hypophysectomized (HIFR) at 21 days of age and implanted subcutaneously with a silastic-encased diethylstilbestrol (DES) pellet. The presence of high concentrations of this estrogenic substance in the absence of gonadotrophins stimulates the proliferation of granulosa cells. Dispersal of such ovaries can be achieved by forcing the tissues through a stainless steel wire mesh and resuspending the granulosal isolates in incubation buffer. Mature animals were also treated with gonadotrophins prior to treatment with bromoergocryptine and/or graded doses of PRL. Microsomal membranes were prepared from homogenates of ovaries from these latter animals and used for PG receptor studies.

An assay procedure for prostaglandin E and F has been devised consisting of organic solvent extraction, silicic acid column chromatography, and specific radioimmunoassay. Serum progesterone levels were determined by specific radioimmunoassay.

Membrane suspensions were incubated with graded concentrations of [^{3}H]$PGF_{2\alpha}$ with or without 1.6 µM $PGF_{2\alpha}$ in 10 mM Tris, pH 7.5, for 2 hr at 22°C to provide binding data for Scatchard analyses. Values of specific binding were obtained by incubating membrane suspensions with 8.5 nM [^{3}H] $PGF_{2\alpha}$ ± 1.6 µM $PGF_{2\alpha}$ for 2 hr at 22°C. After incubation, the membrane-PG mixture was mixed for 5 minutes at 22°C with an equal volume of 2% charcoal-0.05% dextran suspended in 10 mM tris, pH 7.5, to adsorb the unbound PG. A 250 µl aliquot of this membrane-PG-charcoal mixture was then layered atop 150 µl of a 2.2M sucrose, 10 mM Tris cushion, pH 7.5, that had been prepared previously in the microfuge tubes. The samples were then centrifuged for 3 minutes at room temperature and then cut at a point mid-way between the tops of the sucrose buffer and the charcoal pellet. The charcoal-containing lower half, to which the free prostaglandin had absorbed, was discarded while the top portion of the tubes containing the membranes and their bound prostaglandins were counted in a scintillation counter.

Another method of radioreceptor assay was also used to measure $PGF_{2\alpha}$ binding to ovarian tissues. This entails a filtration procedure used in our studies on mammary tumor PG receptors (Z01 CB 05219-12 LPP). After incubation with [^{3}H]$PGF_{2\alpha}$ ± excess unlabeled $PGF_{2\alpha}$ for 75 minutes at 22°C, aliquots of the membrane suspension were placed on a filter disc to separate bound and free PG. The filters were

then washed with buffer and counted in a scintillation counter.

Major Findings:

Patients suffering from hyperprolactinemia are found to be anovulatory and/or amenorrheic. The reason for this association has been unknown. Studies by other investigators have shown that the absence of $PGF_{2\alpha}$ within the ovarian complex prevents release of the ovum in such a manner that would permit subsequent successful fertilization. In addition, Gullino has shown that Prostaglandin E induces neovascularization when implanted in the cornea of the rabbit. Other studies have shown that follicular neovascularization follows the ovulatory surge of LH, a necessary event in forming the well-vascularized corpus luteum, and that women suffering from hyperprolactinemia have an inadequate luteal phase. These observations suggested to us that prolactin might be exerting its effects on the ovary through modification of the PG pathways and prompted the following studies.

Granulosa cells obtained from hypophysectomized, immature, female rats previously treated with diethylstilbesterol implants and various s.c. injections of oFSH, PMSG, hCG, oPRL were incubated at 37°C for 2 hrs $\pm$ 10^{-5} M indomethacin. Prostaglandins E and F_2 were extracted from the suspensions, partially purified by chromatography on silicic acid columns and then quantitated by specific RIA. The amounts of PGE and $PGF_{2\alpha}$ synthesized by the DES-treated controls were not significantly altered by treatments with a single injection of 5 I.U. hCG and/or 100 µg oPRL every 4 hours for 2 days prior to sacrifice. Three injections of 100 µg oFSH, however, markedly increased the rates of synthesis of PGE and $PGF_{2\alpha}$. These rates were further increased by 40-100% when a single injection of hCG was added to the FSH treatment. Addition of hourly injections of 50 µg oPRL to either the FSH or FSH $\pm$ hCG schedule, however, decreased the synthesis of PGE by 79-80% and $PGF_{2\alpha}$ by 35-56% during the two day treatment period. Doses of oPRL ranging from 0-50 µg were also injected hourly for 48 hours into PMSG + hCG treated animals. This revealed that PG syntheses in the 1.0 µg oPRL group being stimulated to 240-290% that of animals receiving no oPRL. Progesterone levels rose from 0 to 1 ng/ml in the sera of PMSG + hCG-treated animals as the dose of PRL was raised from 0 to 50 µg/hr. These data suggest that the euprolactinemic and hyperprolactinemic states modify ovarian steroidgenesis and the ovulatory process by either accentuating or suppressing the FSH-related stimulation of prostaglandin synthesis.

The mechanism by which prostaglandins exert their effects are unknown. However, for these locally active hormones to be effective, they must bind to a receptor site within the ovary. Attempts to study PG binding have resulted in a rapid and highly reproducible PG receptor assay. The method, rapid and reproducible, has yielded Scatchard curves compatible with either negative cooperativity or the presence of both high and low affinity binding sites having K_d = 5.8 and 77 nM, respectively. Under the conditions of assay, free $PGF_{2\alpha}$ was bound irreversibly to the charcoal. There was no significant diffusion of either membrane-associated or charcoal-associated $PGF_{2\alpha}$ into the sucrose layer for up to 20 minutes after centrifugation, thereby removing any urgency for manipulation of the assay tubes. The PG-receptor complex was shown to be stable for at least 30 minutes, PG not being stripped from the membrane receptor. These observations

demonstrate that the speed and stability of the assay are such that large numbers of samples can be studied within a short period of time.

Additional investigations showed that binding to the membranes reached equilibrium within 90-140 minutes when incubated at 22°C and was partially reversible with excess $PGF_{2\alpha}$ displacing ~ 70% of the previously bound $PGF_{2\alpha}$ within a 3 hour period. $PGF_{1\alpha}$ and PGE_1 also displaced $PGF_{2\alpha}$ but to a lesser degree while PGA_2 did not displace $PGF_{2\alpha}$ from its receptor. When unlabeled prostaglandins E_1, A_2, $F_{1\alpha}$ or $F_{2\alpha}$ were mixed with [^{3}H] $PGF_{2\alpha}$ at the beginning of the initial 75 minutes incubation at 22°C, similar results were observed. For $PGF_{2\alpha}$ to bind to ovarian membranes, prior exposure to gonadotrophins in vivo was required, maximal values being obtained 3-5 days after the first injection of FSH. Concomittant daily injections of 1 mg bromoergocryptine caused a two-fold increase in specific binding of $PGF_{2\alpha}$ after three days of treatment. Injection of 1, 10, 100, or 1000 µg PRL every 4 hours for 2 days resulted in a suppression of PG binding when the 10, 100, and 1000 µg doses were used. Further studies demonstrated that alteration of prolactin levels in vivo modulate the ability of rat ovarian membranes to bind $PGF_{2\alpha}$. Mature female rats were either hypophysectomized or injected with 1 mg bromoergocryptine (CB154) every 12h x4d. Graded doses of oPRL (0,0.1, 10, 100µg) were injected s.c. every 4h x3d prior to sacrifice. All animals had received gonadotropin priming before sacrifice. Ovarian microsomal membranes were incubated with graded concentrations of ^{3}H-$PGF_{2\alpha}$+1.6µM $PGF_{2\alpha}$ in 10mM Tris, pH 7.1, for 75 min at 22° to yield data suitable for Scatchard analysis or with 8.5nM ^{3}H-$PGF_{2\alpha}$+1.6µM $PGF_{2\alpha}$ for specific binding data. Specific binding was increased 2.5-fold by CB154-treatment 10 or 100µg oPRL every 4h into hypophysectomized rats decreased binding 2.5- and 4-fold. However, low doses in the intact CB154-treated group 3- and 2-fold, respectively. Similarly, an increase of 50% was observed in the 1µg oPRL-treated hypophysectomized group. These data reflect changes in binding capacity. The data demonstrate that the numbers of $PGF_{2\alpha}$ receptors in ovarian membranes increase when exposed to low or physiologic concentrations of prolactin but decrease when subjected to pathologically high levels of prolactin.

The above studies indicate that prolactin modifies both prostaglandin synthesis and binding in the PRL-responsive ovary. This may be the mechanisms through which prolactin acts and may also prove to be the physiologic mechanism by which PRL receptor modulation is achieved. Extension of these observations to other PRL-responsive tissues is currently under investigation.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of the mechanism by which prolactin acts upon the ovary will enhance our understanding of the role of this mammotrophic hormone in the development of both mammary carcinomata and the pathologic states which predispose individuals to its occurrence.

Proposed Course of Research:

Granulosa cells subjected to various hormone manipulations will be studied in detail with special attention to alterations in the enzymes and intermediates within the prostaglandin cascade and the modulation of prostaglandin receptor activity.

Publications:

Rotondi, A. and Knazek, R.A.: A rapid method for the measurement of prostaglandin receptors. Prostaglandins Leukotrienes and Medicine 9: 45-53, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08249-04 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Hormonal Control of Growth of Normal and Neoplastic Mammary Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: William R. Kidwell, Chief,Cell Cycle Regulation Section LPP, NCI

COOPERATING UNITS *(if any)*

Laboratory of Tumor Immunology and Biology, NCI

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cell Cycle Regulation Section

INSTITUTE AND LOCATION

NCI, NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Studies with proline analogs, which selectively block collagen deposition and thus cause the production of a faulty basement membrane, have been found to cause a regression of mammary tumors that produce basement membranes. Similarly, normal mammary epithelium which also produces this extracellular matrix material is caused to involute by these proline analogs. These studies have suggested that production of basement membranes is important for the growth and survial of normal and neoplastic mammary epithelium. Consequently we have began a series of experiments to evaluate the mechanisms by which production of basement membrane collagen is controlled. The results indicate that at least 3 major mechanisms exist: (1) Modulation of collagen turnover which is suppressed by glucocorticoids (2) increased synthesis as a consequence of cell contact with "foreign" surfaces (3) increased synthesis in response to growth factors and hormones. In the latter category is a new class of growth factors which we have discovered. These are produced by and autostimulate mammary tumor cells to enhance their rate of collagen production. One such factor has been partially purified from rat, mouse and human mammary tumors and human milk. The human milk and human mammary tumor factors have the same PI and are probably identical. The rodent factors have a different PI. These factors differentially stimulate collagen synthesis by as much as 10 fold and are believed to be important in the growth control of mammary tumors. Further studies are underway to develop a radioimmuoassay for the factors for screening for their presence and possible function in mammary tumorigenesis.

Other Professional Personnel:			
	M. Bano	Visiting Fellow	LPP, NCI
	J. Zwiebel	Res. Associate	LPP, NCI
	D. Salomon	Expert	LTB, NCI
	B. Vonderhaar	Res. Biologist	LPP, NCI

Project Description

Objectives:

Studies with proline analogs that selectively inhibit collagen deposition have been found to block the growth of differentiated mammary tumors that make a basement membrane, indicating that this extracellular matrix is important for tumor cell growth and survival. These studies are designed to elucidate the growth factor and hormone requirements for collagen synthesis by tumor cells and whether such factors are autoregulated by growth factors produced by the tumors themselves.

Methods Employed:

Collagen synthesis stimulating activities have been extracted from rat, mouse and human mammary tumors with acidified ethanol. Factor purification included gel filtration, ion exchange column chromatography, isoelectric focusing and gel electrophoresis. Effects on collagen synthesis were assessed by quantitating the relative amount of ^{14}C-proline incorported into collagenase sensitive protein in mammary cell cultures or alternatively measuring the mass of 4-hydroxy-proline proline and hydroxlysine formed by the cell cultures.

Major Findings:

1. The collagen synthesizing stimulating activity of rat mammary adenocarcinomas has been purified to approximate homogeneity by selective extraction, isoelectric focusing and gel electrophoresis. The factor is a protein with a molecular weight of 68,000 and a PI of 5.9. A survey of a variety of mammary tumors indicates that the factor is produced by well differentiated mammary tumors but not be poorly differentiated mammary tumors that do not contain myoepithelial cells and consequently do not synthesize a basement membrane. In vitro translation studies have indicated that the abundance of m-RNA for collagen is not increased in cells by the factor. Since pulse-chase experiments failed to demonstrate any effects of the factor on collagen turnover it appears that the factor enhances the efficiency of collagen m-RNA translation by some mechanism.

2. A collagen synthesis stimulating activity has been detected in spontaneous and virally induced mouse mammary tumors. Utilizing a similar purification method as for the rat mammary tumor factor, we have detected considerable activity in mouse mammary tumors. The activity enhances collagen synthesis by as much as 5 fold over total protein synthesis. Comparison of the effects of the rat and mouse tumor factors indicates that there is a species specificity since mouse cells respond about 2-3 fold more to the mouse tumor factor than to the rat factor.

3. Primary human mammary tumors and human milk contain collagen synthesis stimulating activities. Human mammary tumors were found to possess large amounts of collagen synthesis stimulating activities. The activity was also extractable from human breast tumor cells (MCF-7 and Clouser) grown as solid tumors in nude mice. The human tumor factor has been partially purified by isoelectric focusing. The major peak of activity has a pI of 4.8. A factor with an identical PI has been detected in human milk. This observation suggests that the collagen synthesis stimulating activity may be important for normal mammary cell and tumor cell growth.

4. Both normal and neoplastic mammary epithelium are dependent on proline for for optimal growth. Studies with mammary epithelium in explant culture or as purified epithelial cells has shown that proline stimulates mammary epithelium growth and differentiation (casein synthesis, α-lactalbumin synthesis and galactosyl transferase synthesis) by 4-20 fold. The proline dependency appears to be a consequence of the increased requirement for the formaation of a new basement membrane by the proliferating epithelium since proline supplementation to cultures of these cells causes them to differentially increase collagen and laminin production by 2-7 fold.

5. Formation of basement membrane is one of several pleotrophic responses of mammary cells to growth factors. Fourteen growth factors and hormones have been screened for their effects on collagen production by isolated mammary cells. Of these, 10 stimulate mammary cell growth and differentially enhance the rate of collagen production. Only 1 factor stimulates cell growth without differentially enhancing the rate of collagen production. Three factors which did not stimulate cell proliferation did not enhance collagen production rates. These studies indicate that there is an automatic activation of the collagen synthetic potential as a consequence of initiation of cell growth.

Significance to Biomedical Research and the Program of the Institute:

We have demonstrated that blocking collagen synthesis leads to the growth arrest of mammary adenocarcinomas. Further studies of collagen synthesis may provide a variety of methods by which tumor growth arrest can be accomplished in the breast cancer patient. These studies should provide fundamental information about the role of collagen in the growth and differentiation of normal breast epithelium and the significance of collagen production by neoplastic cells derived from it.

Proposed Course of Research:

Major effort during the coming year will be on the purification of human mammary collagen synthesis stimulating activity. Since the factor is present in abandance in human milk this will be utilized for the purification. Following purification we will attempt to develop monoclonal antibodies to the factor for use in quantitating the factor in human breast tumor tissues.

Publications:

Kidwell, W.R., Liotta, L.A., Zwiebel, J.A., Salomon, D.S. and Bano, M.: Effects of growth factors on mammary epithelial cell growth and basement membrane synthesis. In Sato, G. and Sirbasku, D. (Eds.): Growth of cells in hormonally defined medium. Cold Spring Harbor Series on Growth Regulation, Vol. 9., 807-818, 1982.

Kidwell, W.R., Bano, M. and Salomon, D. Growth of mammary cells on collagen surfaces in serum free medium. In Barnes, D. and Sato, G. (Eds.) Cell culture Techniques. Alan R. Liss, Inc., N.Y. In press.

Bano, M., Salomon, D.S., Zwiebel, J. and Kidwell, W.R.: Detection and partial characterization of collagen synthesis stimulating activities in mammary adenocarcinomas. J. Biol. Chem. 258: 2729-2735, 1983.

Salomon, D.S., Bano, M. and Kidwell, W.R. Isolation and characterization of a growth factor, embryonin, from bovine fetuin. J. Biol. Chem. 257: 14093-14101, 1982.

Salomon, D.S., Smith, K.B., Losonczy, I., Kidwell, W.R., Alessandri, G. and Gullino, P.M.: α_2-macroglobulin, a contaminant of commercially prepared pedersen fetuin: Isolation, characterization and Biological Activity. In Barnes, D. and Sato, G. (Eds.). Cell Culture Techniques. Alan R. Liss Inc., New York. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08250-03 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Fracture-label: Cytochemical labelling of freeze-fractured membranes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: P. Pinto da Silva Chief, Membrane Biology LPP, NCI

COOPERATING UNITS *(if any)*

Dr. C. P. Leblond, Department Anatomy, McGill University, Montreal, Canada

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.72	0.61	.11

CHECK APPROPRIATE BOX(ES)

☐ (a) Human 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 developing a variation of the fracture-label technique--freeze-fracture radioautography--to study the various membrane components of cells and tissues. Cells are fixed in glutaraldehyde, frozen and fractured in liquid nitrogen and subsequently labelled with radioactive lectins or other radioactive cytochemical labels. Radioautograph preparations are examined by light and electron microscopy. The work is in progress and we believe such novel technique will help to reach a better understanding of the molecular architecture of biological cell membranes and the importance of various membrane components to the function of orgon, cells and organelles.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	F. Kan	Visiting Fellow	LPP, NCI
	C. Parkison	Chemist	LPP, NCI

Project Description

Objectives:

Development of a novel technique--Freeze-fracture radioautography-to study the various membrane components of cells and tissues.

Methods employed:

Suspensions of glutaraldehyde-fixed cells (T-lymplocytes and peripheral blood cells) were frozen, fractured in liquid nitrogen and subsenquently labelled with various radioisotopes target for the cell membranes. Cell samples were then dehydrated, embedded in Epon and sectioned. Semi-thin and ultra-thin sections were coated with nuclear emulsion and exposed in the dark at 4°C for reasonable periods of time. Radioautograph preparations were examined by light and electron microscopy.

Major Findings:

Giving continuation to this project, freeze-fracture radioautography--a variation of the fracture-label technique--is now being developed. The preliminary results suggest the feasibility of such method.

Significance to Biomedical Research and the Program of the Institute:

Freeze-fracture and radioautography may be combined to localize the individual membrane components in order to provide a better understanding of the molecular architecture of biological membranes and the importance of various membrane components. (Glycoproteins, glycolipids, etc) to the function of organs, cells and organelleo. Such novel technique may also be used to study the distribution of various lipids in the two leaflets of the unit membrane, what at present is not attainable by other existing techniques.

Proposed Course of Research:

The work is in progress.

Publications:

Pinto da Silva, P.: Freeze-fracture cytochemistry: labelling of plasma and intracellular membranes. Cienc. Biologica (Cell and Mol. Biol.) 7: 1-11, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08251-04 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Growth Factor Production by Neoplastic Rat Mammary Epithelial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
W. R. Kidwell, Chief Cell Cycle Regulation Section LPP NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.	1.5	0.5

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

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

The ability of cells to proliferate independently of a surface substratum is a property that distinguishes transformed cells from normal cells. Current thinking is that this ability of tumor cells is brought about by the production in tumor cells of anchorage-indepent growth conferring factors, or transforming growth factors (TGF). New observations that we and others have made indicate that normal tissues in addition to tumor tissues can make TGF. For example, we have found that TGF activities are made by or accumulate in proliferating bovine mammary gland, in rat, mouse and human adenocarcinomas and in fact are present in large amounts in human milk. The TGF activities in human milk and human mammary tumors have been partially purified. The major species from the two sources have identical PI's and are probably the same protein. TGF activities have also been detected in several clones of MCF-7 and Clouser, human tumor cell lines, grown in nude mice. These findings indicate that tumor cells need not necessarily make their own TGF to express the anchorage-independent growth phenotype but rather upon transformation they can respond to factors normally made by, and probably physiologically important to the host. Consistent with this concept is our finding that some rodent mammary carcinomas and 1 human mammary tumor contained no detectable TGF activity.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	J. Zwiebel	PHS Fellow	LPP, NCI
	M. Bano	Visiting Fellow	LPP, NCI
	D. Salomon	Expert	LTB, NCI

Project Description

Objectives:

Various investigators have suggested that the anchorage-independent growth phenotype which is characteristic of transformed cells is produced by growth factors, transforming growth factors, produced when cells become transformed. This hypothesis is being tested by analyzing for the presence of transforming growth factor activities in both normal and neoplastic human and rodent mammary tissues.

Methods Employed:

Anchorage-independent growth-conferring factor activities (TGF) were measured by suspending normat rat kidney cells, 3T3 cells or chick embyro fibroblasts in soft agar plus or minus TGF activities prepared from various sources. After two weeks growth colonies were stained with NBT and counted on an electronic colony counter. TGF activities were extracted from tissues or fluids by the acid-ethanol technique of Roberts et al. and further purified by gel filtration and isoelectric focusing.

Major Findings:

TGF activities are detectable in some human mammary tumors and in human milk. Isoelectric focusing of human milk (with or without casein removal) revealed a major peak of TGF activity with a PI of 4.0-4.1. The activity was present in 3 of 3 batches of milk tested and appears to be distinct from human EGF (Urogastrone) which has a PI of 4.5. This activity was also seen when extracts of human mammary tumors were isoelectrically focused. Three primary human tumor extracts were tested for TGF activity and two of these were highly positive and 1 negative. A second activity present in large amounts in human mammary tumors and human milk was collagen synthesis stimulating activity (CSSF). This activity had a PI of 4.8. Thus the CSSF and TGF are molecularly distinct from one another. TGF and CSSF activities were also produced by the MCF-7 and Clouser human mammary cell lines, the former in culture or grown in nude mice and the latter grown in nude mice. Ten MCF-7 cell variants supplied by J. Schlom's, Lab. Tumor Immunology and Biology were screened for these two activities and for EGF receptor binding activities. The relative amounts of the activities in the clones varies dramatically and showed no relationship to each other. These results tend to confirm the findings by other laboratories that TGF activities exist that differ from sarcoma growth factor in that an interaction with EGF receptors is not required for achieving an anchorage independent growth phenotype.

Significance to Biomedical Research and the Program of the Institute:

Our results suggest that growth factors produced by normal cells may function as TGF activities. In addition the work suggests that tumors may be dependent on non-tumor derived growth factors for displaying the anchorage-independent growth phenotype. These factors supplied by the host may thus be important, not as initiators but as progression factors.

Proposed Course of Research:

The TGF activities of human and rodent sources will be further purified with the objective of developing radioimmunoassays for the specific entities. When such a reagent is obtained the possible role of the TGFs in mammary tumorigenesis will be evaluated by a) screening human breast tumor tissues and b) by comparing the amount of TGFs present in milk and breast tissue from rodent lines with high and low frequency of development of mammary tumors.

Publications:

Zwiebel, J., Davis, M., Kohn, E., Salomon, D and Kidwell, W.R.: Anchorage-independent growth conferring factor production by rat mammary tumor cells. Cancer Res. 42: 5117-5125, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08264-03 LPP

PERIOD COVERED

October 1, 1982 to March 25, 1983

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

Cyclic Nucleotide and Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: Y.S. Cho-Chung Chief, Cellular Biochemistry Sec. LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cellular Biochemistry Section

INSTITUTE AND LOCATION

NIH,NCI, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	1.50	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

Carcinogenic doses of 7,12-dimethylbenz(α)anthracene (DMBA) failed to induce mammary carcinomas in the rats that have received $N^6,O^{2'}$-dibutyryl cyclic adenosine 3',5'-monophosphate (DBcAMP) (Proc AACR 22:76, 1981). We now report that the anticarcinogenic effect of DBcAMP correlates with its effect on DNA binding of the carcinogen and on gene expression. The log phase mammary epithelial cell cultures were used to determine the effect of DBcAMP on DMBA binding to DNA. We observed that DBcAMP inhibits the covalent binding of DMBA to DNA of mammary epithelial cells of the carcinogen-susceptible 50-day-old rats but not that of the unsusceptible 35- and 110-day-old virgins. The inhibitory effect of DBcAMP was appreciable at the concentration of 10^{-7} M, 1/10 the concentration of [^{3}H]DMBA. DBcAMP at 10^{-6} M concentration exhibited the maximal inhibition of DMBA binding: the binding in the 50-day-old virgin epithelial cells was reduced to the level of the bindings observed in the epithelial cells of 35- and 110-day-old virgins. The inhibitory effect of DBcAMP on DMBA binding to DNA reflected in the genomic expression. The *in vitro* translation products of poly(A)+ RNAs from mammary glands of young virgin rats (50-day-old) differed from those of old virgins (110-day-old). The difference was localized in four protein bands of the electrophoretic pattern that increased in their concentration and one protein band that decreased in the old virgin glands. DBcAMP administered *in vivo* altered the genetic transcripts of the young virgin glands to become similar to that of the old virgin glands. DMBA feeding did not appreciably alter the translation protein pattern of the young virgin glands. However, when DMBA was fed to the young virgins that had received DBcAMP, the translation pattern became half-way between those of the young and old virgin glands. The anticarcinogenic effect DBcAMP appears to involve a modification of both carcinogen binding to DNA and the gene expression.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	T. Hasuma	Visiting Fellow	LPP, NCI
	F.L. Huang	Expert	LPP, NCI

Project Description

Methods Employed:

1. DMBA intubation: Sprague-Dawley female rats (50 days old) were given a single intubation of 7,12-dimethylbenz(α)anthracene (DMBA) (20mg in 1 ml sesame oil).

2. DBcAMP administration: DBcAMP (10 mg/200g rat/day), beginning 1 day prior to DMBA intubation.

3. Cell cultures: Mammary epithelial cell cultures were obtained by the method of Wicha et al. (Cancer Res. 39: 426, 1979). Cells were in MEM containing 4% FCS for 3 days before changing into serum-free medium for the binding studies.

4. DMBA binding: [^{3}H]DMBA was dissolved in DMSO and added to cell cultures after dilution into serum-free medium (final concentration, 10^{-6} M). Cells were exposed to [^{3}H]DMBA in the absence or presence of DBcAMP (10^{-9} - 10^{-5} M) for desired periods of time, then washed with PBS and lysed for DNA extraction.

5. DNA isolation: DNA was isolated following the method of Huberman and Sacks.

6. Mammary epithelial cells: mammary epithelial cells obtained by collagenase digestion of mammary gland were grown in culture and only duct cells were used in the experiments.

7. In vitro translation: Total poly A containing RNA was isolated from mammary gland by the method of Deely et al. (J. Biol. Chem. 252: 8310, 1977). In vitro translation systems of both rabbit reticulocyte lysate and wheat germ extract were used. Total translation products were analyzed by SDS-PAGE.

Major Findings

1. Binding of DMBA to Rat Mammary Epithelial Cells in Culture. Mammary epithelial duct cells growing in culture exhibited a time dependent uptake of [^{3}H]DMBA into their DNA. The maximum DNA-binding of [^{3}H]DMBA was significantly higher in the mammary epithelial cells of 50 day old rats (20 pmol DMBA bound/mg DNA) than those in the cells of the of the 35- and 110-day-old rats (10 and 9 pmol DMBA bound/mg DNA, respectively). Moreover, the DNA-bound radioactivity in the cells of the 50-day-old rats was more resistant to dissociation than those in the cells of the 35- and 110-day-old rats. The results of [^{14}C]DMBA binding to the DNA were similar to that observed with [^{3}H]DMBA binding and all the radioactivity was found to be associated with the DNA as evidenced by the CsCl density gradient centrifugation. These results mimicking that of the in vivo data

showed that DMBA binding to DNA of mammary epithelial cells is age dependent and the binding is highest in the epithelial cells derived from 50-day-old rats having the highest tumor incidence.

2. Inhibitory effect of DBcAMP on DMBA binding to DNA. A greater inhibition of DBMA tumor production was observed in the rats that received DBcAMP treatment starting at 1 day prior to DMBA administration as compared to those that received a delayed treatment of DBcAMP that started at 7 days post DMBA feeding. This suggest possible interference by DBcAMP of the DNA binding of DMBA which has been shown to be complete within a week post DMBA administration.

The log phase mammary epithelial cells were exposed for 48 hr to 10^{-6} M [^{3}H]DMBA in the absence or presence of DBcAMP (10^{-9} - 10^{-5} M). The results showed that DBcAMP inhibits the covalent binding of DMBA to DNA of mammary epithelial cells of the carcinogen-susceptible 50-day-old rats but not that of the unsusceptible 35- and 110-day-old rats. The inhibitory effect of DBcAMP was appreciable at the concentration of 10^{-7} M, 1/10 the concentration of [^{3}H]DMBA. DBcAMP at 10^{-6} M concentration exhibited the maximal inhibition of DMBA binding: the binding in the 50-day-old rat epithelial cells was reduced to the level of the bindings observed in the epithelial cells of 35- and 110-day-old rats. That the specific inhibitory effect of DBcAMP on the DMBA binding to DNA only in the carcinogen-susceptible rats (50-day-old) suggests a direct effect of DBcAMP on the DNA binding rather than the effect of DBcAMP at metabolic activation of DMBA.

3. Effect of DMBA and DBcAMP on Genetic transcript of Mammary Gland. Poly(A) containing RNAs were isolated from mammary glands of the young virgin (50-day-old) and the old virgin (110-day-old) rats of control and those treated with DMBA and/or DBcAMP, and translated in a cell-free protein synthesizing system. The in vivo translation products of mRNAs from mammary glands of young virgin rats differed from those of old virgins. The difference was localized in four protein bands of the electrophoretic pattern that increased in their concentration and one protein band that decreased in the old virgin glands. DBcAMP administered in vivo altered the genetic transcripts of the young virgin glands to become similar to that of the old virgin glands. DMBA feeding did not alter appreciably the translation protein pattern of the young virgin gland. However, when DMBA was administered to the young virgin that received DBcAMP, the protein pattern of either treatment alone was no longer seen, and there appeared a new protein pattern which is half-way between the patterns of the young virgin gland and that of the old virgin gland. The results suggest that DMBA binding to DNA may not alter the genetic transcript per se; instead it may ultimately stimulate the highly proliferative potency of the mammary gland at a young age, preventing its maturation or differentiation whereas, the anticarcinogenic effect of DBcAMP involves a modification of the gene expression: the genetic transcript of the young virgin mammary gland turned into that of the older virgin whose tumor incidence has been shown to be low.

Significance to Cancer Research and the Program of the Institute:

These studies contribute to the understanding of the molecular mechanism of carcinogenesis. The results suggest the antagonistic effect of cAMP at the

DNA-binding of the carcinogen and the gene expression. The suppression of mammary carcinogenesis found with orally administered DBcAMP may be of significance in the prevention studies of mammary cancer in humans.

Proposed Course of Research:

The project discontinued on March 25, 1983 due to the termination of the appointment of the visiting fellow.

Publications:

Cho-Chung, Y.S., Clair, T. and Shepheard, C.: Anticarcinogenic effect of $N^6,O^{2'}$Dibutyryl Cyclic Adenosine 3',5'-monophosphate on 7,12-Dimethylbenz(α)-anthracene mammary tumor induction in the rat and its relationship to cyclic adenosine 3',5'-monophosphate metabolism and protein kinase. Cancer Res. 43: 2736-2740, 1983.

Huang, F.L., Hasuma, T. and Cho-Chung, Y.S.: Anticarcinogenic effect of $N^6,O^{2'}$-Dibutyryl cyclic adenosine 3',5'-monophosphate on 7,12-dimethylbenz(α)-anthracene mammary tumor induction: Modification of DNA binding and gene expression. Cancer Res. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08268-02 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Structure, topology, and dynamics of tight junctions

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: P. Pinto da Silva, Chief, Membrane Biology Sec. LPP, NCI

COOPERATING UNITS *(if any)*

Dr. J. Chevalier, Department de Biologie--LPPC--Biomembranes--Centre de' Etudes--Nucleaires de Saclay--911911 Gif Sur Yvette, France

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.51	1.41	0.11

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

In toad urinary bladder, osmotic shock (application of distilled water in the serosal compartment versus amphibian ringer at the mucosal side) induces massive, fast and partially reversible proliferation of tight junction strands. Two minutes after initiation of the osmotic shock, the proliferation process is 75% complete at 37°C (40% at room temperature). Maximum-proliferation is reached after 10 min at 37°C (60 min at room temperature). Cytoskeleton perturbers (colchicine, cytochalasin), as well as cycloheximide (an inhibitor of protein synthesis) have no effect on the proliferation process. Limited reversal of junctional strand proliferation is a slow process, accelerated by the presence of cycloheximide.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:			
	J. Chevalier	Foreign Scientist	France
	G. Tadvalkar	Visiting Fellow	LPP, NCI
	C. Parkison	Chemist	LPP, NCI

Project Description

Objectives:

To study the structure, topology and dynamics of tight junctions.

Methods Employed:

For studies of tight junction proliferation in toad bladders, paired urinary bladders of Bufo marinus are mounted as sacs at the tip of a glass canula, according to the technique of Bentley. The serosal and mucosal faces of the tissue were bathed with an aerated amphibian Ringer solution (A6 Ringer containing 5 mM glucose). Osmotic shock was induced by immersing the hemi-bladders in distilled water for different periods of time (2, 5, 10, 30, 60 min) at room temperature or 37°C. In some cases, specimens were preincubated, before the application of the osmotic shock, with, in the serosal compartment, cycloheximide (200 µg/ml, 30 min R.T. and 5 min 37°C) or cytoskeleton perturbers (colchicine 10^{-3} M, 2 hr R.T. followed by colchicine 10^{-3} M + cytochalasin B µg/ml, 2 hr R.T.) or Ringer free Ca^{++}, free Mg^{++} + EGTA 2mM (90 min R.T.). In other circumstances, after osmotic shock, the initial serosal (A6 Ringer + 5mM glucose) was restored and the tissue incubated for various period of time (30 min to 3 hr). Epithelial cells were scrapped, fixed in Ringer/glutaraldehyde 2% solution and processed for freeze-fracture.

Major Findings:

In toad bladder, osmotic shock induces massive, fast and partially reversible proliferation of tight junction strands. Maximum-proliferation is reached after 10 min at 37°C (60 min at room temperature) but, after two minutes, the process is 75% complete at 37°C (40% at room temperature). Cytoskeleton perturbers (colchicine, cytochalasin), as well as cycloheximide (inhibitor of protein synthesis) have no effect on the proliferation process. The reversal of junctional strand proliferation is a limited and slow process, that can be accelerated by cycloheximide.

Significance to Biomedical Research and the Program of the Institute:

The zonula occludens plays a major role in the structure and functions of epithelial tissues, binding cells together at their apical pole and sealing the intercellular spaces. Our proposal that inverted cylindrical micelles are principal elements of tight junction structure and that at the junctional site the exoplasmic halves of the plasma membrane are continuous is a radical departure from previous concepts, has important physiological implications, demonstrates the importance of non bilayer lipid configurations in biological membranes and suggests numerous avenues of experimentation. It has been extremely well

received after its publication as a major review article in Cell. Proliferation of tight junction strands is an interesting phenomenon that explains previous instances reported in studies of cellular pathology (the effect of ionizing radiation, for instance). The ability of osmotic shock to induce a massive proliferation of new junctional strands in the basal part of toad bladder epithelial cells, without affecting the apical zonula occludens, provides a useful system that is amenable to physiological study (e.g. water transport) and where the genesis and the dynamic as well as the composition and structure of tight junction strands can be now investigated.

Proposed Course of Research:

Within the next year we plan to investigate the action of lipid perturbers on the structure of tight junctions. This will test our model of tight junction structure that encourages the existence of intramembranous, inverted cylindrical micelles as main structural components of tight junctions. We will also start to generate computer models of tight junction networks as based on structural branching assumptions as well as known junctional networks from various transporting and non transporting epithelial. These projects will involve the participation of Dr. G. Tadvalkar and also of Dr. R. Blumenthal and members of the computer division.

Publications:

Chevalier, J. and Pinto da Silva, P.: Massive, fast assembly of tight junction strands induced by osmotic shock: time study; influence of calcium and cytoskeletal perturbers (In preparation).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08269-02 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Membrane differentiation: Role of Integral Components in Membrane Domains

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: P. Pinto da Silva, Chief, Membrane Biology Sec. LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.71	0.6	0.11

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

We have extended our previous work on the topology of membrane components involved on the expression of chemically-homologous domains at the surface of mammalian cells. As we did in the past, we have used a highly polorized cell (spermatozoa) where regionalization of surface components is extreme. In these cells, the distribution of receptors of lectins (concanavalin A, and wheat germ agglutinin) in plasma and acrosomal membranes was determined in preparations obtained by the "fracture-label" method, conventional freeze-fracture, and surface labeling. Sites of lectin attachment to the membrane were visualized by electron microscopy using protein-coated colloidal gold as the cytochemical marker. In addition to our previous results we show that: a. stable surface domains formed by specific glycoconjugates are primarly conditioned by transmembrane glycoproteins; b. regionalization of glycoconjugates may also occur in intracellular membranes, as we have demonstrated in the acrosomal membrane (where Con A receptors are accumulated in the equatorial segment); c. current methods of membrane fractionation, as well as conventional cytochemical techniques may lead to data on the topology of specific components of intracellular membranes that is tainted by the presence of contaminants.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: A.P. Aguas Visiting Fellow LPP, NCI

Project Description

Objectives:

Characterization of the formation and maintenance of chemical domains on the plasma membrane of differentiated cells.

Methods employed:

We have extended our observations using the same methodology that was described in the previous report. In addition, we have developed a new method--"plasma membrane stripping"--that allows the access of cytochemical markers to intracellular membranes under experimental conditions that virtually abolish contamination. Cells were washed in buffer (PBS), fixed in glutaraldehyde (1.5% in PBS), washed again in buffer, and centrifuged twice in 30% bovine serum albumin (BSA) in PBS. The pellet of BSA-cells was crosslinked with glutaraldehyde, cut into small pieces, and submitted to grinding, at room temperature, with a glass pestle.

Major Findings:

1. Con A and WGA receptors are asymmetrically inserted in the acrosomal membrane of sperm cells, being exclusively partitioned with its exoplasmic membrane half.

2. Transmembrane glycoproteins show different densities on the head and tail segments of spermatozoa.

3. Con A binding sites are accumulated on the equatorial segments of the acrosomal membrane. This represents one of the first examples of regionalization of specific glycoconjugates in an intracellular membrane of a mammalian cell.

4. Comparison of lectin labeling on the cytoplasmic surface of the acrosome obtained by hypotonic disruption of the plasma membrane and by "plasma membrane stripping" indicates that conventional methods (as well as membrane fractionation) of obtaining access of cytochemical markers to intracellular membranes may result in artifactual data, due to adsorption of contaminants to the membranes.

Significance to Biomedical Research and the Program of the Institute:

Our results allow a better characterization of membrane differentiation related to the expression of chemically-homologous domains at the cell surface of mammalian cells. We also provide evidence that intracellular membranes, in the case of highly polarized cells, may also present regionalization of specific components. An extensive description of the general significance of our project to biomedical research is stated in previous reports.

Proposed Course of Research:

We are now studying the dynamics of transmembrane proteins during fusion between the two membrane systems, acrosomal and plasmalemmal, that occurs during the acrosomal reaction. Our preliminary results point to clustering of integral membrane proteins into the equatorial segment of the plasma membrane. This event appears to preceede membrane continuity that is mediated by the formation of cylindrial filaments, probably consisting in lipids in inverted mycellar configurations. inverted mycellar configurations.

Publications:

Aguas, A.P. and Pinto da Silva, P.: Regionalization of transmembrane glycoproteins in the plasma membrane of boar sperm head as revealed by "Fracture-label" (submitted for publication).

Aguas, A.P. and Pinto da Silva, P.: Topology of lectin receptors and chionic charges in the acrosomal membrane of mammalian sperm cells. (In preparation).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08270-02 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

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

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
PI: P. Pinto da Silva Chief, Membrane Biology Section LPP, NCI

COOPERATING UNITS *(if any)*
Dr. Maria Rosaria Torrizi, Instituto de Patologia Generale, First University of Rome, Via Regina Elena 324, Rome, Italy

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, NIH Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.72	1.61	0.11

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

B

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

Thin section fracture-label was used to determine the distribution of wheat germ agglutinin (WGA) binding sites in intracellular membranes of secretory and non-secretory rat tissues as well as in human leukocytes. In all cases, analysis of the distribution of WGA led to the definition of two endomembrane compartments: one, characterized by absence of the label, includes the membranes of mitochondria and peroxisomes as well as those of the endoplasmic reticulum and nuclear envelope; the other, strongly labeled, comprises the membrane of lysosomes, phagocytic vacuoles, and secretory granules, as well as the plasma membrane. The Golgi apparatus was weakly labelled in all studied tissues. This appears to reflect the short lived presence of fully glycosylated membrane proteins in this organelle.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	M. R. Torrisi	Foreign Scientist	Italy
	F. Kan	Visiting Fellow	LPP, NCI
	C. Parkison	Chemist	LPP, NCI

Project Description

Objectives:

To study the distribution of WGA-binding glycoconjugates in Golgi and lysosomal membranes as well as in mitochondria and peroxisomes of a variety of secretory and non secretory tissues from the rat and in human leukocytes.

Methods Employed:

Cells: Liver and ileum tissues, as well as adrenal and salivary glands were excised from adult Sprague-Dawley rats. Cells were isolated by digestion with collagenase type IV or collagenase type II (5 mg/ml in Hank's solution, Worthington Biochemical, Freehold, N.J.) for 5-10 min. at 37°C. Cells and tissues were washed twice in Hank's solution, fixed in 1% or 2% glutaraldehyde in phosphate buffer saline (PBS) pH 7.4 (2 hrs, 4°C), impregnated in 30% glycerol and frozen as described elsewhere (11, 12). Human peripheral blood lymphocytes and monocytes isolated from fresh heparinized blood by Ficoll-Hypaque gradients (17), were washed twice in PBS and fixed in 1% glutaraldehyde (2 hrs, 25°C). Human neutrophils were isolated as above followed by Plasmagel (Roger-Bellon, Paris) sedimentation and lysis of erythrocytes with Tris-buffered ammonium chloride. Isolated neutrophils were washed twice in 0.1 M sodium cacodylate-HCl buffer and fixed in 1.5% glutaraldehyde (2 hrs, 25°C). Some samples were incubated for 30-40 min. at 25°C in Karnovsky's diaminobenzidine saturated solution with H_2O_2 as substrate for the peroxidase reaction (18, 19). All cells were impregnated in glycerol and frozen.

Fracture-labelling: Frozen cells were immersed in liquid nitrogen and crushed with a glass pestle (10-13). The freeze-fractured cells were thawed (1% glutaraldehyde, 30% glycerol in PBS) and deglycerinated in 1mM glycylglycine. Fractured cells were incubated in solutions of WGA (0.25-1 mg/ml in 0.1 M Sorensen phosphate buffer containing 4% polyvinylpyrrolidone pH 7.4) for 1 hr at 37°C. Controls were preincubated in 0.4 M N-acetyl-D-glucosamine for 15 min at 37°C, then treated with WGA in the presence of the sugar (1 hr, 37°C). All samples were incubated in the presence of colloidal gold-ovomucoid complex at 25°C for 60 min. (20). Fractured cells were also incubated and labeled with 1 mg/ml WGA-ferritin conjugates (E-Y Lab., San Mateo, CA) in PBS (1 hr, 37°C). To locate thiamine pyrophosphatase activity, salivary cells, fixed in 2% glutaraldehyde in cacodylate buffer, were processed as above and, after fracture, thawing and deglycerination, treated for thiamine pyrophosphatase reaction (21) and labeled with WGA-colloidal gold-ovomucoid complexes.

Processing for Electron Microscopy: Fracture-labeled cells and tissue fragments were fixed in 1% Osmium tetroxide in veronal acetate buffer pH 7.6 (2 hrs, 4°C), stained en bloc with uranyl acetate (5 mg/ml), dehydrated in acetone and embedded in Epon 812. Thin sections were examined unstained or post-stained with uranyl acetate and lead citrate.

Major Findings:

Analysis of the distribution of WGA leads to the definition of two endomembrane compartments: one, characterized by absence of label, includes the membranes of mitochondria and peroxisomes as well as those of the endoplasmic reticulum and nuclear envelope; the other, strongly labelled, comprises the membranes of lysosomes, phagocytic vacuoles, and secretory granules, as well as the plasma membrane. The membrane of the Golgi apparatus were always weakly labelled. This appears to reflect the short lived presence of fully glycosylated membrane proteins in this organelle.

Significance to Biomedical Research and the Program of the Institute:

Study of the mechanisms of glycosylation of membrane proteins is at present pursued in many laboratories, generally involving biochemical approaches. Because cell fractionation is necessary, cross contamination of membrane fractions makes it difficult to ascribe precise locations to glycosylated products. Cytochemical investigations rely on and labelling of autoradiography (with limited resolution) and frozen thin sections, an approach of unusual difficulty and limited scope. Our "fracture-label" techniques circumvent these problems and permit -- in an easy an straightforward manner -- not only to locate a variety of membrane components but also to learn about their pattern of distribution and differential association with each membrane half. Our study provided new insights about the intra localization of membrane glycoproteins. Having now learned about the distribution and topology of these glycoproteins in the membranes of normal cells, we are now in a favorable position to study any departures that may characterize pathological conditions, particularly those characteristics of malignant transformation.

Proposed Course of Research:

Dr. Frederick Kan is currently investigating the distribution of WGA binding sites on the Golgi and other intracellular membranes of intestinal epithelial cells, including goblet cells.

Publications:

Torrisi, M.R. and Pinto da Silva, P.: Compartmentalization of intracellular membrane glycocomponents is revealed by fracture-label. J. Cell Biol. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08271-02 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

T Lymphocyte Heterogeneity: Labeling of Lectin Receptors of Transmembrane Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: P. Pinto da Silva Chief, Membrane Biology LPP, NCI

COOPERATING UNITS *(if any)*

Dr. P.A. Bunn, Jr. and Dr. C. F. Winkler, N.C. Navy Medical Oncology Branch
Naval Hospital, Bethesda, Maryland

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NIH, NCI Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.72	0.61	0.11

CHECK APPROPRIATE BOX(ES)

☐ (a) Human 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 recently described the partition of wheat germ agglutinin (WGA) receptor sites over the protoplasmic and exoplasmic plasma membrane faces of freeze-fractured human thymus-derived (T) lymphocytes. Our results indicated there is an heterogenous expression of integral membrane proteins (receptor sites over protoplasmic faces) within populations of human T cells. Here, we look for the distribution of WGA receptor sites over fracture-faces of the plasma membranes of neoplastic lymphocytes. We observed that, in cells from the culture lines Molt 4 (from acute lymphoblastic T leukemia) and Hut 78 (from mycosis fungoides), exoplasmic fracture-faces were always labeled, but no WGA receptor site was found on protoplasmic fracture-faces. This project has been temporarily interrupted due to lack of personnel.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	A. Pavan	Visiting Fellow	LPP, NCI
	C. Parkison	Chemist	LPP, NCI

Project Description

Objectives:

To observe the partition and distribution of WGA receptor sites on the plasma membrane faces of freeze-fractured neoplastic lymphocytes.

Material and Methods:

Cultures of the cell lines Molt 4 (from acute lymphoblastic leukemia) and Hut 78 (from Mycosis Fungoides) were obtained from the N.C. Navy Medical Oncology Branch --Naval Hospital, Bethesda. Fracture-label was done as described on previous report (October 1, 1981 to September 30, 1982).

Major Findings:

We observed that, in the neoplastic cell lines, exoplasmic fracture-faces of the plasma membrane were always labeled. However, contrary to results obtained with normal T dLymphocytes, no WGA receptor sites were ever found on protoplasmic fracture-faces. Our results indicate there is a reduction in the expression of integral membrane proteins in neoplastic cells.

Significance to Biomedical Research and the Program of the Institute:

Fracture-label appears to open a new approach to search and characterize lymphocyte subpopulations based on differences in the expression of transmembrane proteins. Heterogeneity of T cell populations, as revealed by fracture-label, retains possible operational value and a significance that is reinforced by the homogeneous labelling patterns so far observed in other cells as well as by the recent finding of multiple WGA binding glycoproteins in the plasma membranes of human T cells. The effects of stimulatory and inhibitory concentrations of WGA, the possible correlation with existent lymphocyte subpopulations (e.g. helper/suppressor), as well as the screening of fracture-labelled lymphocytes from patients with various types of T cell leukemias are, therefore, of immediate concern.

Proposed Course of Research:

This project has been temporarily interrupted due to the lack of personnel.

Publications:

Torrisi, M.R. and Pinto da Silva, P.: T-lymphocyte heterogeneity: wheat germ agglutinin labeling of transmembrane glycoproteins. J. Cell Biol. 95 (2, pt.2) 60a, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08272-02 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Membrane glycoproteins and glycolipids of normal and transformed human cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: P. Pinto da Silva Membrane Biology Section LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

This project has been temporarily interrupted to permit the conclusion of project #Z01CB08275-02 LPP and the development of Project #Z01CB08283-01 LPP the study of cytoplasm compaction.

PHS 6040 (Rev. 1/83) GPO 895-100

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08274-02 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Regulation of Lactogenic Hormone Receptors in Mammary Tissue

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: B.K. Vonderhaar Research Chemist LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

This project is designed to evaluate the nature of lactogenic hormone receptors and the factors (including other hormones) which affect binding of the hormone to this molecule. Studies include 1) purification of the receptor from human tissue and preparation and characterization of an antibody against it; 2) examination of the nature of the interaction of prolactin and human growth hormone with native as well as cryptic forms of the receptor and 3) characterize the selectivity of the effect of changes in the membrane lipid environment on binding of lactogenic hormones to their receptors vs other peptide hormones (such as EGF) binding to their receptors.

PHS 6040 (Rev. 1/83) GPO 895-100

Others Professional Personnel: T.M. Horn Staff Fellow LPP, NCI

Project Description

Objective:

The purpose of these studies is to investigate the nature of the interaction of lactogenic hormones with their receptors. The stability of the hormone-receptor complex, and the nature of the cryptic sites was investigated, as well as the effects of alterations in membrane lipids and membrane aggregation. The selectivity of these effects on lactogenic hormone receptors was determined by examining similar effects on somatogenic receptors or the receptors for EGF. Purification of the lactogenic hormone receptors was attempted in order to prepare antibodies for studies on regulation of synthesis of the molecule.

Methods Employed:

C3H/HeN mice, Sprague Dawley rats and mid pregnant rabbits were used. Hormones were iodinated by a lactoperoxidase method. Microsomal membranes were prepared from mammary glands (MG) or livers (L) of 10-12 day lactating or 10-12 day mid-pregnant mice and human placentas using established procedures. Specific binding of prolactin (PRL), human growth hormones (hGH), or EGF to membrane bound or detergent solubilized receptors was studied by standard competition binding techniques. Solubilization of receptors was performed either in the presence of 1% Triton X-100 or 0.5-1.0% of the zwitterionic detergent CHAPS. Plasma membranes and golgi fractions were purified using standard step-sucrose gradient techniques.

Nb2 rat lymphoma cells (obtained from Drs. Gout and Noble) are propagated in Fischer's leukemic cell medium with 0.15 mM β-mercaptoethanol, 10% horse serum and 10% fetal calf serum. Growth of cells is monitored by direct counting using a hemacytometer.

Major Findings:

In order to understand the regulation of PRL binding and induction of cryptic binding sites, we examined the role of membrane protein aggregation and fluidity changes induced by Concanavalin A (Con A) on subsequent hormone binding. The binding of lactogenic hormones to membranes from either mammary glands (MG) or livers (L) of mice and rats was inhibited by Con A to about 40-50%. Binding to rabbit membranes, however, was stimulated by as much as 2 to 3 fold in the presence of Con A. This difference in sensitivity of rabbit vs mouse or rat receptors to Con A action is probably due to differences in the associated proteins rather than the receptor itself. Triton solubilized pre-bound PRL-receptor complexes from either mouse or rabbit tissues are retained by Con A-sepharose columns. CHAPS solubized pre-bound PRL receptor complexes are not retained by Con A-Sepharose. Ovalbumin binds equally well to Con A-Sepharose in the absence of any detergent or in the presence of Triton or CHAPS. This rules out the detergent interfering with Con A binding. Both mouse and rabbit receptors remain

as large molecular weight complexes in the presence of Triton, but are dissociated to their 37K dalton "core binding unit" in the presence of CHAPS. These data show that it is the nature of the receptor associated glycoproteins which determines the Con A effects on PRL binding.

The lipid environment in which the lactogenic hormone receptors are localized plays an important role in their ability to bind the hormones as well. Membrane associated methyl transferases use S-adenosyl-L-methionine (SAM) as a methyl donor to convert phosphatidylethanolamine to phosphatidylcholine. We have observed a specific effect of SAM on mouse mammary gland and liver microsomes. Binding activity for ^{125}I-hGH as ligand, displaceable by oPRL, is stimulated in radioreceptor assays in the presence of SAM, but when ^{125}I-oPRL or ^{125}I-hPRL are ligands, there is no increased binding.

In addition to this specificity, observed in the lactogenic hormone family, binding of ^{125}I-mEGF displaceable by mEGF is not affected, even though in the same assay binding of ^{125}I-hGH increases 2-fold. The tonicity of the buffer used has no effect, indicating that inversion of the microsomal vesicles is not required.

SAM is stimulatory for hGH binding in lactating and mid-pregnancy mammary glands and livers, as well as in livers from 3 mo. old virgin female and male mice. Lactating liver and mammary glands both show 2 fold stimulation of hGH binding, but liver has 10-50 times more relative methyl transferase activity.

We further characterized the SAM effect on ^{125}I-hGH binding using isotonic buffer (phosphate buffered saline). Previous studies show that SAM is the methyl group donor in the synthesis of phosphatidylcholine (PC) from phosphatidyl ethanolamine (PE). At least two enzyme activities, both called phospholipid-Nmethyl transferases, are required. These were originally distinguished in reticulocytes, based on differences in pH optima, magnesium requirement, Km for SAM, and localization to the inner or outer aspect of the membrane. In other tissues (from other species) the enzymes have different characteristics.

In mouse liver, PC synthesis has a high Km for SAM and no Mg^{2+} requirement. Phospholipid synthesis and SAM stimulation of hGH binding were monitored in parallel assays. From Lineweaver-Burk analysis of the SAM concentration curve it appears, thus far, that the Km for increased binding of hGH is 3x the Km for PC synthesis. However, 3 molecules of SAM are required for each molecule of PC formed.

Lactogenic receptors are present in both the golgi and plasma membrane fractions of liver. SAM can only stimulate hGH binding in the plasma membrane fraction. Binding activity in the golgi is unaffected. This substantiates our earlier suggestion that SAM stimulates hGH binding by activation of cryptic sites in the cell surface membrane. The lack of SAM effect in the golgi indicates that SAM is not involved in transport of lactogen receptors from the golgi to the cell surface.

In order to study the regulation of the lactogenic hormone receptor itself under controlled conditions, we have begun the purification of the prolactin receptor from human tissue. Recently we isolated a receptor from mouse liver that binds the lactogenic hormones ovine prolactin and human growth hormone. We now have isolated the receptor from human placenta using a modification of this procedure. Placenta has a low number of prolactin receptors which localize, both biochemically and histologically to the chorion. Large amounts of human placenta are readily available from Bethesda Naval Hospital.

Chorion is dissected from term placentas (age less than 24h, stored at 4°C). Microsomes are then prepared. The microsomes are "stripped" of endogenous lactogen bound to receptor by treatment with 4M $MgCl_2$. At other concentrations, less binding activity is recovered. The microsomes were then restored to low Mg^{2+} buffer, and solubilized with 0.5% CHAPS. Usually 50% of the binding activity is recovered in the supernatant. The supernatant is then incubated with an ovine prolactin affinity matrix. Unbound or loosely bound material is eluted using 4M urea and 1M NaCl. Receptor is released from the prolactin-matrix using 4M $MgCl_2$ which is quickly removed by dialysis.

The binding activity recovered is very low, but, on SDS-PAGE analysis using silver stain, we observe a single band of MW ~35-37,000 daltons. This is the same size as the "core binding unit" of the prolactin receptor purified from mouse liver. The material purified from human placenta is highly cross-reactive on Ouchterlony test plates with rabbit antiserum raised against purified mouse liver prolactin receptor. This antibody reacts only with mouse and human prolactin target tissues, their solubilized membranes and purified receptors. It inhibits the binding of prolactin to solubilized receptors from mouse liver. It does not inhibit the growth promoting effects of PRL on the rat Nb2 cells nor does it substitute for PRL in these cells. This is consistent with a lack of cross-reactivity on Ouchterlony plates between the antibody and rat mammary gland or liver membranes.

Significance to Biomedical Research and the Program of the Institute:

Prolactin-thyroid interactions are important in growth and differentiation of mammary glands. Altered thyroid status may be implicated in changes in risk of human breast cancer. Our studies are aimed at understanding whether changes in thyroid status play a direct or indirect role in mammary tumorigenesis. Thyroid hormones are known to regulate prolactin action through control of lactogenic receptors. Therefore, all aspects of prolactin binding and control of exposed as well as cryptic sites must be examined. Preparation of antibodies against the lactogenic receptors will readily allow us to study the receptor molecule itself in the absence of the hormone and thus determine if lack of prolactin binding in certain mammary tumors is due to lack of receptors or only their existence in masked forms. Changes in membrane phospholipids can greatly affect the capacity of a target cell to recognize and subsequently respond to specific hormones and drugs. Certain chemotherapeutic agents and drugs such as deazaodenosine, 5-azacytidine and methotrexate interfere with cellular methylation. How these agents affect membrane methylation and hormonal responsiveness of normal vs tumor tissue is of great interest.

Proposed Course of Research:

We will prepare sufficient quantities of prolactin receptors from human tissue (placenta or solid MCF-7 tumors) using the techniques developed so far. We will prepare monoclonal antibody against the human receptor. We will also attempt to use the anti-prolactin receptor antibody already on hand to purify the human receptor if necessary. We will use this antibody to begin screening human tumors for organ specific receptor content. We will examine primary vs metastatic breast tumors for the presence of receptors. Using the antibody we will examine the regulation of receptor synthesis and availability of this molecule to the hormone in cultured human breast cancer cells (MCF-7 and T47D). We will examine the receptor molecule itself to determine if it is a kinase which is autophosphorylated during hormone binding, as many peptide hormone receptors are now being shown to be. Finally we will begin work on the "2nd message" for PRL to determine its character (i.e.: if it is part of the "core binding unit" or one of the specific receptor associated membrane proteins) and on isolation and sequencing the prolactin receptor gene from human tissues.

Publications:

Liscia, D.S., Alhadi, T. and Vonderhaar, B.K.: Solubilization of active prolactin receptors by a non-denaturing zwitterionic detergent. J. Biol. Chem. 257: 9401-9405, 1982.

Liscia, D.S. and Vonderhaar, B.K.: Purification of a Prolactin Receptor. Proc. Natl. Acad. Sci. USA 79: 5930-5934, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08275-02 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Asymmetric topology of glycolipids in membranes of Acanthamoeba castellanii

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: P. Pinto da Silva Chief, Membrane Biology LPP, NCI

COOPERATING UNITS *(if any)*

Dr. Blair Bowers, NHLBI

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.72	0.61	0.11

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

Fracture-label--a method that permits the cytochemical characterization of faces produced by freeze-fracture--was used to determine the partition and distribution of a glycolipid on membrane fracture-faces of Acanthamoeba castellanii cells. After treatment with Con A, the glycolipid--a lipophosphonoglycan (LPG)--was labeled with colloidal gold coated with horseradish peroxidase. The label was abundant over exoplasmic fracture-faces (face E) of plasma membranes but absent from protoplasmic fracture-faces (face P). We conclude that, in A. castellanii, glycolipid molecules are restrict to the outer half of the plasma membrane (see Annual Report for the period October 1, 1981 to September 30, 1982). This conclusion was confirmed by new experiments with cells disrupted by freezing and thawing, where access of label to the cell interior did not result in labeling of the inner surface. Our results are the first to establish the exclusive localization of a glycolipid to the outer half of a plasma membrane. Fracture-label is proposed as a new technique to investigate the distribution and partition of glycolipids in plasma and intracellular membrane halves.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	M. Luiza F. Barbosa	Visiting Fellow	LPP, NCI
	C. Parkison	Chemist	LPP, NCI

Project Description

Objectives:

To study the partition and distribution of a glycolipid on the fracture faces and membrane surfaces of Acanthamoeba castellanii.

Methods employed:

In order to confirm the results described in the Annual Report for the period of October 1, 1981 to September 30, 1982, we performed new experiments with cells disrupted by freezing and thawing, where label had access to the cell interior.

Cells were washed with 0.1M sodium phosphate buffer pH 6.8 (PB), spun down, frozen in liquid nitrogen, rapidly thawed in PB buffer, with or without 1mM quinacrine dihydrochloride and immediately fixed in 3% glutaraldehyde in PB buffer at 25°C for 30 min. After fixation, cells disrupted by freezing and thawing were washed in PB buffer and labeled for detection of Con A binding sites as previously described.

Major Findings:

The new experiments, label of cells disrupted by freezing and thawing, confirmed the conclusion reached with the fracture-label method: in A. castellanii, glycolipid molecules are restrict to the outer half of the plasma membrane. Our results were the first to establish the exclusive localization of a glycolipid to the outer half of a plasma membrane. We propose fracture-label as a new technique to investigate the distribution and partition of glycolipids in plasma and intracellular membrane halves.

Significance to Biomedical Research and the Program of the Institute:

Glycolipids are important components of biological membranes. Among many functions, they serve as receptors, antigen determinants or as regulators of cell behavior. Investigation of the topology of glycolipids in biological membranes is difficult due to the cryptical disposition of some glycosphingolipids. In this work, we propose fracture-label as a new technique to investigate the distribution and partition of glycolipids in plasma and intracellular membrane.

Proposed Course of Research:

This project was concluded with a paper submitted and accepted for publication.

Publications:

Barbosa, M.L.F. and Pinto da Silva, P.: Restriction of Glycolipids to the outer half of a plasma membrane: Concanavalin A labeling of membrane halves in Acanthamoeba castellanii. accepted for publication in Cell, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08277-02 LPP

PERIOD COVERED

October 1, 1982 to March 1, 1983

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

Antibodies as probes of cyclic nucleotide function in human breast cancer cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: Y.S. Cho-Chung Chief, Cellular Biochemistry Sec. LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cellular Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0	1.3	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

Increase of cAMP-dependent protein kinase type II has been assessed as an early event in hormone-dependent mammary tumor regression. In this study we showed, by immunological techniques, the appearance of new species of the regulatory subunits of cAMP-dependent protein kinase type II in the nuclei of regressing as compared to growing MCF-7 tumors in nude mice. We raised monospecific antibodies against the regulatory subunits (R) of type I (R^I) and type II (R^{II}) of cAMP-dependent protein kinases derived from bovine skeletal muscle and bovine heart, respectively. The antibodies were affinity purified using glutaraldehyde cross-linked immuno-absorbents. In the radio-immunoassay, R of MCF-7 tumor cross-reacted with bovine anti-R^I and -R^{II} antibodies as the bovine antigens, suggesting an immunological similarity between R of human MCF-7 tumor and bovine R. In the nuclear extracts of growing MCF-7 tumors, the anti-R^I and -R^{II} antibodies, by immunoprecipitation, detected 47,000-dalton, and 44,000- and 35,000-dalton cAMP receptor proteins, respectively. Following estrogen-withdrawal, new species of cAMP receptor proteins with m.w. of 50,000 and 52,000 appeared in the nuclei of regressing tumors. The 50,000 and 52,000-dalton proteins were specifically precipitated by the anti-R^{II} antibody but not with the R^I antibody. Concomitant with the appearance of 50,000- and 52,000-dalton R^{II} was the disappearance of the 35,000-dalton R^{II} from the nuclei. Indirect immunofluorescence revealed that during regression of MCF-7 tumors, the intensity of immunofluorescence of R^{II} dramatically increased in the nucleoli. The results suggest the regulatory role of type II cAMP-dependent protein kinase in mammary cancer regression.

Other Professional Personnel: C.L. Kapoor Visiting Associate LPP, NCI

Project Description

Objectives:

1. To identify and localize the molecular species of cAMP receptor proteins in growing vs regressing MCF-7 tumor (human breast cancer) in nude mice.

2. To explore the regulation and function of cAMP receptor proteins, R^I and R^{II}, in breast cancer cells during growth and growth arrest in vivo and in vitro.

Methods Employed:

1. Bovine skeletal muscle R^I and bovine heart R^{II} cAMP-dependent protein kinases were purified as described (JBC 245, 12427, 1979) and antisera were raised in rabbits. Antibodies were purified by using cross-linked R^I and R^{II} sepharose (J. Immunol. Methods 57: 215, 1983).

2. Immunocytochemistry of R^I and R^{II} in MCF-7 cells and MCF-7 tumor were carried out as described (Hand Book of Exp. Pharm. 58, 333, 1982; Science, 211, 407, 1981). Unfixed cryostat section (~4μ) of MCF-7 tumor were used for the immunocytochemistry.

3. The cross-reactivity and specificity of R^I and R^{II} antibodies were determined as described (JBC, 245, 12427, 1979; PNAS, 78, 653, 1981).

4. MCF-7 cells, an established cell line of human mammary carcinoma, were cultured as described in (Cancer Res. 43: 295, 1983). The MCF-7 cells were implanted in nude mice bearing a 17β-estradial pellet as described in (J. Natl. Cancer Inst. 67: 51, 1981). Regression of the tumor was induced by estrogen pellet removal and ovariectomy of the host. The tumor was removed 3 weeks after the hormone-withdrawal.

5. Other techniques used were standard methods.

Major Findings:

1. Affinity purified antibodies to type I and type II regulatory subunits (R^I and R^{II}, respectively) of cAMP-dependent protein kinase were utilized to identify and localize the cAMP receptor proteins in growing vs regressing MCF-7 tumor in nude mice. Specific antibodies toward bovine skeletal muscle R^I and bovine heart muscle R^{II} were generated in rabbits. The cross-reactivity of bovine anti-R^I and R^{II} with human breast cancer cells were demonstrated by using sensitive radioimmunoassay and immunoprecipitation.

2. The antibodies of R^I and R^{II} were purified to homogeneity by using cross-linked-immunoabsorbent affinity chromatography. The cross-linking of R^I and R^{II} sepharose prevented the leakage of R^I and R^{II} subunits during the elution of anti R^I and R^{II} antibodies. From original anti-R^I and anti-R^{II}

antisera approximately 500 and 200-fold purifications were obtained, respectively. The affinity purified antibodies were of IgG nature as identified by SDS gel electrophoresis and specific immunoabsorption analysis with goat anti-rabbit IgG and protein A-sepharose.

3. In the radioimmunoassay, R of MCF-7 tumor cross-reacted with bovine anti-R^{I} and -R^{II} antibodies as the bovine antigens, suggesting an immunological similarity between R of human MCF-7 tumor and bovine R.

4. In the nuclear extracts of growing tumor the R^{I} antibody immunoprecipitated cAMP receptor protein of 47,000 daltons, whereas the R^{II} antibody precipitated 44,000- and 34,000- dalton cAMP receptor proteins.

5. Estrogen withdrawal resulted in the appearance of new species of the 50,000- and 52,000- dalton cAMP receptor proteins in the nuclei of regressing tumor. The 50,000- and 52,000- dalton proteins were specifically precipitated by the anti-R^{II} antibody but not by the R^{I} antibody. Concomitant with the appearance of the 50,000- and 52,000- dalton R^{II} was the disappearance of 34,000- dalton R^{II} from the nuclei.

6. The only R^{I} protein of 47,000 dalton was found in MCF-7 tumor and the amount of R^{I} remained same during the tumor regression.

7. Indirect immunofluorescence revealed that the R^{II} of MCF-7 tumor is localized in the nucleoli, whereas the R^{I} is localized diffusely in the nuclei but not in the nucleoli of the tumor. The specific localization of R^{II} of MCF-7 tumor in the nucleoli suggests a regulatory role of R^{II} in cell division. Importantly, the immunofluorescence of R^{II} cAMP receptor in the nucleoli of MCF-7 tumor markedly increased upon estrogen withdrawal indicating an inverse relation between cAMP and estrogen in the regulation of tumor growth. These results suggest a regulatory role of R^{II} cAMP receptor in mammary cancer regression.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the nucleolar and chromosomal compartmentalization of R^{II}, and differential nuclear roles of R^{I} and R^{II} of cAMP-kinases in hormone-dependent and -independent human breast cancer cells may provide a new understanding into the initial events of growth of malignant cells. These studies raise the possibility whether similar distribution and compartmentalization of cAMP kinases exist in hormone responsive and hormone unresponsive human breast tumors.

Proposed Course of Research:

The project discontinued on March 1, 1983 due to termination of the appointment of the visiting associate.

Publications:

Kapoor, C.L. and Cho-Chung, Y.S.: Mitotic apparatus and nucleoli compartmentalization of 50,000-dalton type II regulatory subunit of cAMP-dependent protein kinase in estrogen receptor negative MDA-MB 231 human breast cancer cells. Cell Biol. Int. Reports, 7: 49-60, 1983.

Kapoor, C.L. and Cho-Chung, Y.S.: Compartmentalization of regulatory subunits of cyclic adenosine 3':5'-monophosphate-dependent protein kinases in MCF-7 human breast cancer cells. Cancer Research, 43: 295-302, 1983.

Kapoor, C.L. and Cho-Chung, Y.S.: Affinity purification of antibodies of regulatory subunits of cAMP-dependent protein kinase using cross-linked immuno-absorbent. J. Immunological Methods, 57: 215-220, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08279-02 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effect of proline analogs on normal and neoplastic breast epithelium

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
PI: W. R. Kidwell Chief, Cell Cycle Re. Sec. LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Cell Cycle Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

A series of proline analogs have been analyzed for their effects on collagen synthesis inhibition in cultures of primary DMBA-induced rat mammary tumors and for their effects on mammary tumor growth in tumor bearing animals. Azetidine carboxylate, thioproline and cis-hydroxyproline were found to be potent, selective inhibitors of collagen synthesis, blocking proline incorporation into collagen by 7 to 27 fold more than incorporation into tumor cell protein. In vivo all 3 of these compounds at doses of 50-200 mg/kg S.C. caused tumor growth arrest or regression. Two possible reasons for these effects on tumor growth seem likely: the tumor cells require collagen synthesis for growth and/or tumors are partial proline auxotrophs. Collagen synthesis requirements were assessed by attempting to rescue the tumor cells from proline analog killing by plating tumor cells on various collagen matrices, with negative results. However proline, usually considered a non-essential amino acid was growth stimulatory for tumor cells. A 30% increase in growth rate was observed at 50 µg proline/ml growth medium. Even though a reduced sensitivity to proline analogs was not seen on plating the tumor cells on collagen substrata, there was an effect of the proline analogs on basement membrane collagen formation by the tumors in vivo. The analyses of total tumor protein showed that tumors treated for 10 days had only 1/4-1/2 as much 3 as 4-hydroxyproline, consistent with the fact that basement membrane collagen, rich in 3-hydroxyproline, was reduced relative to stromal collagen which has very little 3-hydroxyproline. Since these tumors produce only basement membrane collagen, the proline analog effects were likely to have been due to effects on basement membrane collagen formation by the tumor epithelium.

Other Professional Personnel:	S. J. Taylor	Medical Staff Fellow	LPP, NCI
	M. Bano	Visiting Fellow	LPP, NCI
	F. Grantham	Bio. Lab. Tech.	LPP, NCI

Project description

Objectives:

Basement membrane collagen synthesis appears to be an essential function for well's differentiated mammary tumors. These studies are designed to evaluate a number of proline analogs for their specificity of inhibition of collagen deposition and the consequences of this inhibition for tumor growth arrest or tumor regression.

Methods employed:

Proline analogs were screened for their effects on collagen synthesis and cell cell growth using primary cultures of normal or tumor epithelium. Collagen production by the cultures was assessed on the basis of the relative amount of ^{3}H-lysine incorporated into a collagenase sensitive form or on the basis of the amount of 4-hydroxyproline or hydroxylysine formed in the presence or absence of the analogs. Effects of the analogs on tumor growth was determined by measuring the change in tumor volume over the time course of treatment. 3 and 4-hydroxy proline were purified from control as proline analog treated tumors by deamination of the primary amino acids in tumor protein hydrolysates by nitrous acid treatment followed by acid reflexing and water-ethyl acetate partitioning. The prolines which are recovered intact were then quantitated on an amino acid analyzer or by NBZ derivitization and chromatography on silica gel plates.

Major findings:

Thioproline, cis-hydroxyproline and 1-azetidine carboxylate arrest tumor cell growth in culture and in vivo. Analyses of the effects of the proline analogs indicate that in short term culture of tumor cells, there is a highly selective effect on collagen production followed by a dramatic inhibition of cell proliferation. Studies of the relative amount of basement membrane collagen present in tumors from animals with and without pretreatment with the proline analogs indicates that these analogs also reduce the amount of basement membrane collagen produced by the tumor cells. These results are consistent with the hypothesis that collagen synthesis is essential for optimum tumor growth. However, studies of the interaction of tumor cells with stromal or basement membrane collagen, the collagen type made by the tumors, show that tumor cells do not selectively recognize any collagen type. This is in marked contrast to the results seen with normal cells in which cell attachment rates to basement membrane collagen is 3-4 times more efficient than is attachment to type I or III collagen, the stromal collagen type. Tumor cells also differed from normal cells in that their sensitivity to the proline analogs was not reduced by plating the cells on basement membrane collagen. These results suggest that proline analogs might cause tumor cell growth arrest by some mechanism other than via blocking collagen production

or that collagen synthesis by tumor cells is coupled to the production of some other factor that is important for tumor growth. Some support for the former postulate was obtained by assessing the effects of proline on tumor cell growth in vitro. Proline at 50 µg/ml growth medium produced a 30% increase in tumor cell growth rate. This is a surprising finding because proline is generally considered to be a non-essential amino acid. Proline analogs may therefore be active on tumor cells in part because of the proline auxotrophy of tumor cells. In any event the proline analogs may be useful as cancer chemotheraputic agents because they arrest tumor growth (at least differentiated tumor) with little deleterous effects for the host animals.

Significance to Biomedical Research and the Program of the Institute:

The formation of the basal lamina with its constituent collagen appears to be a fundamental and essential part of the proliferative process of normal epithelium of the mammary gland and by differentiated tumors derived from it. Because in the adult the production of collagen is not extensive, tumors making collagen can be differentially killed by blocking collagen formation (or secretion) with proline analogs in the absence of toxic effects on normal tissues.

Proposed Course of Research:

Results from this study are currently being prepared for publication. Two important objectives remain to attained before the project is complete. First, recent reports from D. Pitelka's labaoratory indicate that metastatic breast tumor cells synthesize a basement membrane. They may therefore be sensitive to proline analogs and this will be tested. Second, the information attained to date appears to be sufficient to justify clinical trials. We will provide a description of our results and hypotheses to appropriate researchers in attempt to initiate such trials.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08280-01 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Oncogene Expression in Mammary Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

PI: Y.S. Cho-Chung Chief, Cellular Biochemistry Section LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cellular Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.5	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.)*

More than 15 distinct transforming genes have been identified in the genomes of oncogenic retroviruses. Each of these oncogenes has a homologue in the chromosomal DNA of all vertebrate species. Current evidence indicates that this highly conserved set of genes may play a vital role in cell proliferation and/or differentiation. In addition, inappropriate expression of some of these genes has been implicated in the genesis of cancer.

Our efforts have been concentrated on the cellular homologue of the ras gene, the oncogene carried by Harvey and Kirsten Sarcoma viruses. An amplified expression of the cellular ras gene has been correlated with the oncogenesis of human bladder, lung and colon carcinomas.

In this study we are investigating the role of ras gene expression in the induction of rat and human mammary carcinomas. It is our hypothesis that mammary cancer can be triggered by activation of ras gene expression. Enhanced expression might result from genetic changes at the ras locus itself such as a rearrangement involving new promoter sequences, or from changes at other, so-called "regulatory" loci.

The expression of the ras gene will be determined in growing vs regressing mammary tumors, hormone-dependent vs hormone-independent tumors, and the mammary gland of rodents during normal development or chemical or viral carcinogenesis. The ras gene expression will be also determined in hyperplastic alveolar nodules, benign and malignant breast diseases of humans. The goal of this proposal is to provide us a fundamental basis for better understanding the mechanisms by which oncogenes involved in neoplastic growth.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	T. Clair	Chemist	LPP, NCI
	F. L. Huang	Expert	LCCTP, NCI
	M. E. Furth	Memorial Sloan-Kettering Cancer Center, N.Y.	

Project Description

Methods Employed:

1. Tumors: Primary and metastatic human breast tumors and benign breast diseases, and primary, 7-12-dimethylbenz(α)anthracene (DMBA)-induced rat mammary carcinoma and transplantable MTW9, MTW9A, DMBA #1, MT13762 mammary carcinomas in rats were used. Tumor regression was produced by hormone-withdrawal or DBcAMP (10 mg/day/200 g rat s.c.) treatment.

2. MCF-7 cells: The MCF-7 cells (Mason Research Institute) were grown in McCoy's 5A medium supplemented with bovine insuline, penicillin, streptomycin and fetal calf serum (1040) and ± additives.

3. mRNA: Total polyA containing mRNA was isolated from tumors by the method of Deeley et al. In vitro translation systems of both rabbit reticulocyte lysate and wheat germ extract were used. Total translation products were analyzed by SDS-PAGE.

4. p21 Immunoprecipitation: [^{35}S] methionine-labeled tumor cell lysates or the in vitro translation products of tumor mRNAs were incubated for overnight at 4°C with rat monoclonal antibody against Harvey sarcoma virus p21. The reaction mixture was then incubated for an additional 2.0 hr at 4°C with a 10% (vol/vol) suspension of Formalin-fixed Staphylococcus aureus (precoated with rabbit anti-rat immunoglobulin G), then immunoprecipitates were collected by centrifugation, washed, and analyzed by electrophoresis in SDS/polyacrylamide gels.

5. Molecular clones of viral DNAs: Molecular clones containing the transforming sequences of Harvey Sarcoma virus (ras^{H}) and Kirsten sarcoma virus (ras^{K}) in plasmid pBR322 were used as probes to detect cellular ras transcripts in tumors. BS-9 contains a 0.5 kilobase (kb) fragment of ras^{H} and HiHi-3 contains a 1.0-kb fragment of ras^{k} sequences. Plasmid DNAs were ^{32}P-labeled by nick-translation to specific activities of approximately 4 x 10^{8} cpm/μg DNA for use as hybridization probes.

6. Blot hybridization analysis: Procedures for digestion of cellular DNAs with restriction endonucleases, electrophoresis in agarose gels, and transfer to nitrocellulase filters followed the method described in (Cell, 19: 863, 1980). Prehybridization, hybridization, and washing procedures were as described by Hanahan and Meselson (Gene, 10: 63, 1980) except that 10% dextran sulfate was included in the hybridization buffer (Proc. Natl. Acad. Sci. USA 76: 3683, 1979).

Major Findings:

The in vitro translated proteins from poly(A)+ RNAs differed when hormone-dependent rat mammary carcinomas were compared during their growth and regression (Huang, and Cho-Chung, Cancer Res. 43: 2138, 1983). In this study we present evidence that the 22K M.W. protein which shows an amplified translation in the growing tumor as opposed to the regressing tumor, represents transforming gene products associated with the oncogenic expression. A monoclonal antibody, 259 that reacts with the 21K transforming protein (p21) encoded by the v-ras gene of Harvey murine sarcoma virus (Ha-MuSV) specifically immunoprecipitated the 22K translation product of the growing tumors. In contrast, an insignificant amount of p21 was detected in the translation products from the regressing tumors induced by either hormone-withdrawal (ovariectomy) or dibutyryl cyclic AMP treatment. The monoclonal antibody, 238 that efficiently binds only the Ha-ras-p21 species but not Ki-ras-p21 also immunoprecipitated the 22K tumor translation protein suggesting that the tumor p21 is the product of a Ha-ras gene. These results suggest that activation and suppression of a cellular oncogene is associated with the growth and regression of mammary tumors, respectively and cAMP may play a regulatory role in this oncogene expression.

Significance to Cancer Research and the Program of the Institute:

These studies contribute to the understanding of the mechanism by which oncogenes involved in the neoplastic growth. The findings that activation and suppression of cellular ras oncogene correlate with growth and regression of mammary tumors, respectively, are consistent with the hypothesis that ras gene expression plays an important role in oncogenesis of mammary tumors. Moreover, cAMP may play important role in oncogenesis since treatment with dibutyryl cyclic AMP resulted in tumor regression as well as suppression of the oncogene expression. Finally quantitative detection of ras-gene expression may contribute to the early diagnosis of mammary cancer.

Proposed Course of Research:

To extend the investigation on the mechanism of oncogenesis of mammary tumors, the following proposal is made:

1. Assess the relationship between mammary tumorigenesis and expression of ras-gene at the level of both ras-transcript and ras-gene product, p21;

2. Localize p21 in tumor cells by immunoperoxidase staining method;

3. Assess the role of cAMP and its receptor protein in modulation of ras-gene expression during mammary gland development and mammary carcinogenesis;

4. Assess whether DNA of DMBA mammary carcinoma can transform NIH 3T3 cells and the transforming gene of tumor is homologous to the ras gene of Harvey sarcoma virus.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08281-01 LPP

PERIOD COVERED

April 1, 1983 to September 30, 1983

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

Mechanism of Reverse Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI.: Y.S. Cho-Chung Chief, Cellular Biochemistry Section LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Cellular Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

Occasionally, tumor cells differentiate spontaneously and then regress completely. It has been suggested that cAMP may be linked with the morphological differentiation of neoplastic cells since treatment of some tumor cells with dibutyryl cAMP, prostaglandin E_1 and inhibitors of cAMP-phosphodiesterase induces irreversible morphorlogical differentiation. That this differentiation may be a reversion of malignancy is supported by the observation that no tumor is produced when these treated cells are inoculated into animals.

Avian sarcoma virus-transformed mammalian cells also occasionally revert to a normal phenotype. Current information suggests four major categories of mechanisms by which transformed cells may revert to a normal phenotype: (1) loss of the viral genome; (2) mutation in the transforming gene(s) (by deletion, insertion or base change); (3) reduction in transforming-gene expression at either transcriptional, translational, or posttranslational levels; and (4) the appearance of host-cell resistance to the effects of viral transforming genes.

To investigate factors that affect phenotypic reversion of transformed cells, we have chosen a cell line 433 of NIH 3T3 cells containing the transforming ras-gene of Harvey sarcoma virus flanked by LTR of MMTV; the expression of ras-gene in 433 cells is therefore controlled by mouse mammary tumor virus promoter (MMTV-LTR) which is under control of glucocorticoid. Thus, the phenotypically normal 433 cells become transformed and produce the ras-gene product, p21 only upon addition of glucocorticoid. The goal of this study is to investigate the effect of intracellular regulatory factors, such as cyclic nucleotides, hormones, and growth factors on the controlling element, MMTV-LTR to gain knowledge on the mechanism of reverse transformation.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:			
T. Clair	Chemist	LPP,	NCI
B. Bassin	Chief, Biochem. Oncogenes	LTIB,	NCI
C.L. Kapoor	Retina Foundation Fellow	LVR,	EI

Project Description

Method Employed:

1. 433 cells: An established cell line of NIH 3T3 cells carrying MMTV-LTR-v-has. Grown on polylysine-coated plastic substrate in media containing only transferrin, insulin and salts. Under these conditions, the cells respond dramatically to the addition of dexamethasone. Flat, contact inhibited monolayers are observed in the absence of hormone; in the presence of increasing concentration of dexamethasone, the cells become round and refractile and float away from the substratum. Effect of intracellular regulatory factors, such as, cyclic nucleotides, steroid hormones and growth factors will be examined on the expression of ras-gene and cell morphology.

2. p21 Immunoprecipitation: [^{35}S] methionine-labeled cell lysates were incubated for overnight at 4°C with rat monoclonal antibody against Harvey Sarcoma virus p21. The reaction mixture was then incubated for an additional 2.0 hr at 4°C with a 10% suspension of Formalin-fixed Staphylococcus aureus (precoated with rabbit anti-rat immunoglobulin G). Immunoprecipitates were collected by centrifugation, washed and analyzed by SDS-PAGE.

3. Immunocytochemistry: An indirect immunocytochemistry of cAMP receptor proteins of 433 cells was carried out as described in (Kapoor and Cho-Chung, Cancer Res. 43: 295, 1983). Affinity purified cAMP receptor protein antibodies were utilized to reduce the background fluorescence.

4. MCF-7 cells: The MCF-7 cells (Mason Research Institute) were grown in McCoy's 5A medium supplemented with bovine insuline, penicillin, streptomycin and fetal calf serum (10%). Addition of dibutyryl cAMP induces growth arrest along with a change in cell morphology.

5. Transfection: Transfection of NIH 3T3 cells with cellular DNA from growing vs. dibutyryl cAMP-arrested MCF-7 cells is performed. The transformed foci formation is correlated with appearance of tumor cell specific antigens (86K, 70K and 19K proteins) and the effect of cAMP receptor proteins.

Major Findings:

An established cell line in which the gene product responsible for the transformed phenotype is under control of a hormone responsive promoter made possible the studies on reverse transformation. 433 cell line of of NIH 3T3 cells is a stable MMTV-LTR-v-ras gene carrier in which the transforming ras-gene is not expressed until glucocorticoid is added. Flat, contact inhibited monolayers are observed in the absence of hormone; in the presence of dexamethasone (10^{-11}–10^{-6}M) the cells become round and refractile and float away from the substratum.

This effect of dexamethasone was reversed when DBcAMP (10^{-4}M), 8-azido cAMP (10^{-5}M), or 8-Br-cAMP (10^{-5}M) were added simultaneously with dexamethasone (2 x 10^{-6}M): the cells exhibited flat, contact inhibited monolayers just as the cells in the absence of dexamethasone. The effect of dexamethasone in conversion of normal phenotype to transformed phenotype occurs between 10^{-9}M and 10^{-8}M concentrations, in close agreement with the binding constant for the glucocorticoid receptor, confirming that the cell phenotype "switch" is in fact mediated by the normal cytoplasmic receptor. This is also true with the effect of cAMP analogs in reversion of the transformed to normal phenotype since the effective concentration of cAMP analogs was in close agreement with the binding constant for the cAMP receptor. These results suggest that steroid and cAMP receptors counteract each other directly or indirectly on a hormone-responsive promoter that regulates expression of an oncogene.

Significance to Cancer Research and the Program of the Institute:

These studies contribute to the understanding of the mechanism of reverse transformation. The antagonistic action between a steroid hormone and cAMP on a hormone-responsive promoter that regulates the oncogenic expression may be of great importance in the understanding of cancer etiology; thus extension of this knowledge will contribute to prevention and better treatment of cancer.

Proposed Course of Research:

To extend the investigation on the mechanism of reverse transformation, the following proposal is made:

1. Assess the relationship between the effect of cAMP on phenotypic reversion of transformant and expression of the transforming ras-gene transcript and its product p21.

2. Assess whether the antagonism between glucocorticoid receptor and cAMP receptor is exerted directly on MMTV-LTR.

3. Assess intracellular localization of glucocorticoid- and cAMP-receptor by immunocytochemical method.

4. Assess whether growth arrest of MCF-7 cells by DBcAMP reflects a phenotypic reversion using transfection experiments of NIH 3T3 cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08282-01 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

In Vitro Assembly of Gap Junctions

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

PI: Pedro Pinto da Silva Chief, Membrane Biology Section NCI, LPP

COOPERATING UNITS *(if any)*

Dr. Blanchette Mackie, NIADDKD

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Membrane Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.92	0.81	0.11

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

We developed a method to induce rapid assembly of gap junctions in prostate epithelial cells in vitro. Assembly of gap junctions can be induced by incubation at 0°C followed by incubation at 37°C. Colchicine (10^{-5} M, 10^{-3} M) and cytochalasin B (25 µg/ml, 100 µg/ml) at room temperature or at 37°C also induce assembly of gap junctions. Assembly of the junctions proceeds even in the presence of a metabolic inhibitor (dinitrophenol) or of an inhibitor of protein synthesis (cycloheximide). We conclude that assembly of gap junctions can proceed from a pool of pre-existing precursors. The experimental conditions that result in gap-junction assembly involve perturbation of the cytoskeleton. Therefore, we propose that the assembly of gap junctions requires convergent migration of precursor molecules whose positional control in the membrane is released by perturbation of the cytoskeleton. Aggregates of particles and rugosities, whose distribution size and shape is similar to that of gap junctions, may represent intermediate assembly stages. This would indicate that the final stages in the assembly take place only after convergence of the precursor molecules to the junctional site and involve profound conformation changes required for establishment of fully assembled connexons.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: G. Tadvalkar Visiting Fellow LPP, NCI
C. Parkison Chemist LPP, NCI

Project Description

Objectives:

To develop experimental models in which formation of gap junctions, de novo, can be induced under controlled conditions.

Methods employed:

Adult male Sprague-Dawley rats (150-175 g) were lightly anesthetized with ether and the bilobed prostatic tissue was excised. In each instance, one lobe was used to test the experimental conditions and the other used as a control. Each experiment was repeated three times with tissue taken from different animals. Tissue was minced in 0.1 M sodium cacodylate buffer at pH 7.4 and incubated in the same buffer under the desired experimental schedule. Some experiments involved temperature shifts. A rapid temperature transition was assured by placing the tissue within vials with a wire-mesh botton that could be quickly transferred to buffers at different temperatures. The tissues were incubated at 0°C for 2, 5, 15, or 30 min and then transferred to 37°C and incubated for an additional 2, 5, 15, or 30 min. Temperature shift experiments (30 min at 0°C; 30 min at 37°C) were also performed in the presence of cycloheximide, a protein synthesis inhibitor (27), or of 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation (5, 14). These drugs were added to the incubation medium at both temperatures in concentrations of 200 µg/ml and 5 mM, respectively.

To assess the effect of chemical disruptors of the cytoskeleton, we incubated minced tissue (at room temperature or at 37°C) for 60 min in colchicine (10^{-3} M and 10^{-5} M) or in cytochalasin (100 µg/ml or 25 µg/ml). A shorter incubation period (10 min at room temperature) was also tried on higher concentrations of the drugs (colchicine 10^{-3} M, cytochalasin B 100 µg/ml). To test the reversibility of the effects of colchicine and cytochalasin B, we removed some treated samples after 30 min, washed them and incubated them at 37°C for 30 min in buffer without the drugs. Experiments with cytoskeleton disruptors were also performed with temperature shifts as described above (i.e. preincubation with the drug at 0°C for 30 min followed by incubation at 37°C for 30 min).

To rule out the possible toxic effects of arsenic in cacodylate as well as the effect of the absence of Ca^{++}, Mg^{++}, K^{+}, and Cl^{-} ions from the incubation medium, we repeated all experiments using Tyrode buffer bubbled with a gaseous mixture of 95% oxygen, 5% carbon dioxide (24, 25).

All experiments were terminated by fixation for 2 h in 5% glutaraldehyde in cacodylate buffer at the termperature of the last incubation. The specimens were rinsed in buffer, impregnated in 30% glycerol in buffer, placed on gold specimen carriers, and frozen in partially solidified Freon 22. The frozen specimens were freeze-fractured at -- 130°C and shadowed at an angle of 45° with a platinum/carbon electron gun. The replicas were cleaned, mounted on Formvar-coated grids, and observed with an electron microscope at 80 kV.

Morphometry: For each experimental condition we photographed and printed (final magnification, x 32,500) at least 25 views of distinct areas of fractured lateral membranes displaying increase in gap junction frequency. The minimum total area of fractured lateral membrane scanned in each experiment was in excess of 2,000 μm^2. Gap junctions were identified, counted, averaged, and their average frequency was expressed as the number of gap junctions per 100 μm^2. The maximum density of gap junctions observed on fractured faces of lateral membranes (area>20 μm^2) was also recorded.

Major Findings:

We show that it is possible to induce, in vitro assembly of gap junctions. The experimental conditions that induced this assembly can be related to the disruption or perturbation of elements of the cytoskeleton. Since assembly of gap junctions can occur in the presence of concentrations of dini trophenol (inhibitor of cellular metabolism) or of cycloheximide (inhibitor of protein synthesis), we propose the assembly of gap junctions can proceed from moieties already present at the plasma membrane at the onset of the of the experiment.

Significance to Biomedical Research and the Program of the Institute:

Gap junctions are regional membrane specializations that, in animal cells, are responsible for intercellular communications. The mode of formation of gap junctions and the forces responsible for their structural maintenance are largely unknown. A mjaor obstacle for investigations on formation and maintenance of gap junctions is the lack of experimental models, where gap junctions are formed de novo, under controlled conditions. We developed a method to induce, in vitro, notable increases in the frequency of gap junctions in rat prostate.

Proposed Course of Research:

The object of our current research is the ontogenetic and structural relationships as well as functional "linkages" that, we believe, exist between gap and tight junctions and, possible, desmosomes.

Publications:

Tadvalkar, G. and Pinto da Silva, P.: In vitro, rapid assembly of gap junctions is induced by cytoskeleton disruptors. J. Cell Biol. 96: 1279-1287, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08283-01 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of Cytoplasm Compaction by Permeation of Probes into Freeze-Fractured Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
PI: Pedro Pinto da Silva Chief, Membrane Biology Section LPP, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Membrane Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.72	0.61	0.11

CHECK APPROPRIATE BOX(ES)
☐ (a) Human 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 developed a method to study the existence and distribution of protein-free spaces in the cytoplasm, by the exclusion of probes of known dimensions from the chemically cross-linked and freeze-fractured cytoplasm matrix. The results were observed by electron microscopy and the probe utilized to explore the cytoplasm compaction was non-cationized ferritin (100 A° in diameter). We observed that cells in a resting state have a crowded cytoplasm that after cross-linkage by glutaraldehyde is impermeable to ferritin. In contrast, ferritin freely penetrates the cross-linked cytoplasm of growing cells. Variations in cytoplasm permeability to ferritin were observed in single populations of lymphocytes from peripheral blood. These variations are in accordance with the heterogenous character of these populations. Results obtained with lymphocytes activated with Phytohaemagglutinin suggest that lymphocytes with permeable cytoplasms probably correspond to activated cells. Contrary to lymphocytes, homogenous populations of cells (neutophils from peripheral blood and cells from specific stages of differentiation of the fungus Phytophthora palmivora) respond uniformly to ferritin penetration. We also observed that cytoplasm compaction can change during differentiation and that the distribution of protein-free spaces in muscle cells characterizes different physiological states. We conclude the exclusion of probes from freeze-fractured cytoplasm is an easy and convenient method to evaluate cytoplasm compaction and to detect significant changes in the distribution of protein-free spaces at selected cellular states.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: Maria Luiza F. Barbosa Visiting Fellow LPP, NCI
C. Parkison Chemist LPP, NCI

Project Description

Objectives:

To study cytoplasm compaction through examination of the existence and distribution of protein-free spaces at selected stages of cellular development.

Methods Employed:

We utilized an adaptation of the fracture-label technique to evaluate the degree of protein packing in the cytoplasm. Cells or tissues were fixed in glutaraldehyde, frozen and fractured in liquid nitrogen. Due to the cross-linking properties of glutaraldehyde, after fixation, proteins were immobilized into a covalently linked "gel" network. The compaction of the network was a function of the protein concentration and reflected the compaction of the cytoplasm. After fracture, the exposed cytoplasm was put in contact with a solution with probes of known sizes. From the exclusion of these probes from the cross-linked cytoplasm we infered the existence of "void" space and assessed the degree of protein packing. The probe utilized was non-cationized ferritin. The cells utilized were: Lymphocytes and neutrophils from peripheral blood, cells from skeletal muscle of toad and cells from different stages of morphological differentiation of the fungus Phytophthora palmivora.

Major findings:

Technical development: Establishment of an easy and convenient method to evaluate cytoplasm compaction and to detect significant changes in the distribution of protein-free spaces at selected cellular states.

Scientific observations: 1) Cells in a resting state have a crowded cytoplasm, impermeable to ferritin: growing cells are freely penetrated by ferritin. 2) Homogenous populations of cells responded uniformly to ferritin penetration: in heterogenous populations, cytoplasm permeability varied among cells. 3) Cytoplasm compaction can change during differentiation; 4) The distribution of protein-free spaces in muscle cells characterizes different physiological states.

Significance to Biomedical Research and the Program of the Institute:

This work makes possible the utilization of a new criteria, cytoplasm compaction, in the study of important processes as latency, cell growth, differentiation, transformation and aging. The development of probes with new characteristics or different sizes would certainly improve the technique's resolution and expand its applicability.

Proposed Course of Research:

This project is being completed and should be submitted soon for publication.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08284-01 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Expression of Galactosyl Transferase in Normal and Cancerous Mammary Gland Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

PI: Hira L. Nakhasi LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

In order to study the regulation of galactosyltransferase gene in normal and neoplastic mammary glands we set out to clone the cDNA corresponding to this protein. To date we have generated a library of cDNA clones from a rat lactating mammary gland. This library was hybridized to cloned cDNA probes for abundant class of rat mammary gland mRNA. cDNA clones which did not hybridize to these probes (negative selection) were further selected by hybridization to cDNA probes synthesized either from 14 day lactating rat mammary gland RNA and or from 2 day pregnant rat mammary gland RNA. A set of clones were selected by this procedure. These clones were then classified by the protein products synthesized in vitro by their complementary mRNAs. Two sets of cDNA clones were identified which are under hormonal control and have different levels of mRNAs in mammary tumors.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	P. M. Gullino	Chief, Medical Officer	LPP, NCI
	P. K. Qasba	Expert	LPP, NCI

Project Description

Methods Employed:

1. Isolation of RNA from rat mammary gland.

2. Synthesis of cDNAs using reverse transcriptase.

3. Synthesis of double stranded cDNAs, tailing the cDNAs, ligating to tailed pBR 322 and transfecting the E. coli. RR_1 strain.

4. "Positive selection" of the RNA from the nitrocellulose bound cloned DNAs and translating them in vitro in either rabbit reticulocyte lysate or wheat germ translational system.

5. Digestion of the genomic DNAs with restriction endonucleases, separation on agarose gels, transfer onto nitrocellulose filter and then hybridizing to specific radioactive probes.

6. Separation of total or polyA+ RNAs on the agarose gels, transfer on to nitrocellulose filters and hybridization to radioactive probes.

Major Findings:

1. By using the "negative selection" technique we were able to obtain two types of clones. One which represents a casein type of mRNAs but is not one of the known caseins and other which represents a rare class of mRNA.

2. The casein type cDNA clone has been designated as p53 and the rare class type as p13.

3. p53 cDNA clone selects 4 types of mRNAs in "positive selection" which when translated in vitro system synthesize 50K, 39K, 31K and 26.5K proteins.

4. p53 cDNA clone hybridizes to total RNA from 14 day lactating mRNA and is absent in virgin rat mammary gland RNA. Overectomy of the mid-pregnant female rats increases the level of this mRNA.

5. In carcinogen induced rat mammary tumor MTW9 the levels of clone p53 mRNA are almost the same as in 14 day lactating rat mammary gland whereas in other carcinogen induced tumor, 7,12 DMBA the levels are slightly less.

6. Clone p13 represents a rare class of mRNA in rat mammary gland (0.05%).

7. This clone hybridizes to two size class of mRNAs (1080 and 3800 bases) in rat mammary gland RNA and one size class of mRNA (3800 bases) in liver RNA.

8. mRNA selected from mammary gland RNA codes in wheat germ in vitro transtational system 23.5K protein whereas mRNA selected from liver RNA does not code for any protein. However, in rabbit reticuloocyte lysate system even mRNA from mammary gland RNA does not code for any protein.

9. Clone p13 is coded by a single copy gene and this gene has different restriction patterns for the DNA from mammary gland and liver.

10. Expression of this clone is amplified in 7,12 DMBA tumor but it is greatly reduced in MTW9 tumor. There is an increase in mRNA levels corresponding to this clone with the onset of gestation and continues to increase during lactation.

Significance to Biomedical Research and the Program of the Institute:

Clone p53 and p13 can be used as mammary tumor specific markers and can distinguish between different carcinogen induced tumors.

Proposed Course of Research:

1. The nucleotide sequence of these clones will be done which will be useful to determine the nature of these cDNA clones and the proteins which they code for.

2. These clones will be used to analyse various types of carcinogen induced tumors.

3. Finally cDNA clone for both rat and human galactosyl transferases will be isolated, characterized and then used as molecular probes to study the regulation of its gene in both normal and tumor breast tissues.

Publications:

Nakhasi, H.L.: Characteristics of a rare class of mRNA from rat mammary gland. (In preparation).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08285-01 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Purification and Characterization of Rat and Human Milk Galactosyltransferase.

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: Hira L. Nakhasi Staff Fellow LPP, NCI

COOPERATING UNITS *(If any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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.)*

Glycosyl transferases are a group of enzymes which are associated to the cell membrane and have been implicated to play a role for intercellular adhesion and tumor invasion. We are studying one of these enzymes, galactosyl transferase. We have purified this enzyme from both rat and human milk using two rapid chromatography steps, N-acetyl-glucosamine-sepharose and α-lactalbumin-sepharose. This procedure enables the isolation of the rat milk enzyme which is highly labile and has low activity. This enzyme showed three polypeptide bands of MW 59K, 54K and 27K on SDS-polyacrylamide gel. The human milk enzyme on the other hand showed a single band of MW 54K on SDS-polyacrylamide gel. Antisera raised against the 54K form of rat milk galactosyl transferase immunoprecipitated the other forms suggesting that they are antigenically related. However, this antisera did not cross react with human milk enzyme.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:	K. R. Daruwalla	Visiting Fellow	LPP, NCI
	P. K. Qasba	Expert	LPP, NCI

Project Description

Methods employed:

Whole rat milk from wistar rats was obtained from Dutchland Laboratories Inc., Denver, PA and human breast milk was obtained at Georgetown University Hospital, Washington, D.C. Both these milks were defatted and caseins were removed by acid precipitation. The whey was then passed through N-acetylglucosamine-sepharose column. The material bound to this column was passed through α-lactalbumin-sepharose column. α-lactalbumin-sepharose bound material was finally run on SDS-polyacrylamide gel electrophoresis to check for its homogenity.

Major Findings:

1. Galactosyl transferase activity from rat milk is 0.6 units/ml which is 16 fold lower than the enzyme activity in human milk.

2. Rapid loss of enzyme activity from rat milk was observed during the purification steps and on storage.

3. Using the modified coupling procedure for N-acetylglucosamine to sepharose the binding capacity of the rat milk enzyme was enhanced to a great extent.

4. SDS-polyacrylamide gel analysis of purified rat milk enzyme showed three polypeptide bands corresponding to Mw 59K, 54K and 27K. In contrast the purified human milk enzyme showed a single polypeptide band corresponding to Mw 54K.

5. Antisera raised against 54K form of rat milk purified enzyme cross reacts with other forms of rat milk enzyme. It does also precipate enzyme activity with increasing concentration. However, this antisera does not cross react with purified human milk enzyme.

Significance to Biomedical Research and the Program of the Institute:

Purified human milk galactosyl transferases will be used to make monoclonal antibodies. These monoclonals will be used as a diaganostic tools for human breast cancer.

Proposed Course of Research:

1. Purified human milk galactosyltransferase will be subjected to amino acid sequence analysis.

2. From the amino acid sequence synthetic oligo nucleotides will be synthesized and those will be used as probes to isolate the cDNA clones for human galactosyl transferase.

3. cDNA clones for human galactosyl transferase will be used to study the gene regulation in normal and cancerous mammary glands.

4. Monoclonal antibodies will be raised against this protein which will be then used for breast cancer therapy.

Publications:

Daruwalla, K.R., Qasba, P.K. and Nakhasi, H.L.: Purification of rat galactosyl transferase from rat milk. (In preparation).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08286-01 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Regulated expression of a cloned α-LA gene transfected into a mammalian cell

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: P. K. Qasba Senior Investigator LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

Rat α-lactalbumin gene expression in the mammary gland is under the influence of insulin and hydrocortisone. Prolactin, however, enhances this expression and can be replaced by a factor present in bovine serum albumin. We have isolated rat α-lactalbumin gene and constructed various plasmids carrying the regulatory sequences of the gene. We are presently studying the involvement of the individual hormones in the expression of these plasmids in the transfected mammalian cells.

PHS 6040 (Rev. 1/83)

GPO 895-100

Other Professional Personnel: I. K. Hewlett Visiting Fellow NCI, LPP

Project Description

Objectives:

1. To determine the nucleotide sequences involved in the expression of α-LA gene by various hormones and to determine the alterations which have occurred in these sequences upon neoplastic transformation.

2. To evaluate the role of prolactin and hydrocortisone in the regulation of the production of α-LA mRNA.

Major Findings:

This work has just been started in the last five months. We have inserted the rat α-LA gene sequences in the vectors that permits the expression of the genes in the mammalian cells, e.g., pSV2-gpt recombinants carrying neomycin resistant markers.

Methods Employed:

1. Construction of the plasmids carrying α-LA gene in the vectors which contain antibiotic resistant markers, e.g., neomycin.

2. Transfection of the mammalian cells carry hydrocortisone and prolactin receptors, e.g., MCF-7 cells or L-cells (which carry only hydrocortisone receptors) with the α-LA gene constructs.

3. Quantitation of mRNAs: The method is based on hybridization of the RNA linked to DBM-cellulose paper (filters) with a nick-translated [^{32}P] plasmid probe. RNA isolated from fresh tissue is bound to DBM-cellulose filters (usually 2 to 6 µg of total RNA per 11 mm circles). The filters are prehybridized, then hybridized to [^{33}P] plasmids, extensively washed and counted. Standard curves were obtained hybridization of known amounts of pure α-LA mRNA.

4. Agarose gel electrophoresis of RNA: Estimation of the presence of mRNA in total RNA extracted from tissues or cultured cells is also performed after agarose gel electorphoresis of the total RNA. RNA from nick-translated [^{32}P] plasmids. The cellulose paper is washed and then exposed to an x-ray film.

Significance to Biomedical Research and the Program of the Institute:

Studies on the α-LA gene expression during normal differentiation of mammary cell and alterations in these sequences upon neoplastic transformation will help us to understand the underlying relationship of differentiation with transformation of the mammary epithelial cells.

Proposed Course of Research:

The experiments will be further carried out to complete the stated objectives.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08287-01 LPP

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization and cDNA cloning of α-LA-like activity from epididymis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

PI: P. K. Qasba Senior Investigator LPP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Pathophysiology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

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

C

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

α-lactalbumin, a modifier protein that changes the substrate specificity of galactosyltransferase, to promote the synthesis of lactose, is found in the mammary glands of lactating mammals and in milk. Molecules similar to mammary gland α-lactalbumin but distinct in their modifier activity have been found in the epididymal fluid. This activity differs from mammary gland α-LA activity in that it transfers galactose from UDP-galactose to either glucose or myo-inositol with equal efficiency. Using rat mammary gland α-lactalbumin cDNA clone as a hybridization probe, RNA sequences homologous to α-lactalbumin mRNA were also detected in total RNA from rat epididymis. This finding suggests that α-lactalbumin or similar molecules, in addition to regulating lactose synthesis in the mammary gland, may have other important functions, e.g., synthesizing specific oligosaccharide sequence on the cell surface glyco-proteins which are recognized as new antigenic determinants specifically in the male reproductive tract, where lactose is absent and free glucose levels are barely detectable, α-LA-like activity may modulate sperm surface glyco-proteins which may play an important role in sperm-egg surface interactions during fertilization.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: I. K. Hewlett Visiting Fellow NCI, LPP

Project Description

Objectives:

Characterize α-LA-like activity in epididymis and isolate a cDNA clone corresponding to this protein.

Major Findings:

1. α-LA-like activity was found in the extracts from epididymis. Lactose synthetase activity in the epididymal extracts was about 30-50 times less than in the comparable extracts from 5 day lactating mammary gland. Furthermore, epididymal extract, in contrast to rat mammary gland extract or pure α-LA, promotes the transfer of galactose from UDP-galactose to either glucose or myo-inositol with nearly equal efficiency.

2. The products of these reactions, lactose and galactinol were characterized by paper chromatography.

Methods Employed:

1. Purification and measurements of α-LA-like activity.

2. Identification of the enzymatic products either by paper chromatography or by high pressure chromatography.

3. Isolation of the epididymal α-LA mRNA, synthesis of double stranded DNA, synthesis of recombinant plasmids and isolation of the cDNA clone corresponding to epididymal α-LA.

Significance to Biomedical Research and the Program of the Institute:

The characterization and cloning of α-LA-like activities from other tissues are essential in understanding the function of the modifiers of galactosyltransferase which may be involved in the alterations of cell surface antigenic determinants during the neoplastic transformations.

Proposed Course of Research:

The experiments will be further carried out: 1) To isolate and sequence the cDNA clone corresponding to the epididymal α-LA, and 2) to find if α-LA-like activities are present in other tissues.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08288-01 LPP

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

cDNA cloning of cow galactosyltransferase

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

PI: P. K. Qasba LPP, NCI

COOPERATING UNITS *(if any)*

Dr. K. Brew, Dept. of Biochemistry, Miami Medical School, Miami, Florida

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

C

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

α-Lactalbumin modifies the activity of galactosyltransferase in such a way that it inhibits the transfer of galactose from UDP-galactose to N-acetyl-glucosamine either free or linked as a terminal sugar of a glycoprotein, but facilitates the transfer to glucose or myo-inositol. To understand the modulation of galactosyltransferase activity essential for generating specific cell surface antigenic determinants, we have first isolated and characterized cDNA clones corresponding to α-lactalbumin. Isolation and sequence analysis of the cDNA clones corresponding to the galactosyltransferase is now essential in understanding this molecular mechanism of the modulation of the transferase.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: H. L. Nakhasi Staff Fellow LPP, NCI

Project Description

Objectives:

The purpose of this work is to isolate and characterize cDNA clone corresponding to bovine galactosyltransferase.

Major Findings:

The project was started only a month ago.

Methods Employed:

1. A cDNA library will be prepared from total poly(A) RNA isolated from bovine mammary gland and vectors which permit expression of cDNA inserts in mammalian cells (H. Okayama and P. Berg vectors).

2. Synthetic polynucleotides corresponding to the polypeptides of cow galactosyltransferase (peptides isolated and sequenced by K. Brew) will be used as probes to select for the cDNA clones corresponding to cow galactosyltransferase.

3. The cDNA clones will be sequenced. Protein sequence derived from the DNA sequence will be compared with the partial protein sequence of the cow galactosyltransferase which will be provided to us by K. Brew.

Significance to Biomedical Research and the Program of the Institute:

Protein sequence of galactosyltransferase and a cDNA probe for it will help in understanding the modulations of the cell surface glycoproteins essential for cell-cell interactions.

Proposed Course of Research:

See Major Findings

Publications:

None

LABORATORY OF MATHEMATICAL BIOLOGY

SUMMARY

October 1, 1982 through September 30, 1983

Summary Report

The activities of the Laboratory of Mathematical Biology (LTB) fall into several broad areas: macromolecular structure and function, membrane structure and function, immunology, pharmacokinetics, and computational and modeling methodology. Most of the work is theoretical, but experiments are also carried out in the laboratory. Much of the theoretical work and modeling is done in collaboration with experimental groups at NIH and elsewhere.

The studies in the section on Membrane Structure and Function (R. Blumenthal) deal with the the insertion and organization of molecules (proteins, lipids) in membranes and changes in membrane organization both in the lateral and perpendicular directions. We use spectroscopic techniques (fluorescence, circular dichroism) to study lipid-protein interactions, and measure conductance across planar black lipid membranes (BLMs) to approach questions of membrane protein topology.

The following systems are investigated: 1) Interactions of cytoskeletal proteins (clathrin, tubulin) with lipid vesicles 2) The effect of charge clusters and the membrane potential on the disposition of membrane proteins. We are carrying out BLM studies with the hepatic asialoglycoprotein receptor and with melittin. We are also looking at the interaction of apocytochrome c with membranes as a model for mitochondrial protein transport. 3) Fusion of lipid bilayers induced by Ca^{2+}, pH and/or such proteins as clathrin and tubulin using resonance energy transfer between two lipid fluorophores incorporated into the same bilayer. We have reconstituted VSV G protein in lipid vesicles and are studying the mechanism of fusion induced by that protein (O. Eidelman, R. Blumenthal, P. Greif). 4) Lateral organization of membrane components between apical and basolateral surfaces in epithelial cells.

Work on liposomes has centered on three ways to direct liposome-encapsulated agents to tumor targets: (1) Antibody-mediated targeting to tumor cells, with immunoglobulin attached to the liposome covalently or through binding sites; (2) Temperature-sensitive liposomes, so designed as to "self-destruct" and release encapsulated drug selectively in a tumor; (3) Compartmental delivery of liposomes and their contents to lymph node micro-metastases (J. Weinstein).

We have developed 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 (J. Weinstein, D. Covell). 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 of the antibody were combined with in vivo pharmacokinetic parameters to develop SAAM compartmental models of the delivery system. Later,

specific uptake of antibody via the lymphatics was obtained in lymph node micrometastases of guinea pig line 10 tumor. The principle is currently being extended to antibody-bearing, radionuclide-containing liposomes as a means of amplifying effect. The cell biology of monoclonal antibodies after binding is also being explored, by methods similar to those with which we previously examined the cellular fate of antibody-bearing liposomes. To this point, our studies suggest that the lymphatic route will provide higher sensitivity, higher efficiency, and less cross-reaction than intravenous injection for diagnostic imaging of early lymph node tumors. The combined theoretical/experimental program is aimed at the rational design of such approaches for clinical use.

Work continued on the analysis and modelling of experimental data bearing on molecular mechanisms of endocytosis (M. Gex-Fabry, C. DeLisi), exocytosis (M. Wastney, C. DeLisi) and chemotaxis (C. DeLisi) of various cellular systems. The former project involves a comprehensive analysis of data related to the early and late stages of cellular responses to epidermal growth factors. From this study we hope to gain a better understanding of, among other things, the effects of forbyl esters and various other tumor promotors on cellular division. In some respects the system can also be viewed as a single cell analogue of the B-cell mitogenic response to T independent antigens. Studies of exocytosis are at present confined predominently to mechanisms contolling the initial stages of release of histamine from mast cells and basophils, and the antigen specific and non specific desensitization of these systems. The processes occurring in these one cell - one ligand systems also serve as models for corresponding processes in the more complex B and T cell systems.

Fundamental theoretical studies of the chemotactic responses of leukocytes and bacteria continued, in an effort to elucidate the role of random errors due to thermal noise, on the ability of cells to respond accurately to chemotactic signals.

Work continued on analysis and experimentation designed to elucidate the maintenance phase of the immune response and impairments in that phase. The insights gained from simpler systems are being brought to bear in the formulation of a mathematical model whose purpose is to help plan and analyze experiments on complex time dependent behavior characteristic of immune response maintenance. A number of other dynamical systems are also being systematically studied and methods for analyzing them are being developed.

The development of a comprehensive data base containing primary, secondary and tertiary structural information in all sequenced nucleic acids and proteins continued (M. Kanehisa). Algorithms are being developed and used for the retrieval and analysis of large amounts of information related to sequence and structural homologies. Methods are being developed for predictive classification of the location and general function of proteins, such as oncogene products, which should serve as a guide to locating them and planning experiments to elucidate their function (M. Kanehisa, P. Klein, and C. DeLisi).

Research within the laboratory encompasses biological macromolecules and their properties. Stabilities of macromolecular conformations are determined by interatomic interactions; the relative importance, for proteins, of various classes of interactions, short range and long range, is being assessed in detail

(R. Jernigan and S. Miyazawa). Short and medium range interactions appear to determine locations of regular α-helices. Calculation of approximate free energies are used together with optimal selection methods to yield secondary structure predictions. Several simple models of long range intramolecular interactions have been formulated to facilitate investigations of protein folding pathways. A simple method, similar to a helix-coil theory, has been developed to describe the folding process. Methods for "trapping" folding intermediates are necessary because they are usually not present in substantial concentrations. From calculated probabilities of each residue in the native conformation at each stage of folding, folding-unfolding pathways have been constructed. Typically, folding is observed to proceed by growth from only a few centers within the molecule. Also models of membrane receptor proteins are being developed (R. Guy). This structural modeling proceeds by combining the limited experimental data with calculations of preferred locations, orientations of helices with respect to the membrane boundaries, helix-helix packing, and disulfide bond formation. The result for acetylcholine receptor protein is a polar channel composed of one amphipathic helix from each subunit. Mechanisms for channel opening and closing, as well as toxin and drug binding, are being investigated.

Modeling of the endocrine system has continued on lipoproteins, the glucose-insulin system and receptors (D. Covell).

Detailed modeling of ketone bodies has also been carried out (M. Wastney) in collaboration with Dr. S. Hall of Ottawa, who carried out the experiments. This relates to other modeling carried out by this group on intermediate metabolism. Studies on insulin secretion in various populations and its variation as a function of glucose load have also been studied (D. Covell).

The modeling techniques have been further extended by adding a number of new features in Conversational SAAM (CONSAM) on our VAX 11/780 computer (R. Boston, and P. Greif). This is a continuing project as the power of the SAAM program is being extended.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08300-11 LTB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Development (Previously SAAM, Modeling and Applications)

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Charles DeLisi, Acting Chief, LTB, NCI

COOPERATING UNITS *(if any)*

LaTrobe University, Australia

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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.)*

Continuing development of a computer system (SAAM) for the simulation analysis and modeling of bio-kinetic systems. Further development of a conversational model of operation, increased versatility of applications and automation in modeling are in progress.

Analysis of various kinetic and metabolic systems by the use of mathematical models carried with other investigators.

Other Professional Personnel:

Raymond C. Boston, Ph.D., Visiting Scientist, LTB, NCI
Peter C. Greif, M.D., Staff Fellow, LTB, NCI

Project Description:

Objectives: To develop a general purpose computer program for modeling bio-kinetic systems that may be readily used by investigators not sophisticated in mathematics or programming. Initiated in 1959, the program continues to be expanded and revised as new features are added.

Analysis of data on metabolic systems, and further development of modeling theory and techniques through the applications of the SAAM computer program.

Major Findings: Through the visits of Dr. Raymond Boston from LaTrobe University, Australia, new features have been incorporated in conversational SAAM (CONSAM). These include the abilties to interface with the least squares convergence procedures and to modify them to compensate for non linearities. The differential equations soluiton algorithms are also being expanded to take advantage of the latest state of the art in this area (Schwartz).

The SAAM programs are being applied to a variety of problems. A model was constructed for the kinetics of warfarin in dogs (Covell); HDL apoA kinetics were studied in the dog in collaboration with Dr. A. Scanu (Univ. of Chicago) (Chu), and in monkeys -- in collaboration with Dr. Peter Herbert (Univ. of Rhode Island) (Chu). Various other transport problems have also been modeled in collaboration with other investigators, or as port of a training activity.

A pilot study was initiated this year to develop data bases for various metabolis systems to serve as bases for the development and testing of models. The data bases will be constructed partly from available data and partly from literature data. Formatting of the literature data is now in progress in collaboration with Dr. Naomi Sager, who heads a computer linguistics group at New York University, NY.

Significance to Biomedical Research and the Program of the Institute: The methodology of modeling is most essential for studying the behaviour of systems for both normal and abnormal states. The SAAM computer program has been a most essential tool in the modeling of various systems described elsewhere in this laboratory report. Its wide use in other centers in the United States and elsewhere is further evidense of its value in biomedical research.

Proposed Course: This is a continuing process for the development of theory and for the application of modeling techniques.

Publications:

Boston, R., Greif, P., and Berman, M.: CONSAM-Conversational SAAM as a Modeling Tool. In Berman, M., Grundy, and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. 1983, 468 pages.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08303-11 LTB

PERIOD COVERED: October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Movement of Molecules in Membranes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation) Robert Blumenthal, Ph.D., Chief, Membrane Structure and Function Section, LTB, NCI

COOPERATING UNITS *(if any)*

M. Henkart, Ph.D., P. Henkart, Ph.D., & R. Schlegel, M.D. IB, NCI; A. Walter, Ph.D, & J. Hendler, M.D., LB, NHLBI; C.J. Steer, M.D. & R.D. Klausner, M.D., LBM, NIAMDD; S.J. Morris, Ph.D., IRP, NINCDS

LAB/BRANCH: Laboratory of Mathematical Biology

SECTION: Membrane Structure and Function Section

INSTITUTE AND LOCATION: NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

We study the organization and changes in organization of membrane components (lipids and proteins), both in the lateral and in the perpendicular direction. (1) We follow the insertion of a protein into a preformed lipid bilayer (either in the form of a planar bilayer or of a lipid vesicle), and study the factors which determine the protein's orientation. We measure electrical properties of Black Lipid Membranes (BLM) to study: (a) mechanisms of ion transport; (b) properties of transport systems isolated from natural cell membranes; (c) mechanisms of cytotoxicity; (d) the effect of the membrane potential on the disposition of membrane proteins. (2) We have developed model systems in which fusion of phospholipid vesicles is induced Ca^{2+}, pH, and/or by such proteins as tubulin, clathrin, apocytochrome c and VSV G protein. We study this fusion process using an assay involving resonance energy transfer between two fluorophores incorporated into the vesicle bilayer. (3) We observe lateral organization and movement of fluorescently - labelled molecules on cell surfaces by fluorescence microscopy. We study the mechanism by which asymmetry is maintained between apical and basolateral surfaces in epithelial cells.

PHS 6040 (Rev. 1/83) GPO 895-100

Project Description:

Objectives: To study the physical mechanisms of ion transport in reconstituted membranes. To develop the lipid bilayer membrane as an assay for transport systems isolated from natural cell membranes. To study mechanisms of cytotoxicity. To study the effect of the membrane potential on the disposition of membrane proteins. To study the role played in cell membranes by the mobility and distribution of cell surface receptors. To study the physiological significance of domains of lipid in membranes. To study factors which constrain the movement of membrane protein and lipids to specific areas of the cell surface. To study the mechanism of membrane fusion.

Methods Employed: The bilayer membranes are formed from natural membrane extracts, oxidized cholesterol, or pure lipids in a aperture between two electrolyte solutions. The electrical properties of the membranes are measured before and after application of an activating factor. Lipids vesicles are formed by sonication, reverse-phase evaporation and detergent dialysis. Leakage from vesicles is assayed by measuring the increase in fluorescence as vesicle-encapsulated self-quenched carboxyfluorescein is released into the medium and diluted. Spectroscopic changes upon interaction of proteins with lipid vesicles are studied by fluorometry and circular dichroism. Movement of fluorescently labelled molecules (proteins, lipids, carbohydrates) on cell surfaces is monitored by fluorescence microscopy. Other techniques to measure movement of molecules in membranes are fluorescence polarization, fluorescence energy transfer, and fluorescence stopped-flow kinetics.

Major Findings: 1) N-terminal-acetylated melittin showed similar conductance changes in BLMs as unmodified melittin. The dependence of the conductance change on voltage indicate movement of one charge as opposed to two charges with the unmodified melittin. This indicates that the N-terminal glycine is involved in the gating of melittin. 2) Stable clathrin- vesicle complexes are formed by incubating the protein with phosphatidylcholine vesicles at pH 6.0 or below. The interaction is accompanied by structural changes in the bilayer as shown by Raman Spectroscopy. At pH 6.5 and below clathrin induces dioeyol phosphatidylcholine bilayers to fuse, as shown by electron microscopy, increased trapped volume, and changes in resonance energy transfer between two fluorescent lipid probes incorporated into the same vesicle. 3) Stopped-flow kinetics of Ca^{2+}-induced fusion of phosphatidylserine vesicles indicates that the rate-limiting step is stable aggegation. With small vesicles the fusion is leaky, whereas with large vesicles it is not. 4) Apo cytochrome c undergoes a structural change from random coil to α helix upon interaction with phospholipid vesicles containing phosphatidylserine. At pH below 5.0 the protein causes those vesicles to aggregate and fuse. 5) VSV G protein is reconstituted in phosphatidylcholine vesicles by dialysis of octylglucoside. At pH 6.0 and below those vesicles (virosomes) fuse with acceptor veicles containing phosphatidylserine as shown by electron microscopy, increased trapped volume, and changes in resonance energy transfer between two fluorescent lipid probes incorporated into the same vesicle. 6) Apical surfaces of epithelial cells are labelled with fluorescent gangliosides. Those gangliosides are endocytosed but do not pass to the basolateral surfaces.

Significance for Biomedical Research and the Program of the Institute: Mobility, distribution and expression of cell surface components are considered to have important implications for cell transformation and for many aspects of the physiology of normal and tumor cells.

Proposed Course: A) The BLM will continue to be used as an assay for conductance - inducing materials from cells. The notion of voltage - dependent assembly of proteins in membranes will be further explored with different lipid compositon, and with peptides with specific charge cluster distribution. We hope to study the voltage - dependent effects of leader sequences on lipid bilayers, and to develop an in vitro protein assembly system using the BLM. B) Further studies on the mechanism of membrane fusion will involve manipulation of the lipid composition, stopped-flow kinetics, ^{31}P-NMR and freeze - cleavage. We plan to identify an intermediate fusion complex (inverted micelle) using our fluorescence energy transfer techniques. C) We will continue studying the mechanism of establishing polarity in epithelial cells using fluorescent lipid probes, and fusion techniques with the VSV G protein virosomes.

Publications:

Blumenthal, R., and Klausner, R.D.: The interaction of proteins with black lipid membranes. In Poste G., and Nicholson G.L. (Eds.): Cell Surface Reviews. 8: 43-82, 1982.

Blumenthal, R., Kempf, C., van Renswoude J., Weinstein, J.N., and Klausner, R.D., Voltage-dependent orientation of membrane proteins. J. Cell Biochem. in press.

Weinstein, J.N., Leserman, L.D., Henkart, P.A., and Blumenthal, R.: Antibody-mediated targeting of liposomes. In Gregoriadis, G., and Papahadjapoulos, D. (Eds): Targeting of Drugs. New York, Plenum, 1982 pp. 185-202.

Blumenthal, R.: Membrane transport: In Colombetti, L. (Ed.): Radiotracers in Biology and Medicine. Chapter IV, 1982 CRC Press, Baton Rouge, FL pp. 49-77.

Klausner, R.D., Kempf, C., Weinstein, J.N., Blumenthal, R., and van Renswoude, J.: The folding of ovalbumin; in vitro renaturation vs. in vitro biosynthesis. Biochem. J. 1983, in press.

Steer, C.J., Klausner, R.D., and Blumenthal, R.: Interaction of liver clathrin coat protein with lipid model membranes. J. Biol. Chem. 257: 8533-8543, 1982.

Kumar, N., Blumenthal, R., Henkart, M., Weinstein, J.N., and Klausner, R.D.: Aggregation and calcium-induced fusion of phosphatidylcholine vesicle-tubulin complexes. J. Biol. Chem. 257: 15137-15144, 1982.

Klausner, R.D., Berman, M., Blumenthal, R., Weinstein, J., and Caplan, S.R.,: Compartmental analysis of light-induced proton movement in reconstituted bacteriorhodopsin vesicles. Biochemistry. 21: 3643-3656, 1982.

Dragsten, P.R., Handler, J.S., and Blumenthal, R.: Asymmetry in epithelial cells: Is the tight junction a barrier to lateral diffusion in the plasma membrane? In Bolis, S., Hoffman, J.F., and Giebisch, G. (Eds.): Membranes in Growth and Development. 1982 New York, Alan R. Liss, pp. 525-536.

Blumenthal, R., Ralston, E., Dragsten, P., Leserman, L.D., and Weinstein, J.N.: Lipid vesicle-cell interactions: Analysis of a model for transfer of contents from adsorbed vesicles to cells. Membrane Biochemistry 4: 283-303, 1982.

Blumenthal, R., Henkart, M., and Steer, C.J.: Clathrin-induced pH-dependent fusion of phosphatidylcholine vesicles. J. Biol. Chem. 258: 3409-3415, 1983.

Kempf, C., Klausner, R.D., Blumenthal, R., and van Renswoude, J.: Transport of proteins from the sites of genetic expression to their sites of functional expression: Protein conformation and thermodynamic aspects. Horizions in Biochemistry, in press.

Weinstein, J.N., Ralston, E., Leserman, L.D., Klausner, R.D., Dragsten, P., Henkart, P., and Blumenthal, R.: Self-quenching of carboxyfluorescein: Uses in studying liposome stability and liposome-cell interaction. In Gregoriadis, G. (Ed.): Liposome Technology CRC Press, in press.

Morris, S.J., Smith, P.D., Gibson, C.C., Haynes, D.M., and Blumenthal. R.: Rapid kinetics of Ca^{2+}-induced fusion of phosphatidylserine/phosphatidylethanol vesicles. J. Biol. Chem., in press.

Schwartz, C.C., Berman, M., Halloran, L.G., Swell, L. and Vlahcevic, Z.R.: Cholesterol Disposal in Man: Special Role of HDL Free Cholesterol. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Liproprotein Kinetics and Modeling. New York, Academic Press. 1982, pp 337-350.

Wastney, M.E., Riemke, R., Malmendier, C.L, and Berman, M.: Heterogeneity of Low Density Liproprotein: Kinetic Analyses of Asialated Liproproteins. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Liproprotein Kinetics and Modeling. New York, Academic Press. 1982, pp 139-144.

Berman, M., Beltz, W.F., Riemke, R., and Grundy, S.M.: VLDL-RG Exchange with HDL-TG In Vivo. In Berman, M., Grundy, S.M., and B.V. Howard (Eds.) Liproprotein Kinetics and Modeling. New York, Academic Press. 1982, pp 299-308.

Le, N-A., Grundy, S.M., and Berman, M.: A Reduced Model for Very Low Density Liproprotein Triglyceride Metabolism. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Liproprotein Kinetics and Modeling. New York, Academic Press. 1982, pp 461-470.

Chu, M.: and Berman, M., A Possible Interpretation of ApoE Kinetics in Man. In Berman, M., Grundy, and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. 1982, pp 411-416.

Berman, M.: Kinetic Analysis and Modeling: Theory and Applications to Lipoproteins. In Berman, M., Grundy, S.M., and Howard B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. 1982, pp 4-36.

Schwartz, C.C., Berman, M. Vlahcevic, Z.R., and Swell, L.: Multicompartmental Analysis of Cholesterol Metabolism in Man. Quantitative kinetic evaluation of pre cursor sources and and turnover high density lipoprotein cholesterol esters, J. Clin. Inves. 70: 863-876, 1982.

Schwartz, C.C., Vlahcevic, Z.R., Berman, M., Meadows, J.G., Nisman, M, and Swell, L.: Central hole of high density lipoprotein in plasma free cholesterol metabolism. J. Clin. Inves. 70: 105-116, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08320-08 LTB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Macromolecular Conformations

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Robert L. Jernigan, Ph.D., Theoretical Physical Chemist, LTB, NCI

COOPERATING UNITS *(if any)*

W. R. Church, G.A. Gochman and R.A. Reisfeld
Dept. of Immunology, Scripps Clinic & Research Foundation
La Jolla, CA 92037

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	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.)*

Protein conformations have been investigated by calculations which include either short and medium range or long range interactions. These include 1) predictions of regular secondary regions, with a method which is not dependent on X-ray crystals, and 2) investigations of folding pathways of proteins with a simple model in which the native conformation is favored. Conformational invariance in spite of larger sequence variations has been investigated for several families of proteins. Conformational agreement for pairs of residues was observed to be greater than sequence agreement by 20 to 50%. From the second calculations which include long range interactions, definite folding pathways are obtained for pancreatic trypsin inhibitor, myoglobin, ribonuclease, concanavalin A, and lysozyme. The activated state for the folding-unfolding process is found to correspond to the appearance of long range interactions. In all cases only one or two regions of growing nuclei are observed. More detailed models of the specific nature of tertiary interactions are being developed.

PHS 6040 (Rev. 1/83)

GPO 895-100

Other Professional Personnel: Sanzo Miyazawa, Ph.D., Visiting Associate, LTB, NCI

Objectives: To develop theoretical methods adequate to determine the most significant macromolecular conformations. The principal molecules of interest are proteins. We wish to predict protein conformations from their sequences and to determine the relative importances of various classes of intramolecular interactions. At present, methods are limited and cannot be expected to yield complete three dimensional conformations of large proteins. Also we develop simple models for generating conformations at different stages of folding and subsequently construct folding pathways.

Methods Employed: Approximate methods for calculating conformational energies, based on electrostatic energies and hard sphere atom representations, have been utilized to generate input for methods to select best sets of regular secondary conformational regions.

Molecular conformations are generated for all extents of folding; this permits a detailed treatment of folding pathways, by considering most probable protein conformations in samples which are denatured to different extents. The folding-unfolding process is investigated with an idealized model employing approximate free energies. Intra-residue energies consist of an empirical energy taken from the observed frequency distributions in conformational angles (ϕ, ψ). Inter-residue interactions are simplified by assuming that there is an attractive energy operative only between residue pairs which are in close contact in the native structure. These are utilized in a "helix-coil" type model in which long range interactions are included only within native globules. The most probable native residues are indicated at all extents of folding. From these results, probable equilibrium pathways through the folding-unfolding transition can be constructed.

Major Findings: The secondary calculation methods previously developed have been applied to investigate the invariance of conformation for families of sequence variable proteins. For five species of β2-microglobulins, it was found that the average sequence variation of 30% corresponded to an average conformation variation of only 12%. Even more striking agreement was observed for a globin family of nine proteins; there was an average sequence difference of 68% of the residue pairs and yet predicted mean conformational variation of only 19% of the residue pairs.

For this idealized folding model all protein folding transitions appear to be an "all-or-none" type; such behavior can be attributed to the highly specific long range interactions. It is found that turns and α helices appear at an early stage but long range interactions, including those found in β sheets, usually appear near the maximum in the free energy as an activated state in a cooperative step. Results are similar to those obtained in more detailed Monte Carlo generations of conformations with volume exclusion. Most probable conformations are found to be relatively insensitive to the exact value of the contact energy parameter. Calculations were performed for trypsin inhibitor, myoglobin, lysozyme, concanavalin A and ribonuclease A. In all cases, one or two nuclei for growth of native regions were found. Calculations for myogloblin, with and without the heme group, indicate the method to be sensitive to the details of the contact map and consequently physical constraints during folding. Although the heme-protein contacts represent only 6% of the total number of

native contact pairs, their inclusion possibly shifts the folding process from a two center growth process to one center growth.

Proposed Course: Detailed studies of correlations between sequences and known conformations in a large group of proteins are planned. These should lead to improved secondary prediction methods.

More detailed evaluation of the effects on the pathways of the strengths of intramolecular interaction energies will be pursued. More realistic residue-residue energies will be developed in an attempt to include the possibilities of favorable non-native conformations. This should permit consideration of reported folding intermediates with shuffled disulfide bonds. This method is based on a lattice like representation of residues and solvent. Development of methods to treat densely packed protein conformations is anticipated. In order to comprehend the appearance of favorable long range interactions, probabilistic studies of the effects of classes of interactions on ring closure are anticipated.

Publications:

Miyazawa, S., and Jernigan, R.L.: Equilibrium folding and unfolding pathways for a model protein. Biopolymers, 21: 1333-1363, 1982.

Miyazawa, S., and Jernigan, R.L.: Most probable intermediates in protein folding-unfolding with a non-interacting globule-coil model. Biochemistry, 21: 5203-5213, 1982.

Jernigan, R.L., and Miyazawa, S.: Equilibrium folding-unfolding pathways of model proteins: Effect of myoglobin heme contacts. Biopolymers, 22: 79-85, 1983.

Miyazawa, S., and Jernigan, R.L.: Equilibrium folding pathways for model proteins. J. Stat. Phys., 30: 549-559, 1983.

Miyazawa, S.: Cooperative ligand binding on multi-dimensional lattices: Bethe approximation. Biopolymers., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08323-08 LTB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Assay Quantitation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Charles DeLisi, Ph.D., Acting Chief, Math. Biol, NCI

COOPERATING UNITS *(if any)*

John Inman Ph.D., Laboratory of Immunology, NIAID; Irwin Chaiken, Ph.D., Laboratory of Chemical Biology, NIAMD; Jan Cerny, University of Texas.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	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.)*

A physical chemical analysis of affinity chromatography has led to the design of new techniques for the quantitative study of macromolecular interactions. In particular new methods have been proposed which should allow rapid, accurate determination of thermodynamic and kinetic parameters. These methods are now being tested experimentally.

Other Professional Personnel: Herbert W. Hethcote, Ph.D. IPA, Mathematics, University of Iowa; Rodney Walters, Ph.D., Iowa State Univ., Irwin Chaiken, Ph.D., NIAMDD.

Project Description:

Objectives: To develop a simple, fast, widely available method for obtaining quantitative physical chemical information for complex reaction systems. To develop quantitative methods for obtaining kinetic and thermodynamic information on antigen antibody reactions at a single cell level. To develop methods for increasing assay reliability and precision.

Methods employed: Mathematical models; mathematical analyses of data.

Major findings: The chromatography theory is still in its early stages of development, but the equations derived have been applied by Dr. John Inman, NIAID to determine the equilibrium constant for an anti TNP Myeloma. He obtains a value within 5% of the accepted value that had previously been determined by dialysis. Work on applications of the plaque assay, especially as a method for analysis of anti-idotypic antibodies, continued but at a slow pace. Most effort assays whose use as replacements for radio immunassays is continuing to increase. Equations were derived that will allow assay optimization including an analysis of error structure of the system.

Significance to Biomedical Research and the Program of the Institute: The chromotography project is the basic component of projects related to the physical chemistry of cellular recognition and regulation. It will provide the thermodynamic and the kinetic data required to develop a quantitative understanding of cellular regulation. The work on plaques is intimately related to the project on a B cell regulation. It makes possible a method for studying cellular selection. It also provides a potentially new and valuable method for quantitating under appropriate conditions, the antiidiotypic antibody response. Immunoassays are important, for among other things, the detection and quantitation of low concentration of ligands in the serum. Aside from the ability to detect abnormally low or high values of hormones that are normally present, they are potentially useful in the detection of tumor associated antigens.

Proposed Course: Experimental tests of the method are currently in progress. Assuming these are positive and the methods are useful in their present form, further theoretical studies will not be necessary. The project will then be phased out.

Publications

DeLisi, C. and Hiernaux, J.: Mathematical analysis of augmentable plaque forming cells: A quantitative method for monitoring auto antiidiotypic antibody. In DeLisi, C., and Hiernaux, J. (Eds.): Regulation of Immune Response Dynamics. Boca Raton, FL, CRC Press, 1982. pp 43-57.

Hethcote H. and DeLisi C.: Quantitative Affinity Chromatography: Methods for Kinetic and thermodynamic characterization of macromolecular interactions. Proceedings of the 5th International Symposium on Affinity Chromatography and Biological Recognition. 1983, In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08331-07 LTB

PERIOD COVERED: October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
An Analysis of Oscillations in the Glucose - Insulin System in Humans

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
David Covell, Staff Fellow, LTB, NCI

COOPERATING UNITS *(if any)*
Rubin Andres, NIH, GRC
Darish Elhai, SUNY

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

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

B

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

Glucose homeostasis in biological systems is highly regulated. In response to a glucose load, a complex series of hormonal secretions occurs to return plasma glucose concentration to normal. Although these hormonal control mechanisms are poorly understood, experimental data suggest they are the result of mutual effects of both glucose and hormone upon the kinetics of each substance. The complex response dynamics for these hormonal secretions appear to contribute in some organized fashion to glucose homeostasis. To investigate this complex interrelationship we have focused our analysis on the timing of the hormonal secretions during a glucose response. In a normally functioning system such a response exhibits kinetic behaviour characteristic of systems controlled by feedback loops (i.e. damped oscillations to a stable steady state). In certain diseased states or under excessive glucose loads that tax the control mechanisms the dynamic behaviour often appears uncontrolled. The analysis of these states may provide information on the mechanisms involved in glucose homeostasis. Such analysis may also be applicable to a broader class of hormone systems.

PHS 6040 (Rev. 1/83) GPO 895-100

Project description:

Objectives: 1) Establish a quantitative basis for the observed oscillations in glucose and insulin during normal and hyperglycemic conditions. 2) Use the above information to explore the mechanisms involved in the control of glucose and insulin under normal and disease conditions.

Methods Employed: Model development, simulations and optimizations are being done using software available in the IMSL statistical computing package and with the SAAM computing program.

Significant Findings: 1) Plasma glucose concentration oscillates about normoglycemia with a dominant period of approximately 70 minutes; 2) The sustained oscillations described above can be shown to be the result of time delays between the counterinteracting influences of glucose and insulin.

Significance to Biomedical Research and the Program of the Institute: Research investigations into the numerous mechanisms involved in the control of normoglycemia have for the most part concentrated on a single aspect of the total system. A global evaluation of the total system obtained by assembling the important subsystems is useful for evaluating rate controlling proceses. This understanding will become useful for determining those unit processes that are affected during disease states involving altered carbohydrate metabolism.

Proposed Course: The proposed research represents a continuing effort to understand glucose metabolism. The investigation is a collaborative effort with the NIA (Dr. R. Andres) and Downstate Medical Univ. of New York (Dr. D. Elahia). Both investigators are supplying data from hyperglycemic clamp experiments in humans (250 patients at clamps of +54, +98, +143 and +231 mg% glucose, Andres, NIA) and pancreatic perfusion preparations (Elahia, St. U. N.Y.)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08335-07 LTB

PERIOD COVERED

October 1, 1981 to September 30, 1982

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

John N. Weinstein, M.D., Ph.D. LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	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 studied three conceptually different ways of "targeting" liposomes:

(1) Antibody-mediated targeting. We find 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.

(2) Physical targeting. We have 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 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 are being extended to antibody-bearing liposomes.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: Robert P. Blumenthal, Ph.D., Chief, Membrane Structure and Function Section, LTB, NCI; Oscar Dile Holton, III, Ph.D., Expert, LTB, NCI; Michael A. Steller, Biologist, LTB, NCI

Objectives: To investigate the use of liposomes both in cell biology and in clinical therapy. Within this broad context, to (1) explore the use of antigen-antibody interactions to achieve selective association of liposomes with particular cell types; (2) develop synergistic interactions between "temperature-sensitive" liposomal drug carriers and hyperthermic treatment; (3) identify the mechanisms of spontaneous, serum-induced and osmotically-induced release of solutes from liposomes; (4) deliver liposomes and their contents to tumor in lymph nodes -- for nonspecific and antibody-mediated binding.

Non-standard Methods Employed: (a) Preparation of liposomes, by bath and probe sonication; (b) investigation of liposome-cell interactions using the fluorescence-activated cell sorter; (c) dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory for this purpose; (d) measurements of release of solute from liposomes by "fluorescence self-quenching"; (e) determination of the mobility of vesicles bound to cells, using fluorescence photobleaching (f) microwave heating of tumors; (g) quasi-elastic light scattering measurements of liposome size; (h) covalent coupling of antibodies to liposomes.

Major Findings: Objective 1: (a) Bivalent antibody selectively binds TNP-bearing liposomes to TNP-bearing lymphocytes, but the binding does not increase delivery of liposome contents to the cell interior (beyond the amount internalized spontaneously). (b) Endogeneous surface IgA on cells of the murine myeloma MOPC 315 can bind liposomes bearing the appropriate hapten (DNP) to the cell surface. However, as in the lymphocyte system, binding does not increase delivery to the cytoplasm. (c) Lipid vesicles containing fluorescent molecules are potentially useful as markers for sparse or low-affinity cell surface determinants. They can be made to contain many fluorophore molecules, thus amplifying the signal. They give very low-nonspecific background since the fluorophore is sequestered, and appropriate lipids are not very "sticky". (d) Liposomes are removed from the circulation of a mouse much faster if the mouse carries a myeloma secreting antibody to a hapten on the liposomes. (e) Antibody-mediated binding of methotrexate-containing vesicles to MOPC 315 and TEPC 15 myeloma cells did not lead to entry of drug into the cells and inhibition of their metabolism. (f) IGG opsonized DNP-vesicles are bound in large numbers to F_c receptor-bearing cells (murine $P388D_1$), and are then endocytosed. Liposome-encapsualted MTX can escape the pagolysosomal system to reach a cytoplasmic target (dihydrofolate reductase) and affect the physiology of the cell. (g) IgG, protein A, avidin, and other ligands can be coupled efficiently to liposomes by use of the heterobifunctional cross-linking agent N-hdroxysuccinimidy 3-(2-pyridyldithio) propionate. This method of coupling results in only minimal aggregation and little leakage of vesicle contents. Liposomes bearing covalently coupled mouse monoclonal antibody against human α_2-microglobulin bind specifically to human cells, but not to mouse cells.

Objective 2: (a) Small Uniamellar vesicles of 3:1 dipalmitoylphosphatidylcholine - distearoylphosphatidylcholine release their contents very slowly at 37°C, much faster at 41 - 46°C. Such "temperature-sensitive" liposomes appear useful in achieving high drug concentrations selectively in local areas of hyperthermic treatment, for example, in the treatment of tumors. (b) The temperature-dependence of the release can be enhanced by increasing the rate of temperature change, by using multilamellar vesicles in place of the unilamellar ones, and most markedly, by the presence of serum in the medium. Ratios of greater than 100:1 can be obtained for release (of a fluorescent marker) at 43 and 37°. (c) The effect of serum is largely due to interaction of serum lipoproteins (VLDL, IDL, LDL, and HDL) with the liposomes. (d) Four times as much methotrexate was delivered to subcutaneous Lewis lung tumors heated to 42° as to unheated controls in the same animals at 36°; with L1210 tumor the ratio was 14:1. (e) Growth of th L1210 tumors was delayed by such treatment more than could be accounted for by the separate effects of heating and liposomal drug administered separately. (f) Large unilamellar vesicles are stable below T_c but release their contents within a few seconds upon passage through T_c with serum.

Objective 3: (a) Release of carboxyfluorescein from small unilamellar vesicles takes place by "leakage", not by an all-or-nothing "rupture" of the vesicle. (b) The rate constant for leakage increases in inverse proportion to the hydrogen ion concentration of the medium. (c) Liposomes of dioleoyl lecithin leak their contents and form structures with a characteristic appearance in negative-staining electron microscopy when allowed to interact with HDL or LDL. The interaction is faster and more pronounced with isolated HDL apolipoprotein than with the whole lipoprotein particle. (d) Liposomes bearing the DNP-hapten can be made to release carboxyfluorescein in the presence of complement and IGG anti-TNP. Fluorescence self-quenching provides the most sensitive technique available for continously monitoring such processes.

Objective 4: (a) Liposomes can pass intact (and without release of contents) from the peritoneum to the bloodstream. This finding relates to the possible clinical instillation of liposomes intraperitoneally. (b) Passage from peritoneum to blood takes place largely through the lymphatics. (c) After subcutaneous injection, liposomes pass to regional nodes and then to the bloodstream. (d) The only lymph node cells which take up significant amounts of unmodified liposomes are the macrophages.

Significance to Biomedical Research and the Program of the Institute: The three objectives listed relate to the possibilities of using liposomes in diagnosis and tumor therapy. A major barrier to such efforts has been the difficulty of directing liposomes to particular cells or anatomical sites. The studies of antibody-mediated "targeting" suggest a way to achieve selectivity but also demonstrate an additional problem: how to get the liposome and its contents into the cell after binding. The studies of synergism between liposomes and hyperthermia indicate a new way to achieve selective delivery. Use of the liposome as a hapten-carrier for analysis and sorting of cell populations may be useful in a number of areas of cellular and tumor immunology. The covalent attachment of immunoglobulin and ligands to liposomes will make possible a wide range of cell biological studies. Liposomes may be able to carry diagnostic and therapeutic agents to tumor in the lymph nodes.

Proposed Course: Each of the objectives specified earlier will be pursued. In the case of the hyperthermia system, emphasis has now shifted to the design and testing of new liposome types and also to theoretical analysis of the pharmacokinetics.

Publications:

Blumenthal, R., Ralston, E., Dragsten, P., Leserman, L.D., and Weinstein, J.N.: Lipid vesicle-cell interactions: Analysis of a model for transfer of contents from adsorbed vesicles to cells. Membrane Biochemistry 4: 283-303, 1982.

Magin, R.L., and Weinstein, J.N.: Delivery of drugs in temperature-sensitive liposomes. In Gregoriadis, G., Senior, J., and Trouet, A. (Eds.): Targeting of Drugs. Plenum, N.Y., 1982, pp. 203-221.

Weinstein, J.N.: Target-direction of liposomes: Four strategies for attacking tumor cells. In Chabner, B.A. (Ed.): Rational Basis for Chemotherapy. Liss, N.Y. 1983, pp. 441-473.

Chused, T.M., Sharrow, S.O., Weinstein, J.N., Ferguson, W.J., and Sternfeld, M.: XRITC: A new dye for two-color immunofluorescence. J. Histochem. and Cytology., in press.

Magin, R.L, and Weinstein, J.N.: The design and characterization of "temperature-sensitive" liposomes. In Gregoriadis, G. (Ed.): Liposome Technology. CRC press, in press.

Weinstein, J.N., Ralston, E., Leserman, L.D., Klausner, R.D., Dragsten, P., and Blumenthal, R.: Self-quenching of carboxyfluorescein fluorescence: Uses in studying liposome stability and liposome-cell interaction. In Gregoriadis, G., (Ed.): Liposome Technology. CRC press, in press.

Weinstein, J.N., and Leserman, L.D.: Liposomes as drug carriers in cancer chemotherapy. Pharmacology and Therapeutics. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08340-05 LTB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Physical Chemistry of Antibody Effector Functions

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Charles DeLisi, Ph.D. Acting Chief, Laboratory of Mathematical Biology.

COOPERATING UNITS *(if any)* Dr. Ruben Siraganian, Clinical Immunology Section, NIDR; Prof. George Barisas, Biochemistry Dept. Univ. of St. Louis Med. School; Alan Perelson, Ph.D., Los Alamos National Lab., Los Alamos, NM; Dr. David Segal, Immunology Branch, DCBD, NCI; Dr. Steve Dower, Immunology Branch, DCBD, NCI.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	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.)*

Work on the basophil system was continued with the development of a new theory of the kinetics of activation and specific desensitization of cells from allergic and immunized individuals. The theory has been applied to the analysis of a wide variety of data. We also developed new methods, based on measurements of the kinetics of cell activation for determining whether or not descending limb of biphasic dose response curve falls because of insufficient cross-linking. We studied equilibrium and kinetic properties of IgG diagomers of defined size interacting with Fc receptors on a macrophage-like cell line. The results of the equilibrium studies provided the first experimental evidence in support of our predictions that receptor cross-linking can lead to non linear Scatchard plots. In addition, the data suggested two different types of binding sites for dimeric and trimeric oligomers, but only a single type for monomers. The maximum affinity enhancements -- 200 for dimeric relative to monomeric and 2.5 for trimeric relative to dimeric, indicate considerable strain or large differences in the entropic parts of the equilibrium constants for solution phase as opposed to cell surface reactions. The dissociation kinetics of dimer and trimer were biphasic and the rate of dissociation was accelerated by high concentrations of monomer.

PHS 6040 (Rev. 1/83)

GPO 895-100

Project Description:

Objectives: (1) To develop an understanding of the relationship between cell surface events and the biochemical pathway which a cell follows. In the basophil system this means sorting out the parameter domains corresponding to specific desensitization, non specific desensitization, histamine secretion, and inactivity. (2) To develop a detailed understanding of Fc receptor redistributions upon the binding antigen-antibody complexes. (3) To determine and study the relation between cluster size and distribution and cellular activity. (4) To develop an understanding of the relationship between the thermodynamics and kinetics of ligand cell-bound receptor interactions, and the requirements for lymphocyte activation.

Methods Employed: Mathematical modeling; mathematical analysis of experimental data.

Major Findings: Basophilis sensitized to penicillin degranulate and release histamine when incubated with multivalent penicillin derivative but not with monovalent hapten. The dose-response curve is biphasic with maximal release at a concentration of dimeric hapten of about 1 nM. The chacteristics of the response generated using mixtures of monovalent and divalent derivatives, as well as a variety of other evidence, suggest that the rise and fall in the dose-response curve reflects the rise and fall in the concentration of receptors cross-linked by the multivalent hapten.

Extracellular Ca^{2+} is required for histamine release and cells may be desensitized to different degrees by incubation with various concentrations of ligand in the absence of Ca^{2+}. Washing and rechallenging cells with an optimal dose of ligand and Ca^{2+} indicates that the dose dependence of desensitization is also biphasic and is most pronounced at a ligand concentration which ordinarily stimulates maximal release. The implication is that cross-linking, which, for the thermodynamic parameters characteristics of this system, will lead primarily to dimers and trimers, initiates signals for both degranulation and desensitization.

Kinetic studies reveal release curves which are sigmoidal, having delays in the onset of release that vary with ligand concentration. The magnitude of the delay is a biphasic function of concentration with a maximum at approximately the concentrations the peak in the dose-response curve. The results are interpreted in terms of a model in which cross-linked receptors are converted to an active, unstable intermediate which facilitates an increase in cytoplasmic Ca^{2+}, but which decays spontaneously into an inactive product. Dependence of histamine release on the concentration of the intermediate is nonlinear, suggesting either a positive feedback loop stabilizing the intermediate or the interaction of several aggregates. A fit of a simple mathematical formulation of the model indicates that it is qualitatively and quantitatively explains the dose-response, desensitization, and release patterns.

Scatchard plots for IgG oligomers are convex to the origin. The result provides the first direct experimental evidence that receptor clustering leads to such plots. The rate of dissociation of labeled ligand is faster in the presence of cold ligand than in its absence. The result again provides the first direct experimental evidence that receptor clustering can cause such acceleration. Both effects are widely observed in cell systems, and they have previously been attributed to negative binding cooperativity coused by conformational reassesment of the interpretation of a wide variety of data. The method in principle should be applicable to any cell that respond via a receptor clustering mechanism. For such systems it is important to know whether failure of the cell to respond at high ligand concentrations is simply the result of some desensitization signal induced by large aggregates. We have currently identified AgE and human basophils as a system in which a desensitization signal prevents release at high concentrations.

Significance to Biomedical Research and the Program of the Institute: Aside from the obvious bearing on allergic reactions, the basophil system, because it is a one ligand-one cell system which responds in minutes, permits the type of quantitative analysis which is not possible for antibody production and secretion. It is therefore a model system for studying cellular activation.

This project is related to the projects on lymphocyte activation, complement activation and insulin binding. For the first two, binding of antibodies to cells via the Fc receptor must precede biological activity. A detailed understanding of the nature of the complexes formed is a necessary component of developing an understanding of the biological processes. The problem of determining and quantitating the parameters that effect the initial states of signal transduction in basophils is an important aspect of the attempt to understand the nature of the immediate allergic reaction. More generally, however, the basophil system may serve as a model from which to build a deeper understanding of the relation between the binding of ligand to cell surface receptors and subsequent biochemical events and factors that regulate them.

Proposed Course: This project was discontinued during FY '83 except for the publication of manuscripts.

Publications:

DeLisi, C., DelGrosso, G., and Marchetti, F.: A theory of measurement error and its implications for spatial and temporal gradient sensing during chemotaxis. Cell Biophys. 4: 211-229, 1982.

DeLisi, C., and Wiegel, F.: Theory of the effect of membrane fluidity and the probabilty of complement fixation. J. Theor. Biol. 102: 307-322, 1983.

Perelson, A., DeLisi, C., and Wiegel, F.W. (Eds): Cell surface: Concepts and Models. Marcel Dekker. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08341-05 LTB

PERIOD COVERED

October 1, 1982 to September 30, 1982

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

Physical Chemical Studies of Lipid - Protein Interactions

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

John Weinstein, M.D. Ph.D., Investigator, LTB, NCI

COOPERATING UNITS *(if any)*

T. Innerarity and R. Pitas, University of California at San Francisco;
Richard Klausner, LBM, NIAMDD

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	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.)*

We have investigated the interaction of lipoproteins with liposomes to form recombinant particles. A number of lipoprotein fractions (VLDL, IDL, LDL, and HDL) all disrupt liposome stucture by an essentially irreversible and qualistoichometric process. In the case of HDL, the major apoprotein, A-I, recombines with dimyristoyl phsophatidyl choline vesicles 40:1 lipid-protein to form discs approximately 100 Å in diameter and 32 Å in thickness, with proteinon the rim. These structural results were obtained by a combination of neutron scattering, electron microscopy, and column chromatography.

With dipalmitoyl phosphatidylcholine, A-I also forms what we term "vesicular recombinant" particles in a process which may relate to ppysiological mechanisms by which proteiins are assembled into membranes and lipoproteins. To study thie process we have developed a technique called "phase transition release" (PTR) which is also being applied to study incorporation of tubulin into membranes.

Lipoproteins were labelled with the fluorescent lipid 3,3 dioctadecylindocarbocyanine for studies of interaction will cell surface lipoprotein receptors. The lipoproteins are also being labelled with NBD lipids for two-color fluorescence identification of cells in atherosclerotic plaques.

Other Professional Personnel: Robert P. Blumenthal, Ph.D., Chief, Membrane Structure and Function Section, LTB, NCI

Project Description:

Objectives: To investigate the interaction between liposomes and lipoprotein and between lipoproteins and cells. More specifically, (1) To define the mechanism by which liposomes are broken down by serum components, principally the lipoproteins; (2) to determine the relative efficacies of different purified apolipoproteins in breaking up liposomes; (3) to develop fluorescently labelled lioporoteins and liposome-apoprotein recombinants for use in studying lipoprotein-cell interactions; (4) to use the methods developed for objectives (1) - (3) to assess the physical chemistry of proteins-lipid interaction in lipoproteins; (5) to extend to other bilayer-protein interactions the concepts thus developed; (6) in particular, to investigate the assembly of tubulin and actin into membranes.

Non-standard Methods Employed: (a) Preparation of liposomes, by probe and bath sonication: (b) Dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory, i.e., "phase transition release" (PTR); (c) Determination of the mobility of lipoproteins and liposomes bound to the cells, using fluorescence-photobleaching recovery; (d) Neutron scattering studies of lipoprotein size and shape; (e) Aqueous collisional fluorescence quenching studies of lipid-protein interactions; (f) Derivatization of proteins with trinitrobenzene sulfonate; (g) Labelling of lipoproteins with 3,3'-dioctadecylindocarbocyanine (diI) dye.

Major Findings: (1) Liposomes are broken down and their contents released by an interaction with serum, most rapidly at the lipid phase transition. (2) The serum effect is mostly due to low density (LDL) and high density (HDL) lipoproteins, but very low density and intermediate density lipoproteins also play a part, as also does some other, unidentified component of the serum. (3) Heparinized plasma, EDTA plasma, and serum all have the same effect. (4) At least in the case of apo-HDL the mechanism appears to be a quasi-stoichiometric, all-or-nothing breakdown of the vesicle into a small disc of lipid rimmed with protein. The process is largely complete within a second or two at the phase transition of the liposome lipid and is essentially irreversible on that time scale. (5) By neutron diffraction and electron microscopic studies the discs appear to be about 100 Å in diameter and 32 Å in thickness, with a protein rim. They probably consist of a single bilayer. (6) Cholesterol at 40 mole percent in the liposomes severely restricts the interaction with serum components, as does the formation of liposomes from lipids which are below their phase transitions at the temperature of study. (7) At molar ratios of about 2,000:1 dipalmitoyl phosphatidylcholine:Apo A-I, a stable vesicular recombinant particle (VR) is formed below the lipid phase transition temperature (Tc). As the temperature is raised through Tc (in PTR), a new type of recombinant (VR-Tc) is formed. By physical measurements of several types, the A-I's conformation and disposition in the lipid change at Tc. The protein probably becomes trans-membrane. These findings may relate to physiology processes for formation

of HDL and to the assembly of intrinsic proteins into membranes. (8) Purified tubulin interacts with vesicles at Tc to form vesicular recombinants. The interaction is accompanied by structural changes in lipid and protein. (9) HDL, LDL, and apoE-HDL can all be labelled efficiently and irreversibly withthe fluorescent lipid analogue, diII. The lipoproteins are unchanged in physical properties and specific binding to cell surface receptors. The labelled lipoproteins were acetoacetylated for studies of phagocytic and lipoprotein-specific uptake in arterial walls. (10) Studies of the interaction with bilayers of a hepatic membrane receptor for asialoglycoprotein are described in another report (Z10 CB 08343-01 LTB).

Significance to Biomedical Research and the Program of the Institute: (1) A major barrier to the effective use of liposomes as carriers in cancer chemotherapy and diagnosis has been a lack of understanding of their interactions with serum. Our studies define the interaction and indicate what type of liposomes must be used to avoid it. (2) Our studies of HDL-liposome recombinants contriibute to (1) and may also be useful in delineating mechanisms of atherosclerosis. Our fluorescently labelled lipoproteins are currently being used by collaborators to study atherogenesis. (3) Breakdown fo liposomes in serum is essential to the combination of liposomes with hyperthermia to achieve selective release of drugs in the area of a tumor.

Proposed Course: This project is to be terminated during FY 1984, except for writing of manuscripts.

Publications:

Kumar, N., Blumenthal, R., Henkart, M., Weinstein, J.N., and Klausner, R.D.: Aggregation and calcium-induced fusion of phosphatidylcholine vesicle-tubulin recombinants. J. Biol. Chem., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08342-04 LTB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Theory of Receptor-ligand Biophysics

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Charles DeLisi, Ph.D., Acting Chief, Lab. of Mathematical Biology, NCI

COOPERATING UNITS *(if any)*

Dr. Alan Perelson, Theor. Div. Los Alamos National Lab., Los Alamos, NM; Prof. Frederik Wiegel, Dept. of Physics, Twente Univ. of Tech., Enschede, Netherlands; Prof. Federico Marchetti, University of Rome, Rome, Italy.

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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.)*

Rate constants for ligands interacting with cell bound or dispersed receptors have a diffusive part and an intrinsic part. The former depending on geomety, receptor distributions, and diffusion coefficients; the latter on electronic redistributions. We have been focusing on the former and have obtained expressions for diffusion limited association and dissociation rate constants when (1) ligand bind directly and specifically to receptors that are distributed over a spherical surface; (2) ligands bind indirectly by a path that includes non specific association with the cell and diffusion in the surface, toward or away from a specific receptor. We have also developed a formalism that permits calculation of the complete equilibrium and rate constants for cell bound receptors, given the equilibrium or rate constants for dispersed receptors.

Mathematical methods are also being developed to describe aggregation on a two dimensional fluid surface.

Project Description:

Objectives: To obtain expressions that can be used to analyze kinetic data for reactions between ligands and cell bound receptors. To obtain analytic expressions for the time evolution of the distribution function describing the growth of aggregates subsequent to the binding of ligand to laterally mobile cell surface receptors.

Major Findings: The kinetic problem for the entire distribution function, with and without loop closure (cyclic complexes) was solved for the first time for a bivalent-bivalent system. Because the formulation of the problem leads to an infinite system of coupled non-linear equations, numerical solutions are not possible. The analytical results thus allow applications that were previously not possible.

The theory is being used to answer a variety of questions of biological interest. One of its applications, the analysis of ligands dissociating from cell surface receptors, predicts that accelerated dissociation by cold ligand and multi exponential decay of label, is expected even in the absence of negative cooperativity or cross-linking. The conditions under which such observations are expected have been derived, and a consideration of known rate constants indicates that they are likely to be met in a large number of cases.

Clustering of receptors is responsible for cell activation and/or desensitization in a variety of systems. Moreover, since very few clusters - perhaps three or four - are needed to induce activity in some systems, the question of how cells prevent spontaneous activation arises. Equilibrium calculations based on the entropic loss in constraining receptors to be adjacent, indicate that hundreds of pairs are expected on cells having 10^5 - 10^6 receptors.

The results of this project shed light on this problem. The main idea is that receptors must remain within some minimum distance of one another for some required amount of time to transduce a signal. The equations indicate that receptors will diffuse out of this required distance very rapidly, on the order of 10^{-5} seconds. Thus for transduction time (the central parameter) is in the rate of 10-100. One can then show that the probability of spontaneous transduction can easily be as low or lower than 10^{-30}. With even a weak affinity ligand, the probability changes to close to one. Thus a control mechanism based on a residence time requirement can easily lead to virtually infinite amplification of transduction.

Significance to Biomedical Research and the Program of the Institute: The methods are required to develop an understanding of the physical chemistry of the regulation of cell responsiveness at the cell surface level. Aspects of these ideas may be relevant to a wide range of biological processes including enzymic enhancement of catalysis. The results will also allow correlations to be drawn about cellular activity and the size of the aggregates which form on the cell surface, i.e., previously it was possible only to connect the mean number of cross-linked receptors with activity; now the importance of the way

they are distributed in various sized aggregates can be assessed. This project is a necessary aspect of all other research projects.

Proposed Course: This project was discontinued during FY '83 except for the publication of manuscripts.

Publications:

DeLisi, C.: Toward a dynamical theory of membrane organization and function. In DeLisi, C., Perelson, A., and Wiegel, F. (Eds.): Physical Chemistry of Cell Surface Phenomena. Marcel Dekker, In press.

DeLisi, C., and Wiegel, F.: Rate constant enhancement by reduction in dimensionality is likely to be of little consequence in cellular systems. Amer. J. Physiol. In press.

Wank, S., DeLisi, C., and Metzger, H.: Analysis of rate limiting step in a ligand-cell receptor interaction: The IgE system. Biochemistry 22: 955-959, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08357-02 LTB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Cell Interactions

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Charles DeLisi, Ph.D. Chief, Theoretical Immunology Section LTB NCI

COOPERATING UNITS *(if any)*

Dr. Jacques Hiernaux, LTB

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The number of antibody secretion cells in normal and nude mice was studies as a function of time after a single injection of either bacterial levan or lipopolysaccharide. The number of cells producing antibodies against the dominant anti BL idiotype was also studied.

GPO 895-100

Other Professional Personnel: Jacques Hiernaux, Visiting Fellow, LTB, NCI

Project Description:

Objectives: To elucidate the mechanism(s) regulating the maintenance phase of the immune response.

Major Findings: The immune response to a single injection of bacterial levan (BL) or lipopolysaccharide (LPS) persists for months, the number of antibody producing cells oscillating synchronously in some cases and asychronously in others. The pattern occurs in both euthymic and athymic BALB/c suggesting that auto antiidiotypic antibodies and T-cells do not play a significant role in their generation. Moreover, we found no relation between the suppressive property of auto antiidiotypic antibody (in normal mice) and either the induction or maintenance of tolerance to BL.

Significance to Biomedical Research and the Program of the Institute: The mechanisms responsible for long term maintenance are of major importance to the understanding of a number of disease related problems; e.g. allergy, arthritis, graft rejection. Since attempts to modulate the response in these instances can occur weeks or even years after induction, understanding the regulation of the maintenance phase is crucial for successful intervention.

Proposed Course: This project was discontinued over FY '83 except for the publication of manuscripts.

Publications:

DeLisi, C., and Hiernaux, J. (Eds): Regulation of Immune Response Dynamics. Boca Raton, FL, CRC Press, Vols. 1 and 2. 1983.

DeLisi, C., and Hiernaux, J.,: Some comments on dynamic complexity in immunologically related systems. In DeLisi, C., and Hiernaux, J. (Eds.): Regulation of Immune Response Dynamics. Boca Raton, FL, CRC Press, 1982, pp 1-4.

Hiernaux, J., Baker, P., and DeLisi, C.: Oscillatory immune response to lipopolysaccharide. In DeLisi, C., and Hiernaux, H. (Eds.): Regulation of Immune Response Dynamics. Boca Raton, FL, CRC Press, 1982, pp 121-136.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08359-02 LTB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

John N. Weinstein, M.D. Ph.D., Investigator LTB, NCI

COOPERATING UNITS *(if any)*

A. Keenan, NMCC; R. Parker, S. Sieber, DCCP; S. Sharrow, IB, DCBD;
R.K. Oldham, K.M. Hwang, M.E. Key, NCI, FCRC; G. Morstyn, O. Gansow, LCP, DCT
R. Knop, NIAMDD

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.2	2.7	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 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. Imaging studies are currently being followed up with attempts at therapy. For diagnosis of early metastatic tumor in the nodes, the lymphatic route can be expected to 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. We are extending the lymphatic approach to include antibody-bearing liposomes.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel: Mones Berman, Ph.D. Chief, LTB, DCBD, NCI; David Covell, Staff Fellow, LTB, NCI; Michael A. Steller, Biologist, LTB, NCI; Oscar D. Holton, III, Expert, LTB, NCI

Project Description:

Objectives: (i) Establish a quantitative basis (experimental and theoretical) for lymphatic delivery of monoclonal antibody to lymph nodes; (ii) Use that information for optimization of diagnostic imaging of animal tumors; (iii) Treat the tumors with radio-labeled and/or toxin-labeled antibodies (using experimental parameters obtained from the diagnostic studies); (iv) Extend the principles of lymphatic immunodiagnosis and immunotherapy to the clinical setting; (v) Extend our theoretical and experimental models to the intravenous administration of monoclonals; (vi) Extend the lymphatic approach to antibody-bearing liposomes.

Non-standard methods employed: (a) Two-color fluorescence microscopy and fluorescence cell-sorting on cell populations labeled in vivo with monoclonal antibodies; (b) Image-correction techniques for quantitative analysis of gamma camera studies; (c) Use of SAAM modeling system to analyze the pharmacokinetics of antibody distribution and binding in vivo; (c) filtration and centrifugation methods for determination of equilibrium and kinetic binding parameters for monoclonal antibodies; (d) labeling of antibodies for imaging by nuclear magnetic resonance

Significant findings: 1) Antibodies can be visualized selectively on very small aggregates of cells in the face of significant antigen expression on all cells of the animal; 2) Target cells become surface-labelled with fluorescent antibody.

Significance to Biomedical Research and the Program of the Institute: Radiolabelled monoclonal antibodies may be of major use in the diagnosis and staging of tumors. Monoclonals also may prove of therapeutic use in any of several ways: (i) by mobilizing body defenses against target cells; (ii) by delivering drugs, toxins, or radionuclides selectively to tumors; (iii) by "targeting" carriers (e.g., liposomes) for amplified effect of drug, toxin, or radionuclide. All of these endeavors require more pharmacokinetic information than is now available. We plan to develop that background in normal and tumor systems, with emphasis on the detection and treatment of metastases.

Proposed Course: Continue work with the murine histocompatibility antigens and T-cell markers; establish guinea pig hepatoma lines 1 and 10 as solid tumor models; use congenic retrovirus-associated murine B-cell lymphomas (expressing GP70) as a hematogenous model. In each of these systems we will combine fluorescence techniques (microscopy and cell sorting), radioisotope techniques (gamma camera and organ isolation), and ultrastructural techniques (ferritin-labelling) to investigate the anatomical, histological, and cytological localization of the antibodies. Against this background we will consider conjugation of toxins and/or liposomes to the antibodies and graduation to a clinically usable imaging nuclide, probably ^{111}In.

Publications:

Weinstein, J.N., Leserman, L.D., Henkart, P.A., and Blumenthal, R.: Targeting of liposomes. In Gregoriadis, G., and Papahadjopoulos, D., (Eds.): Targeting of Drugs. N.Y., Plenum, in press.

Chused, T.M., Sharrow, S.O., Weinstein, J.N., Ferguson, W.J., and Sternfeld, M.: XRITC: A new dye for two-color immunofluorescence. J. Histochem. and Cytology. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08361-01 LTB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Development of a Kinetic Model of GABA Metabolism in Rabbits With Hepatic Coma

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

David Covell, Staff Fellow, LTB, NCI

COOPERATING UNITS *(if any)*

Peter Ferenci, E. Anthony Jones, Liver Unit, NIAMDD

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.10	0.10	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human 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 systemic metabolism of the neurotransmitter GABA was investigated under normal and coma conditions. The plasma levels of GABA are known to be elevated by an order of magnitude over normal in patients with coma resulting from fulminant hepatic failure. To investigate the mechanism(s) for this elevation a kinetic model was developed to describe GABA metabolism during various stages of coma in a rabbit model. The major finding of the analysis was that a defect in GABA catabolism could not explain the elevations in plasma levels and additional sources for GABA production must be postulated. Subsequent experimental studies have supported these results by showing that gut bacterial production of GABA is substantially elevated during hepatic coma. The research data were obtained in collaboration with Drs. T. Jones and P. Ferenci of the Liver Unit at the NCI.

PHS 6040 (Rev. 1/83) GPO 895-100

Project description:

Objectives: 1) Establish a quantitative assessment of GABA metabolism in normal rabbits and rabbits in hepatic coma. 2) Use the above information to evaluate the mechanism for increased plasma GABA concentrations in the plasma during hepatic coma.

Methods: Model development is being done with the SAAM computing program.

Significant Findings: 1) Elevated plasma GABA levels during hepatic coma appear to be the result of increased GABA synthesis by gut bacteria rather than inhibition of GABA metabolism; 2) Breakdown of the blood brain barrier during hepatic coma and leakage of brain GABA into the plasma does not contribute significantly to elevated plasma GABA.

Significance to Biomedical Research and the Program of the Institute:
Investigations into the metabolism of the neurotransmitter GABA during hepatic coma are necessary to assess metabolic status of the organism while the disease progresses.

Publications:

Ferenci, P.K., Covell, D.G., Jones, A.M. and Berman, M.: Metabolism of the neurotransmitter γ-aminobutryc caid in rabbit. Model of fulimant hepatic failure. Hepatology 1983. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08362-01 LTB

PERIOD COVERED: October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Kinetics of 6-Mercaptopurine in the CSF Following IT & IV Administration

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
David Covell, Ph.D., Staff Fellow, LTB, NCI

COOPERATING UNITS *(if any)*

David Poplack, NCI, Pediatric Oncology; P.K. Narang, Clinical Pharmacology, NCI

LAB/BRANCH
Laboratory of Mathematical Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.10	0.10	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human 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 intrathecal administration of the anticancer agent mercaptopurine (i.e. directly into the cerebrospinal fluid, CSF, of the central nervous system) may provide an effective method for the treatment of acute lymphocytic leukemia (ALL). Such treatment requires careful control of drug levels in the CSF. With high speed digital computers it may be possible to use a sophisticated model of mercaptopurine kinetics in conjunction with a mathematical algorithm for dosage selection to rapidly and effectively control the CSF concentration of mercaptopurine. Towards this goal the metabolism of 6-MP has been investigated in monkeys following intrathecal and intravenous administration of mercaptopurine. The major findings of the research have been the development of a physiological-pharmacokinetic model of mercaptopurine kinetics in the CSF and the development of statistical methods to determine subject specific model parameters. The latter result permits 'real time' evaluation of the appropriate dosage amount and duration of administration. The methodology is currently being tested on monkeys. This research is being conducted in collaboration with Dr. P. Narang of the Clinical Pharmacology Unit and Dr. D. Poplak of the Pediatric Oncology Unit.

PHS 6040 (Rev. 1/83) GPO 895-100

Project description:

Objectives: 1) Establish a quantitative basis for the treatment of acute lymphocytic leukemia (ALL) with 6-Mercaptopurine; 2) Use the above information to design optimal dosing strategies to maximize 6-Mercaptopurine delivery to the brain following intravenous and intrathecal administration.

Methods: Model development, simulations and optimizations are being done using software available in the IMSL statistical computing package and with the SAAM computing program.

Significant Findings: 1) Proper dosage selection and site of administration are important to maximize 6-Mercaptopurine delivery to the the surface of the brain. 2) Entry and exit of drug in the CSF appear to be mediated by plasma filtration of newly formed CSF and bulk flow clearance, respectively.

Significance to Biomedical Research and the Program of the Institute: Investigations into the pharmacokinetics of anticancer agents delivered via different routes are necessary for evaluating treatment efficacy and designing dosing strategies.

Proposed Course: The efficacy of treatment with 6-Mercaptopurine may be improved significantly by the coadministration of blockers of enzymes involved in drug metabolism. The quantitation of such effects are being evaluated by studying 6-Mercatopurine kinetics in humans and monkeys. This information will be used for therapeutic design.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08363-01 LTB

PERIOD COVERED

February 23, 1983 to September 30, 1983

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

Theoretical Analysis of Membrane Protein Structure

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

H. Robert Guy, Expert LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.75	.75	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 structural model of the transmembrane portion of the acetylcholine receptor has been constructed. Sequences of all its subunits were analyzed using partition energy calculations to determine locations and orientations of transmembrane α helices. Side chain packing calculations were used to stack the transmembrane helices together. In the model, each subunit has the following structures in order along the N terminal: a large extracellular domain of undetermined structure, a short apolar α helix that lies on the extracellular lipid surface of the membrane, three apolar transmembrane α helices (I, II, and III); a cytoplasmitc domain of undetermined structure; an amphipathic transmembrane helix (L) that forms the channel lining; a short extracellular helix; another apolar transmembrane helix (IV) and a small cytoplasmic domain formed by the C terminal of the chain. This model differs from others principally in that the channel lining is comprised of a bundle of five amphipathic L helices. These are surrounded by a bundle of ten alternating II and III helices. Mechanisms for conformational changes that open and close the channel are being studied. Applications to other proteins, both to verify method and to extend the applications are anticipated.

PHS 6040 (Rev. 1/83)

GPO 895-100

Objective: Many cellular functions require membrane spanning proteins. These proteins include binding sites for many pharmaceuticals an toxins. Transmembrane portions of many membrane proteins appear to contain α helices; however, further details are not available. The present goal is to develop methods permitting the designs of structural models of membrane proteins. From these subsequently, we hope to develop a deeper knowledge of structure function relationships and drug-receptor interactions.

Methods Employed: Structural models of membrane proteins are developed in several steps. The protein sequence is first evaluated to indicate which segments could form transmembrane α helices and which portions of these helices might be in contact with lipid, with proteins, or with water inside a membrane channel. This method is based on calculations of energies required to move amino acid side chains from water to either a protein or a lipid environment. Predictions of this analysis are combined with experimental information about the protein structure in such a way to be consistent with general features of protein conformations including packing of amino acid side chains between one another, alignment of the ends of transmembrane α helices with membrane surfaces, and formation of disulfide bridges, salt bridges, and hydrogen bonds.

Major Findings: The proposed structure of the acelylcholine receptor channel differs from models proposed by other groups in having a very polar lining formed by one amphiphathic α helix from each of the five subunits. A molecular mechanism for opening and closing the channel is proposed. Concrete proposals are made for the location in the sequence of drug and toxin binding sites, disulfide bridges, and extracellular, transmembrane, and intracellular domains.

Proposed Course: We plan to validate and refine the methods by applying them to proteins of known structurs. We intend to refine the acetylcholine receptor model and models of colicin El and δ hemolysin membrane channels developed earlier by utilizing more detailed molecular calculation, as well as incorporating additional experimental results. It is anticipated that the present methods will be applied to other membrane proteins as appropriate sequences and structural information become available. Proteins under study include apolipoprotein involved in arteriosclerosis, oncogene proteins implicated in cancer, bacteriorhodopsin, and various transport proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08364-01 LTB

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Exocytosis/endocytosis - Data Analysis and Modelling

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Charles DeLisi, Ph.D. Acting Chief, LTB, NCI

COOPERATING UNITS *(if any)*

Drs. R. Singanian, I. Pastan

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	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.)*

A mathematical model has been developed to describe the interaction of peptide hormones with cells. It considers a two step binding at the surface, interalization through coated pits, lysosomal degradation of hormone and possible recycling of the receptors. The model has been applied to a wide variety of kinetic data on EGF, with excellent agreement between theory and experiment. Equilibrium studies lead to linear or curviniear Scatchard plots depending on the model parameters and suggests that the saturation of coated pit proteins rather than receptor heterogeneity could be responsible for experimental concave plots. Furthermore, the interpretation of slopes and intersection in terms of affinities and number of receptors is not immediate if internalization occurs.

We are developing a model for the release of histamine following the binding of IGE to basophils. The model accounts for the kinetics of several biochemical events including methylation of phospholipids and the release of arachidonic acid. These reactions are linked to the influx of calcium into the cell which was also incorporated into the model. The model predicts the time course of these responses, as well as those of regulatory membrane enzymes, and the release of histamine from the cell.

Data from mutant strains have been incorporated, while data on sensitization and desensitization have yet to be modeled.

This system has features which are common to many excretory pathways, such as hormone release, and the model should therefore be generally useful of exocytosis of compounds from cells.

373

Other Professional Personnel: Marianne Gex-Fabry, Visiting Associate, LTB, NCI; Meryl Wastney, Ph.D., Visiting Fellow, LTB, NCI

Objectives: To develop a mathematical model for the binding, internalization and degradation of the epidermal growth factor (EGF) and to test it by comparison with experimental data. To describe the sequence of surface and intracellular events leading to the milogenei effect of EGF. To suggest a mechanism by which agents like tumor promotes interact with EGF binding and endocytosis.

Methods Employed: Mathematical modeling of experimental data using the computer programs SAAM and CONSAM.

Major Findings: The model predicted curves for surface bound and intracellular EGF verses time superimpose with good accuracy to data from Haigler et al., (J. Chem. Biol. 255: 1239-41, 1980). EFG was initially found on the surface but after 5 minutes there was a decrease of surface bound EGF and a simultaneous increase of interalized EGF over 45 minutes. The association of EGF with cells over 6 hours, in the presence or absence of tumor promoter PMA was also obtained from the model (King et al., J. Biol. Chem. 257: 3053-62, 1982). The parameter values suggest that PMA might increase the release of intact EGF to the medium at early times and partly prevent subsequent degradation. The time scale ranging from some minutes for surface events to several hours for intracellular processing requires time dependent transfer coefficients. The steady-state study of the model shows that linear as well as non linear Scatchard plots are predictable. A limited number of coated pit proteins compared to the number of receptors leads either to a negative cooperatively effect or to a positive cooperativity effect, depending on the dissociation and internalization rates. Thus the model suggests an alternative mechanism for the concave Scatchard plots that are generally attributed to non homogeneous receptor sites. Analytical expressions for the slopes and intersections are complex functions of the model parameters. In internalization and degradation occur, estimation of the affinities and number of receptors from Scatchard plots would then lead to errornous values. (1) We were able to fit the data on methylation of phospholipids, arachidonic acid release, calcium influx into the cell and histamine release, using known pathways and enzymes. (2) It was necessary to include several pools for methylated phospholipids, only one of which controlled calcium influx. (3) The system appeared regulated such that the reactions were cyclic in nature.

Significance to Biomedical Research and the Program of the Institute: The project is aimed at elucidating through mathematical analysis of quantitative data, the mechanisms underlying growth, differentiation and secretion in normal and pathological studies. As such it is central to the programs in the Institute.

Proposed Course: The amount of data on the kinetics and concentration dependence of EGF binding and uptake by the cell is enormous and further work is needed to test the proposed model and to modify it accordingly. Particular attention will be given to the influence of tumor promoters and different types of inhibitors. The correlation between the interaction of EGF with the cell and its short and long term effects will be investigated. The significant signal leading to DNA synthesis and cell proliferation is of special interest. The model will

eventually be extended to other systems, aiming at a better understanding of hormone action. Desensitization mechanisms will be analyzed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08365-01 LTB

PERIOD COVERED

October 1, 1982 to September 20, 1983

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

Sequence and Structure Analysis of Proteins and Nucleic Acids

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Charles DeLisi, Ph.D., Chief, LTB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.8	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

The key macromolecules of living systems are linear biopolymers, and the sequence of amino acids along a protein or of nucleotide bases along a nucleic acid is the principal determinant of its structure and molecular associations, and therefore of its function. The information about DNA sequences, and also about translated protein sequences, is being collected at an explosively rapid rate. According to the figures compiled at Los Alamos nucleic acid sequence database, the number of bases determined is doubling every year since 1978 and the total is alreay over two million bases. Despite this valuable body of information, our approach to exploit this is far behind. We are developing algorithms and software for analyses of proteins and nucleic acids, which are integrated on our VAX11/780 computer together with the databases for nucleic acid sequences, protein sequences, and protein structures. The analytical methods we are developing fall into three categories: (i) statistical analysis, (ii) sequence analysis, and (iii) structure analysis. We have started using this entire system for analysis of oncogenes. In view of the recent exciting developments in this area, our theoretical approach would complement experimental approaches and will provide experimentally testable predictions.

PHS 6040 (Rev. 1/83)

GPO 895-100

Other Professional Personnel: Minoru Kanehisa, Ph.D., IPA, Los Alamos National Laboratory and LTB, NCI; Petr Klein, Ph.D., Visiting Fellow, LTB, NCI

Objectives: To develop theoretical methods for identifying functional and structural properties of protein molecules from the amino acid sequence. To analyze oncogenes and mechanisms of cancer transformation by sequence and structure analyses of nucleic acids and proteins.

Methods Employed: We have most up-to-date versions of the three major databases: the Los Alamos nucleic acid sequence database, the protein sequence database from National Biomedical Research Foundataion at Georgetown University, and the Brookhaven Protein Data Bank for both protein and nucleic acid structures. The contents of the three databases are reorganized and integrated into a relational database, which also contains additional information, such as cross references of the original databases, sequence homologies, and other relevant biological data. We have developed three types of analytical methods: (i) discrimination analysis based on statistical properties of proteins and nucleic acids, (ii) sequence analysis, especially homology searches to identify structural and functional relationships, and (iii) structure analysis for predictions of secondary structures and of membrane spanning segments of proteins.

Major Findings: Fifteen or so oncogene encoded proteins have been compared against the protein sequence database. In addition to the known homologies, e.g. src gene product and protein kinase, several potentially important homologies have been found. A particularly interesting point is that different portions of the oncogene product seem to be homologous to different proteins. This may be a result of extensive gene reshuffling during protein evolution. In any case our analysis shows the importance of assessing structural and functional properties locally along the sequence.

As the sizes of the sequence databases are already very large, a reliable criterion is required to efficiently find biologically interesting features from many potential similarities. The homology search algorithm we developed contains a statistical formula to screen out apparent similarities according to a given threshold value for the goodness of matches. We have verified this formula by Monte Carlo calculations.

Using the published classification of protein superfamilies, which is based on sequence homology, we have tried to discriminate various groups of proteins according to the amino acid composition. We could not obtain reasonable discrimination when all twenty different composition data were used. But when the data were somehow combined, for example, by using hydrophobicity and polarity scales, the discrimination was possible. This seems to suggest intrinsic degeneracy of the properties of amino acids.

Significance to Biomedical Research and the Program of the Institute: DNA sequencing has become a routine procedure in wide areas of biomedical research. It is often the case that proteins identified in the determined DNA sequence are not isolated, or even if they are isolated their properties are not well characterized. The computer system we have been developing is based on the

theoretical approaches which make good use of the rapidly expanding sequence and structure databases. It will provide experimentally testable predictions in various problems. Especially, our approach will contribute to our understanding of oncogenes, their normal cellular functions and possible breakdown mechanisms.

Proposed Course: We will extend our discrimination analysis to identification of protein coding regions in DNA sequences. Criteria for discrimination are nucleotide and codon frequencies. Since different strategies are used for degenerate codons in different organisms, this will identify origins of the sequence or of its fragments. The discrimination at the amino acid sequence level will provide additional information about protein structure and function.

We will perform thorough database searches for oncogenes and their protein products both at the DNA and protein sequence levels. DNA sequence analysis will include signal sequences flanking the protein coding region. We will classify oncogenes according to their possible functions. We will collect more biological data on oncogenes and design a relational database for them.

We will put more emphasis on the structure prediction of proteins. We will first collect various statistical data from known protein structures in the Brookhaven Protein Data Bank. We will examine previous prediction methods and assess their limitations. We will then develop our algorithm for prediction, which will also make use of homology information obtained by sequence analysis.

SUMMARY REPORT

LABORATORY OF GENETICS, IMMUNOLOGY INTRAMURAL RESEARCH PROGRAM

DCBD, NCI

October 1, 1982 through September 30, 1983

The Laboratory of Genetics began its first year in 1982 from a division of the Laboratory of Cell Biology and consists of a group of independent investigators whose primary interests are in genetic systems that control neoplasia and antibody structure.

A. Genes and gene products associated with neoplasia

Using the mouse plasmacytoma system as a model it has been now conclusively shown from collaborative work with Francis Wiener, George Klein and Shinsuke Ohno that 95% of the pristane induced tumors have non-random chromosomal translocations rcpT(12;15) and rcpT(6;15). These involve two immunoglobulin gene bearing chromosomes 6 and 12 with a specific region on chromosome 15. The break sites occur in immunoglobulin gene complex loci on chromosomes 6 and 12 and a specific location between D/E bands on chromosome 15. Shen-Ong and Cole demonstrated that this was the location of the mouse c-myc oncogene. Drs. Fred Mushinski and Prekumar Reddy showed that increased transcription of c-myc RNA occurred in mouse plasmacytomas. The process of the chromosomal translocation appears to have activated c-myc oncogene expression.

These findings have now provided an exciting system for studying the role of a specific oncogene in tumor development and maintenance. Little is known about the c-myc gene and its surroundings on chromosome 15. Work is underway to find the genetic basis for the translocation in the c-myc locus.

The finding with pristane plasmacytomas led to a study of B-cell lymphocytic tumors and plasmacytomas induced in BALB/c mice by Abelson virus and pristane. Abelson plasmacytomas appear to be a distinct group of tumors, some 4 of which lack translocations. These tumors express large amounts of v-abl, indicating their transformed state probably depends upon the activity of this oncogene.

Abelson lymphosarcomas also express large amounts of v-abl. However a distinct morphological subset of these tumors has been identified - the Abelson plasmacytoid lymphosarcomas (ABPL), many of which have lost the A-MuLV proviruses. These tumors have the remarkable characteristics of having rearranged c-myb genes.

In summary, 3 of the major oncogenes associated with hematopoietic neoplasms in the chicken and the mouse have been implicated in B-cell neoplasm in the mouse.

A major effort is being made to identify the genes that determine the resistance to plasmacytoma induction in the DBA/2 mouse. Over 30 marker gene differences are known between the plasmacytoma susceptible BALB/c and DBA/2 (including a number on chromosome 15 GPD-1). A number of BALB/c congenic

strains that carry DBA/2 markers have been developed. Many of these have now been tested. Two minor resistance gene loci linked to Fv-1 and Qa2 have been found and possibly one stronger locus Tol-1. This work is progressing.

Another model for plasmacytoma resistance has been discovered in a study of 6 BALB/c sublines. BALB/c Jax is uniquely resistant to plasmacytoma development and we are currently attempting to find the loci responsible for this change.

Dr. Michael Rogers is studying a 75 kd T-cell glycoprotein antigen isolated from the RBL-5 lymphoma. He has succeeded in isolating this highly antigenic material and preparing antibodies to it. The 75 kd protein is probably a degradation product of a 175 kd protein. That is commonly expressed in many normal cells The working hypothesis is that a mutant form of the 175 kd protein is expressed in RBL-5. Dr. Rogers is actively trying to sequence this protein and recover the gene that controls it. The role of somatic mutations in neoplasia has been a subject of considerable controversy in the past. This theory could not be evaluated without primary structural evidence. The 175 kd protein system may have very important implications for neoplasia in general.

Drs. Ruscetti and Wolff are working with the Friend virus system. Two retroviruses that lack cellular oncogene inserts induce erythroleukemia in genetically susceptible strains of mice - these are the spleen focus-forming virus (SFFV) and the Friend mink cell focusing-inducing virus MCF. The erythroleukemia appears to be associated with the expression of the viral env gene glycoprotein products. Drs. Wolff and Ruscetti have determined the nucleotide sequence of the env gene from SFFV and deduced the primary structure of the gp52 protein product.

Dr. Ruscetti is studying the genetic basis of susceptibility to Friend erythroleukemia development and has found a unique mechanism for resistance. The resistant DBA/2 strain produces an MCF/xenotropic related env gene product, that competes with the erythroleukemogenic viral env gene products, and hence renders in some way DBA/2 mice resistant to erythroleukemia development. The localization of this gene, and the mechanism of resistance are under investigation.

Dr. Hayden Coon is studying a line of thyroglobulin producing cells (FRTL) and is selecting mutant cell lines that have modified ability to secrete thyroglobulin (TG). Reconstitution experiments have been done to determine if FRTL cells, can upon retransplantation, regenerate thyroid tissue in vivo.

Dr. Coon and Dr. Nelson Sinback are studying the properties of single ion channels in the FRTL membrane which control the movement of Na+, K+, Ca++, Cl-, and I-. Dr. Sinback is determining the voltage sensitivity, chemosensitivity, channel open time, opening frequency, and ion conductances of the single channels. He can record the current through channels by sealing the tips of blunt electrodes (1{ in diameter) to the external surface of the FRTL cell membrane. By making a high resistance seal between the electrode and membrane (> 10E9 Ohms) the electrode will respond to current going through single channels surrounded by the tip circumference. This technique

allows us to measure ion flow directly and not be forced to infer it from changes in the membrane voltage. It also allows us to record current and voltage from very small cells which are difficult to impale with micro electrodes (see 2 below). Since these patches of membrane can be excised from the cells membrane without breaking the seal, he can also study how intracellular Ca++ acts on the internal surface of the cell membrane to control Na+ and I- channels following stimulation of the cells with noradrenaline.

In collaborative studies with Dr. L. Miller NIAID, Dr. Coon has cloned a malaria plasmodium knowlesi organism by using micromaniplation. This has provided a means for assaying antigenic variations that arise during the course of one infection by malaria.

Dr. Sinback hopes to extend his studies in the forthcoming year to lymphocytes. The "patch" technique offers many interesting opportunities to study membrane physiology. For example, the patches represent small pieces of membrane and the specificity of the ability of specific molecules to transport substances into the cell can be approached by using monoclonal antibodies.

Immunoglobulin genes, antibody structure

This laboratory continues a number of investigations on antibody structure.

Dr. Rudikoff (and M. Pawlita, formerly with this laboratory) have determined the complete variable region sequences for 12 monoclonal antibodies that specifically bind β1,6-D-galactan structures. All of the 12 proteins are apparently products of the same V_L and V_H genes. However, all are different. The major structural differences are determined by the D region. As shown by E.B. Mushinski, these changes are associated with changes in antigenicity (idiotypy) but not functional differences. Dr. Rudikoff has found evidence of somatic mutation in IgM β1,6-D-galactan binding antibodies implying a mechanism other than the error proneness associated with DNA rearrangement is involved.

Dr. Rudikoff continues to study the evolution of immunoglobulin V-gene families and the underlying mechanisms for their evolution. He also is assessing the importance of the process of gene conversion in antibody diversity by studying genomic and rearranged v-genes in specific antibodies. Evidence favoring gene conversion as a mechanism for the somatic generation of diversity was produced with the phosphorylcholine system.

Dr. Mushinski has continued to sequence the region 3' of the mouse ϕ and α C_H genes to demonstrate how RNA splicing mechanisms generate multiple RNAs for membrane IgD and IgA.

Models of the J539 and other galactan binding monoclonal antibodies have been extensively refined using computer modeling energy minimization and physical model construction. The binding site for β1,6-D-galactotetraose has been deduced from the model.

Dr. Sandra Smith-Gill has sequenced V_H regions from two antilysozyme monoclonal antibodies, HyHEL 8 and HyHEL 10. These two proteins appear

to have V_L and V_H structures from closely related genes. The V_L chains from these molecules lack methionines and the isolation of large peptide fragments for sequencing has been laborious and difficult. Accordingly, using partial amino acid sequences the productively rearranged genes can

be identified in cells cDNA clones have been developed in collaborative studies with Thomas Lavoie and William Drohan. These two proteins appear to have V_L and V_H structures from closely related genes.

In collaborative studies with Dr. Davies' laboratory the Fab fragments of HyHEL 10 have been crystallized. Further, crystal structures of HyHEL 10 Fab and lysozyme have been obtained. These data are being actively pursued to develop models of protein-Ab interactions.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB05596-14 LGN

PERIOD COVERED: October 1, 1982 through September 30, 1983

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Pathogenesis of plasma cell neoplasia: characterization of antigen-binding proteins

PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.)
(Name, title, laboratory, and institute affiliation)
M. Potter, Chief, Laboratory of Genetics, NCI

COOPERATING UNITS (if any) G. Klein, F. Wiener, Karolinska Institute; Dr. Elizabeth Blankenhorn, Dept. of Microbiology, Univ. Pennsylvania; T. Roderick, Jackson Laboratory, Bar Harbor, Maine; S. Ohno, Kanazawa Univ., Japan

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.00	3.00	4.00

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.)

Plasmacytomas can be induced in 60% of BALB/c mice by the intraperitoneal injection of mineral oils. The induction procedure requires a conventional closed facility in which mice can be maintained in good health for 1 year. The primary effect of mineral oil is to induce the formation of a chronic granulomatous tissue. One effort of the work is to identify the specific cells and their products in this pathological microenvironment that influence plasmacytoma development. Most inbred strains of mice do not develop plasma cytomas, thus the BALB/c strain has a special genetic predisposition. Identification of specific genes and the functions they govern which determine susceptibility, is one approach to identifying critical factors in tumor development. We are actively pursuing this by a genetic analysis of BALB/c sublines and BALB/c congenic mice. Ninety-six percent of BALB/c plasmacytomas have non-random translocations involving chromosome 15 near the location of the oncogene. A focus of our research is to determine the role of the myc oncogene product in plasmacytoma physiology. Essential to this work is the need to develop monoclonal antibodies to myc gene products. Studies continue on the structure of the kappa and lambda Ig light chain loci in the mouse.

PHS 6040 (Rev. 1/83) GPO 895-100

Plasmacytomagenesis

Using the LBI-closed colony environment we have been able to carry out plasmacytomagenesis experiments that involve maintaining mice free of intercurrent debilitating infections for one year after the first injection of oil. This is essential for the quantitative determination of plasmacytoma incidence. We have standardized the methods of induction so that susceptibility and resistance can be determined more rapidly. The standard treatment of three 0.5 ml doses of pristane at days 0, 60 and 120 gives a 60% yield of plasmacytomas. A single 1 ml dose gives a 35-44% yield of plasmacytomas.

Pathophysiology of the oil granuloma

The principle effect of pristane and mineral oil on the process of plasmacytomagenesis is the formation of a chronic pathological tissue on peritoneal surfaces called an oil granuloma. This tissue develops within a few days after the first injection of oil or pristane and continues to develop as long as free oil remains in the peritoneum. The oil granuloma is composed chiefly of macrophages, but neutrophils appear early and lymphocytes appear later in the peritoneal space. Plasmacytomas arise in the oil granuloma after long latent periods (4-12 months). Primary tumors require this microenvironment for growth. Following transplantation the tumor cells become independent of the oil granuloma, in one or two transfer generations.

In collaborative experiments with Mr. R. Nordan we have studied the effects of chronic indomethacin (administered in the drinking water) on the induction of plasmacytomas by a single injection of pristane. Indomethacin is an anti-inflammatory agent. Mice given indomethacin at the time of the pristane injection or as late as 60 days later have developed a negligible or very low incidence of plasmacytomas as compared to controls. We plan to continue this study to look for the effects of indomethacin on the histological formation of the oil granuloma. Possible targets of indomethacin are macrophages andd PMNL. These granulocyte cells are a source of H_2O_2, O^-_2, and OH^- radicals, which are in turn potential mutagens. We will attempt to demonstrate how indomethacin blocks plasmacytomagenesis.

The peritoneal oil granuloma also provides an essential microenvironment for the growth of primary (early) plasmacytomas, this could include physical features such as adherent cells or soluble factors elaborated by the inflammatory cells.

In studies with Mr. R. Nordan primary plasmacytoma cells (PCT-C) have been propagated in millipore diffusion chambers (MDC). It has been possible to make preparations of primary plasmacytomas that contain a very few macrophages, and these have grown in MDC in normal and pristane conditioned mice. There has been a consistent increase in the proliferation of primary PCT in MDC implanted in pristane conditioned mice as compared with normals strongly suggesting that diffusable substances influence primary plasmacytoma growth. We have not been able to identify the factor that influences primary plasmacytoma growth, chiefly because of the lack of an in vitro assay system. Mr. Nordan has been able to isolate a factor-dependent transplantable plasmacytoma cell line. (XRPC24) and to show, a factor of macrophage origin, permits colony growth of XRPC24 cells in soft agar.

We have yet to demonstrate that this factor influences primary plasmacytoma growth.

We have very recently demonstrated an accumulation of lymphocytes occurs in the peritoneal space, 80 days after the injection of pristine. Thus lymphocytosis appears to be associated with the development of plasma cell tumors.

In collaboration studies with Dr. H.C. Morse III NIAID, we have studied these lymphocytes in the cell sorter and shown they are B-lymphocytes most of them carry IgA molecules on their plasma membranes (Bα). Since the predominant IgA class in BALB/c plasmacytomas is IgA, this population of Bα lymphocytes are potential precursors of plasmacytomas. We plan to culture these cells; attempt to transform them in vitro with Abelson virus and determine their karyotypes.

In genetic studies with Dr. Carol Cowing described below, we have developed a BALB/c congenic strain called BALB/c.DBA/2 Tol-1. The Tol-1 gene determines the susceptibility to tolerance induction. Essentially when DBA/2 mice are injected with 2 mgm of bovine gammaglobulin (BGG) and then challenged 1 week later with BGG in complete Freund's adjuvants they do not form antibodies. In contrast, when BALB/c mice are similarly treated with BGG and challenged, they actively form antibodies. It is thought that the Tol-1 gene determines the ability of macrophages to present antigen. In the first test of these mice at N8 it was necessary to use (N8 x BALB/c)F_1 hybrids, but strikingly we obtained a very low incidence of plasmacytomas in these mice indicating, they carry one of the resistance genes of DBA/2 origin. This is further evidence of the role of macrophages in plasmacytomagenesis. We shall compare the process of oil granuloma formation in BALB/c, DBA/2, and C.D2 Tol-1 mice to look for further evidence of an association of a specific macrophage and plasmacytoma development.

Arthritis

In addition to inducing plasmacytomas pristane also induces the formation of a chronic arthritis in BALB/c mice. This affects the ankle joints, and feet but can involve other joints. Some mice develop ankylosis. In any given experiment from 10 to 30% of the mice develop arthritis. The interesting feature of this arthritis is the long latent period beginning 4 to 5 months and lasting throughout the time when plasmacytomas develop and lack of evidence of mycoplasma infection. A possible mechanism is an autoimmune process beginning with a destructive tissue change in the peritoneum. The evidence to date indicates the development of arthritis is dependent on the amount of pristane, i.e., 0.5 ml induces a very low incidence, 1.0 to 1.5 ml of oil produces a relatively high incidence.

We are attempting to find genes that control susceptibility to arthritis. DBA/2 is resistant. Analysis of BALB/c.DBA/2 congenic lines so far suggests a gene on chr 11 linked to ES.3 may be important as these congensis have not developed arthritis in contrast to the other C.D2 congensis.

Karyotypic analysis of plasmacytomas

In collaboration with Dr. G. Klein and Francis Wiener of the Karolinska Institute, a karyotypic analysis of primary and early transfer generation plasmacytomas has been carried out using G-banding to identify specific chromosomes. Twenty-seven plasmacytomas induced by the i.p. injection of pristane in BALB/c and BALB/c congenic mice have been analyzed: 22 have rcpt(12;15), 3 have rcpT(6;15) and 1 has no translocations. Among 16 plasmacytomas induced in mice given 0.5 ml pristane and subsequently infected with Abelson virus, 9 had rcpT(12;15), 3 had rcpT(6;15) and 4 had no translocations. Abelson virus appears to transform cells that do not carry translocations, and further that Abelson plasmacytomas may have a different spectrum of neoplastic phenotypes than is seen when the mice are induced by pristane alone.

Francis Wiener has made the exciting observation that those tumors which lack chromosomal translocations have another cytological defect characterized by, an interstitial deletion of a band in chromosome 15 near the D/E junction near the location of c-myc. This may be an antecdant of the chromosome 15 translocation process, or a new type of myc gene disorder.

Continuing our collaboration with Francis Wiener we are attempting to induce plasmacytomas in BALB/c hybrids that carry chr. 15 translocations from a plasmacytoma resistant strain; e.g., T6(T14; 15+ del 15) from a CBA/H background; Rb6;15 which is from an AKR background; and Rk21 which is a paracentric inversion of chr. 15. We backcrossed T6 onto BALB/c and tested N1 (to BALB/c) and obtained 6/24 plasmacytomas (an unexpected high yield). Five PCT's occurred in the mice but only one in a heterozygote. This tumor had a translocation involving the chr. 15 of CBA/T6T6 origin. We have now tested CBA/T6T6 and (BALB/c x CBAT6T6)F1 hybrids; both are resistant to plasmacytoma induction by pristane. We are planning to repeat the original observation using a second backcross population in which all of the mice will be heterozygous for T6.

We have been more successful with AKR Rb6;15. We constructed a first backcross population to BALB/c by crossing a (BALB/c x AKR Rb6;15)F1 mouse homozygous for Rb6;15 to BALB/c. The progeny developed a high incidence of plasmacytomas. Of 9 tumors so far examined, 2 have had chromosomal translocations involving the Rb6;15 chromosome of AKR origin and 7 involved the BALB/c chr. 15. This experiment was designed to determine if the chr. 15 of BALB/c origin is more likely to translocate than a chromosome 15 derived from a strain that is resistant to plasmacytoma development. The sample is still too small to draw conclusions and more tumors are being generated. The unexpected high incidence of tumors in the first backcross mice prompted us to test AKR Rb6;15 (BALB/c x AKR)F1 as well as (BALB/c x AKR Rb6;15)F1. Thus far, the incidence of tumors in AKR x BALB/c F1 is plasmacytoma resistant. We are generating more tumors to get better quantitative data. If the trend continues we shall conclude that some gene not on chr 15 influences the rate of chromosome 15 breaks - possibly some DNA repair enzyme or polymerase.

We are testing conventional chr. 15 markers from resistant strains caracul, Ca and belt, bt (from C57BL/6), and Gdc-1 (from DBA/2) for susceptibility to plasmacytomagenesis. Both caracul and belt from C57BL are not linked to a resistance gene.

In collaboration with Dr. Fred Mushinski and E.P. Reddy we have examined the role of abl, myc and myb oncogenes in lymphosarcomas, plasmacytic lymphosarcomas (ABPL) and plasmacytomas (see report by F. Mushinski). The ABPL tumors that now lack A-MuLV, are being examined karyotypically.

Identification of genes confering susceptibility to mineral oil plasmacytomagenesis in BALB/c mice. Most inbred strains of mice are not susceptible to peritoneal plasmacytomagenesis. The remarkable susceptibility of strains BALB/c and NZB suggests susceptibility is determined by specific genes. We are attempting to identify by linkage tests the loci involved in susceptibility of the BALB/c An strain. The approach is at first indirect, and is concerned with identifying resistance (R) genes in other strains. Two R strains are being studied in detail, DBA/2 and C57BL/6.

A second approach to finding genes that are associated with susceptibility and resistance is to compare the susceptibility of BALB/c sublines. We have obtained BALB/c Boy, BALB/c Jax, BALB/c ORNL, BALB/c Arg, and BALB/c wt. The JAX, ORNL, and wt lines were separated from BALB/c An over 40 years ago. Of these mice BALB/c Jax develops only 10-20% plasmacytomas i.e. is partially resistant. All others are highly susceptible. This indicates that, resistance in the BALB/c Jax line probably represents a genetic change that occurred in this subline. Over 50 different genes have been examined by various investigators between BALB/c Jax and other sublines. The best single genetic difference that has been described so far is the Raf-1 (regulator of alpha fetoprotein AFP). In BALB/c Jax the adult levels of AFP are ca. 900-1000 ng/ml in contrast to other inbred strains which are in the 50 ng/ml range. In collaboration with Dr. Elizabeth Blankenhorn of the University of Pennsylvania we have determined the AFP levels of all of the other BALB/c sublines and all are low. Thus high adult AFP levels correlate with relative resistance to plasmacytoma development. Accordingly, we are now crossing BALB/c π xBALB/c Jax, and will select from the F2 mice with high AFP levels (i.e., homozygous for the Raf-1 alleles of BALB/c Jax origin and will determine if these mice are more susceptible to induction of plasmacytomas by i.p. pristane, than F_2 mice with lo Af? levels.

Fl hybrids of BALB/c and the resistant DBA/2 strains are resistant to plasmacytomagenesis suggesting resistance genes are dominant. We are attempting to identify resistance (R) genes in DBA/2 by backcrossing a number of DBA/2 loci onto the BALB/c genotype to produce BALB/c congenics carrying R genes (C.D2).

The following C.D2 (congenics listed by genotype) have been tested and found susceptible:

CCAA (chr. 7)	Igh^c (chr. 12)	$Es\text{-}3^c$ (chr. 11)
CCaa (chr. 7,2)	McF^R,$Env\text{-}1^-$ (chr. 5)	$Es\text{-}3^c$-Hba (chr. 11)
$Idh\text{-}1^b$-Ity^R-$Pep\text{-}3^b$ (chr. 1)	$Pgm\text{-}1^b$ (chr. 5)	Lyt-2, Lyt-1 (chr. 6)

In this group the most remarkable finding is the susceptibility of the MCF^R-Emv-1. This congenic (constructed in collaboration with Dr. Janet Hartley, NIAID) carries a resistance gene that limits the spread of MCF-type retroviruses, and also completely lacks any loci for ecotropic proviruses. This has now been confirmed by hybridizing genomic DNA with an ecotropic virus specific probe. The findings with this congenic virtually eliminate known infectious retroviruses as essential components in plasmacytoma development in BALB/c. It does not exclude activation of endogenous xenotropic type C or intracisternal A particles (IAP) as possible mutagenic agents in plasmacytoma development.

Three C.D2 congenics have shown partial resistance: $Fv\text{-}1^{nn}$ (chr. 4) tested at N9; $Qa2^-$ (chr. 17) tested at N6 and Tol-1 (unknown linkage) tested at N8. All three represent interesting genetic problems. C.D2 $Fv\text{-}1^{nn}$ mice develop between 30-40% plasmacytomas with a longer mean latent period. The $Fv\text{-}1^{nn}$ gene product has not been identified and so it is not clear whether this gene or another closely linked to it are the genes determining resistance.

In constructing the C.D2 $Fv\text{-}1^{nn}$ congenic we backcrossed the $Fv\text{-}1^{nn}$ gene onto BALB/c for 7 generations and then mated two heterozygous N7 mice to each other to produce progeny that were $Fv\text{-}1^n/Fv\text{-}1^n$ as well as a control stock $Fv\text{-}1^b/Fv\text{-}1^b$. When we tested the C.D2 $Fv\text{-}1^{bb}$ at N6 we found it was also partially resistant. We then tested these mice for all other available markers and discovered they were $Qa2^-$. $Qa2^-$ is a chromosome 17 marker. This finding has been confirmed in two laboratories. Dr. Michael Rogers has shown this is not an MHC recombinant. This may be an example of quasilinkage, wherein two chromosomes from the same parental origin stay together at meiosis. Thus when C.D2 $Fv\text{-}1^n/Fv\text{-}1^b$ mice were mated to each other, the chr. 17 with $Qa2^a$ was dissociated from chr. 4 with $F\text{-}1^n$.

The Tol-1 gene governs induction of high dose tolerance as explained above. These mice have developed ca. 35% incidence of plasmacytomas in 330 days. BALB/c $Tol\text{-}1^{aa}$, DBA/2 = $Tol\text{-}1^{bb}$. These mice were tested at N8 and only 50% of the mice actually carry the $Tol\text{-}1^b$ gene as we have no test for homozygosity. Thus this gene may be a strong resistance gene. We are examining these mice for other DBA/2 genes.

Experiments are underway to combine the genes. The evidence suggests at least 3 resistance genes so far exist between BALB/c and DBA/2.

Genetics of Immunoglobulins

The size and gene order of V-region genes in the Ig κ locus of the mouse has not been determined. It is estimated there are between 100 and 300 V_K genes that are organized into families of closely related genes. We have obtained from other investigators a number of V_K probes. These strongly hybridize with several (ca. 1 to 10) restriction fragments (that represent a family) from genomic mouse DNA. The number and size of individual restriction fragments varies in different mouse genotypes and there is considerable variation among wild mice. We are establishing a profile of differences between BALB/c and wild mouse stock called CNV (now being inbred). (BALB/c x CNV)F x BALB/c backcross mice will be examined for recombinants. From these data we should be able to determine the relative gene order of the V_K genes. We wish to be able to estimate how frequent crossing-over occurs in the kappa locus of wild mice.

Publications:

Potter, M., Newell, J.B., Rudikoff, S. and Haber, E.: Classification of mouse V_K groups based on the partial amino acid sequence to the first invariant tryptophan: Impact of 14 new sequences from IgG myeloma proteins. Mol. Immunol. 19: 1619-1630, 1982.

Potter, M.: Current problems in antibody diversity: Introductory remarks. In Cruse, J.M. (Ed.): Survey of Immunologic Research, Vol. 2, Basel, Switzerland, S. Karger, 1983, pp. 25-27.

Potter, M.: Structural correlates of immunoglobulin diversity. In Cruse, J.M. (Ed.): Survey of Immunologic Research, Vol. 2. Basel, Switzerland, S. Karger, 1983, pp. 27-42.

Scott, C.L. and Potter, M.: Diversity of immunoglobulin structural gene loci. In Cruse, J.M. (Ed.): Survey of Immunologic Research, Vol. 2. Basel, Switzerland, S. Karger, 1983, pp. 43-51.

Potter, M.: Immunoglobulins and immunoglobulin genes. In Foster, H.L., Small, J.D., Fox, J.G. (Eds.): The Mouse in Biomedical Research, Vol. 3. New York, Academic Press, 1983, pp. 347-380.

Potter, M. and Wax, J.S.: Peritoneal plasmacytomagenesis in mice: Comparison of three pristane dose regimens. J. Natl. Cancer Inst., in press, 1983.

Potter, M., O'Brien, A., Skamane, E., Gross, P., Forget, A., and Kongshavn, P.: A BALB/c congenic strain of mice that carries a gene locus (Ity^r) controlling resistance to intracellular parasites. Infection and Immunity, in press, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08727-06 LGN

PERIOD COVERED
October 1, 1982 through September 30, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
J.F. Mushinski, Medical Director, Laboratory of Genetics, NCI

COOPERATING UNITS *(if any)*
Laboratory of

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.00	2.00	2.00

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.)*

It is the long range purpose of this project to study the control mechanisms important in regulating protein synthesis in normal and malignant lymphoid cells. The organization of immunoglobulin light and heavy chain genes and the control mechanisms that select those to be transcribed into RNA and then expressed in protein synthesis are being studied. Particular attention is being given to the molecular genetics of membrane bound IgD and IgA since membrane Igs may be receptors for antigens. We have prepared cDNA clones of mouse mIgD and are attempting to do the same for mIgA. We have undertaken a study of the expression of various oncogenes in mouse and human plasmacytomas in hopes of finding clues to the mechanisms responsible for their carcinogenesis. We have discovered that the myc oncogene is genetically altered and transcribed in unusually large amounts in plasmacytomas. We have further discovered that the myb oncogene is altered and transcribed into unusual sized RNAs in certain lymphomas induced by Abelson leukemia virus but from which the transforming virus was lost. We are investigating whether the alteration of the myb proto-oncogene may explain the "hit and run" transformation seen with some oncogenic viruses.

Major Findings

A. Within the past year we have begun a major long term effort to study how important the the expression of proto-oncogenes such as c-myc, c-myb and c-abl may play in neoplastic transformation. We found that there was an unusually large amount of myc RNA in mouse plasmacytomas compared to normal lymphoid tissues such as spleen or thymus. This RNA usually took the form of a 2.4 kb myc RNA, but some plasmacytomas expressed myc in the form of a tumor specific 1.8 kb myc RNA. The plasmacytomas with the 1.8 kb myc RNA always showed a rearrangement in the myc DNA, most commonly seen as an EcoRI myc fragment smaller than the 21 kbp band found in normal tissues and embryos. Extensive studies of myc RNAs and DNAs from plasmacytomas induced in BALB/c mice by intraperitoneal injection of mineral oil or mineral oil plus Abelson leukemia virus (A-MuLV) showed that there was frequently an association of these myc RNA and DNA changes with the chromosome translocations (12;15 or 6;15) commonly seen in plasmacytomas. However, there is not a one-to-one correlation because not all cells with translocations involving the myc-bearing chromosome 15 showed an alteration in myc restriction fragments. Further, not all tumors with myc DNA alterations showed tumor-specific myc RNA transcripts, but virtually all the plasmacytomas, with or without chromosome translocations contained elevated levels of myc RNA. Thus we are convinced that chromosome 15 translocation is only one of several possible ways that myc transcription can be elevated in these tumors. Future studies will attempt to determine which of these mechanisms are, in fact, acting in plasmacytomas.

B. In a related study we discovered the first tumor system associated with permanent alterations in DNA and RNA from the myb oncogene. This finding came in a study of oncogene RNA expression in the three types of B-lymphocytic neoplasms that arise in BALB/c mice injected intraperitoneally with pristane and then infected with the defective transforming retroviruses, Abelson virus (A-MuLV): undifferentiately lymphosarcomas (ABLS), plasmacytomas (ABPC), and a subset of lymphosarcomas morphologically distinguishable by their plasmacytoid appearance (ABPL). As expected, ABLS's and ABPC's contained A-MuLV proviruses integrated in their genomes and large amounts of abl RNA in the form of 6.7 kb A-MuLV RNA genomes. Unexpectedly, ABPL's contained neither integrated A-MuLV provirus nor 6.7 kb abl RNA transcripts. Whereas particularly abundant myc RNA transcripts characterized ABPC's, an abundant amount of myb RNA was seen in ABLS's and ABPL's but not ABPC's. This usually took the form of a major 3.8 kb and a minor 4.2 kb myb RNA, both of which can be found in small amounts of normal cells, most prominently in thymus. The ABPL's, however, contained, in addition, even more abundant myb RNA of a larger size, usually about 5.0 kb. What is more, the ABPL's uniquely showed an alteration in the DNA restriction fragments that hybridized with myb. Since these tumors have remained neoplastic in the absence of A-MuLV transforming virus which presumably initially induced the tumors, we have postulated that the altered myb DNA and RNAs are evidence of a "hit and run" transformation by A-MuLV. It is possible that the abundant, altered myb transcripts preserve the transformation of the cells in the absence of transforming virus.

C. Our studies into the RNA splicing patterns responsible for generating multiple mRNAs for membrane-binding IgD and IgA heavy chains are progressing. A combination of DNA sequence studies, R looping and S1 nuclease mapping have indicated approximately how the multiple RNAs differ from one another. We are now obtaining cDNA clones of many of these forms of RNA, and DNA sequence studies should tell precisely how the major species of RNAs differ from the minor ones.

Significance and Proposed Course

We think that the findings of myc and myb oncogenes expressed in unusual abundance in different types of tumor cells is potentially very important in understanding mechanisms of carcinogenesis in lymphoid cells. It is not clear at this point whether the apparent increase in myc RNA levels in plasmacytomas and the apparent increase in myb RNA in lymphomas is really an elevation over normal levels since no source of normal plasma cells or lymphoblasts has been identified and tested. The findings of DNA alterations or rearrangements at the gene level for both myc and myb are likely to be tumor related, and they are not thought to happen in normal cells. Furthermore, the RNA products of these rearranged myc and myb genes are clearly abnormal and probably are tumor-specific. Yet their relationship with tumorigenesis is still unclear.

We intend to study the molecular basis for these myc and myb DNA rearrangements and to determine the precise nature of the abnormal RNAs synthesized from them. We also would like to understand the mechanisms responsible for elevation of RNA transcription in tumor cells with and without such rearrangements. It is also important to determine whether or not there is a causal connection between these RNA abnormalities and tumorigenesis. To pursue these aims we are preparing bacterial clones of cDNAs which are copies of the usual and unusual mRNAs for myc and myb found in our lymphoid tumors. Structural determinations on these DNAs will show whether point mutations have made these RNAs abnormal even when no changes in overall size can be detected as well as allow us to see how the unusually sized RNAs differ from the normal. Genomic cloning of the myc and myb genes from selected tumors is also under way in order to determine the precise nature of the DNA rearrangements seen by Southern blotting. The biological significance of these changes will be assessed by attempts at transformation of cells with fragments of tumor DNA or clones of these DNAs, for it has yet to be demonstrated that these genetic changes are directly related to carcinogenesis.

Publications

Mushinski, J.F.: Review and discussion of differentiation of IgA-B cells: Molecular mechanisms involved in the generation of cells that secrete IgA. In W. Strober, L.A. Hanson and K.W. Sell (Eds.), Recent Advances in Mucosal Immunity, Raven Press, New York, N.Y., 1982, pp. 187-197.

Blattner, F.R., Richards, J.E., Shen, A., Knapp, M., Strober, S., Gilliam, A.C., Jones, S., Cheng, H.-L., Mushinski, J.F., and Tucker, P.W.: Genetic aspects of IgD expression: I. analysis of Cμ-Cδ complex in committed and uncommitted DNA. Ann. N.Y. Acad. Sci. 399: 1-14, 1982.

Mushinski, J.F., Thiele, C.J., Owens, J.D., Shen, A., Blattner, F.R., Tucker, P.W., and Fitzmaurice, L.: Genetic aspects of IgD expression: II. Multiple forms of δ chain mRNA in normal mouse spleen, mouse B cell lymphomas and mouse and human myelomas. Ann. N.Y. Acad. Sci. 399: 15-26, 1982.

Tucker, P.W., Cheng, H.-L., Richards, J.E., Fitzmaurice, L., Mushinski, J.F., and Blattner, F.R.: Genetic aspects of IgD expression: III. Functional implication of the sequence and organization of the C_δ gene. Ann. N.Y. Acad. Sci. 399: 27-42, 1982.

Scott, C.L., Mushinski, J.F., Huppi, K., Weigert, M., and Potter, M.: Amplification of Ig Cλ genes in populations of wild mice. Nature 300: 757-760, 1982.

Kuchino, Y., Borek, E., Grunberger, D., Mushinski, J.F., and Nishimura, S.: Changes of post-transcriptional modification of Wye base in tumor-specific $tRNA^{Phe}$. Nucl. Acid. Res. 10: 6421-6432, 1982.

Mushinski, J.F., Bauer, S.R., Potter, M., and Reddy, E.P.: Increased expression of myc-related oncogene mRNA characterizes most BALB/c plasmacytomas induced by pristane or Abelson murine leukemia virus. Proc. Natl. Acad. Sci. USA 80: 1073-1077, 1983.

Word, C.J., Mushinski, J.F. and Tucker, P.W.: The murine immunoglobulin α gene expresses multiple transcripts from a unique membrane exon. Embo J. in press, 1983.

Mushinski, J.F., Potter, M., Bauer, S., and Reddy, E.P.: DNA rearrangement and altered RNA expression of the c-myb oncogene in mouse plasmacytoid lymphosarcomas. Science, in press, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB05553-14 LGN

PERIOD COVERED

October 1, 1982 through September 30, 1983

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 on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

S. Rudikoff, Microbiologist, Laboratory of Genetics, NCI

COOPERATING UNITS *(if any)*

S. Clarke, Postdoctoral Fellow, Institute for Cancer Research, Philadelphia, PA

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	4.5	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.)*

I. Immunoglobulin structure and diversity. 1) Complete variable region amino acid sequences have been determined for 12 antibodies with specificity for β (1,6) galactan. Analysis of these sequences has permitted an assessment of diversity among IgM proteins and a definition of the structural basis of a number of idiotypes expressed on these molecules. 2) Sequence analysis of antibodies to phosphocholine has presented data suggesting gene conversion in immunoglobulin variable regions. The rearranged gene postulated to have undergone conversion has been cloned and sequenced and the nucleic acid data is consistent with the originally postulated conversion event.

II. Cell membrane proteins: A number of genomic clones encoding porcine major histocompatability antigens have been isolated and are presently undergoing characterization. The nucleotide sequence of an expressed gene is nearing completion and appropriate comparisons are being made with MHC antigens from other species. These genes are also being tested for expression by DNA transfer experiments using mouse L cells.

PHS 6040 (Rev. 1/83) GPO 895-100

I. Immunoglobulin structure and diversity

This laboratory has, for a number of years, been involved in studies addressing the questions of immmunoglobulin structure function and diversity. These experiments have involved amino acid sequence analysis of groups of myeloma and hybridoma proteins reacting with the same chemically defined haptenic determinant. More recently we have employed recombinant DNA technology to begin to examine the organization, structure and family interactions of immunoglobulin genes involved in these responses.

a) One of the immunoglobulin systems under investigation in our laboratory consists of myeloma and hybridoma proteins specific for β(1,6)-D-galactans. These molecules are being investigated in terms of the genetic mechanisms involved in their origins, binding specificities, and structure of idiotypes. In the previous report we described the analysis of complete light (L) chain variable (V) regions from 10 of these monoclonal antibodies. These L chains were found to be extremely similar in all proteins and displayed evidence of an unusual, but reproducible, V_k - J_k joining event. The invariance of these L chains allowed us to conclude that all phenotypic differences attributable to these proteins would reside in sequence variation found in the heavy (H) chains. We have now completed V region sequence analysis of 12 of these H chains and the major results are as follows 1) A limited, but significant, sequence variation is found in the V_H segment (amino acids 1-94). Southern blot analysis using a cDNA probe made to one of the anti-galactan H chains reveals two hybridizing bands suggesting that no more than two V_H genes are likely to encode these proteins. The sequence variation found in the 8 IgM hybridoma V_H segments is greater than that which could be accounted for by two genes. We have concluded that this additional variation is due to somatic mutation in these IgM producing cells and that somatic mutation is not linked to class switching as has been suggested by others. 2) Most of the sequence variation in these H chains is found in the third complementarity determining region (CDR-3) and results from V_H-D joining, D gene sequence, and subsequent D-J_H joining. The number of amino acids contributed by the D gene in the various proteins ranges from 2-6. However, the length of CDR-3 is invariant and this consistency of length is maintained by the random addition of amino acids on the NH_2 and carboxy sides of D. We have proposed a novel mechanism based on the use of repair enzymes, such as terminal transferase, as a means for introducing these extra amino acids. 3) The extreme sequence variation in CDR-3 has allowed us to conclude that D region sequence is relatively unimportant in terms of specificity or L-H pairing. However, the conserved CDR-3 length may be a critical structural characteristic of these molecules. 4) Serologic studies, using anti-idiotypic reagents to these proteins, in conjuction with the protein sequence analysis has permitted the assignment of a series of idiotypic determinants to the CDR-3 region. We have defined the structural basis of a number of both shared and unique determinants at the molecular level. The structural definition of these markers has raised interesting and provocative questions for proposed schemes of network regulation involving idiotypes.

The question raised above as to the occurrence of somatic mutation in IgM producing cells is a controversial issue, and is in disagreement with conclusions drawn from other studies. It is thus necessary to prove that the V_H sequence variation we have observed is, in fact, due to somatic mutation. In this regard, we are currently cloning multiple isolates of the genes encoding these V_H segments. Nucleotide sequence analysis will then permit a determination of the origins of the observed V_H sequence differences.

b) In previous reports we have described a second immunoglobulin system consisting of antibodies to phosphorylcholine (PC) which have been employed in studies similar to those described above. In an analysis of CBA/J hybridomas we characterized an H chain which had apparently arisen from gene interactions involving three members of the PC-V_H gene family. We proposed that a novel process such as gene conversion was involved in the generation of this structure. We have now cloned the rearranged gene encoding this H chain and DNA sequence comparison with the PC-V_H gene family reveals a pattern consistent with our proposed mechanism of gene conversions. The PC-V_H gene family structure has been determined only for the BALB/c strain. Since the protein and gene we have studied is of CBA/J origin, we cannot determine whether the proposed gene conversion has occurred somatically or in the germline. We are thus cloning the PC-V_H family from CBA/J to answer this question.

The observation of gene interaction in immunoglobulins has stimulated our interest in the mutational events occurring during the evolution of multigene families. Our laboratory is in a unique position to explore this question as Dr. Michael Potter has established a wild mouse colony containing representatives of various species and sub-species spanning the evolution of the species Mus. Southern blot analysis reveals that we can identify and trace the families we have studied in inbred strains throughout this colony. We have thus begun to generate and screen genomic 'libraries' in order to isolate particular gene families and evaluate germline mutational events occurring during evolution.

II. Cell membrane proteins

In previous reports we have described studies involving the characterization of major histocompatability (MHC) antigens in a large organ transplant model in miniature swine. Experiments have continued in the chemical characterization of these proteins and several peptides have been isolated and sequenced from Class I antigens. These sequences are being used to generate synthetic peptides which will then be tested for immunogenic and biologic activity.

Concurrently, we have isolated a number of genes encoding the porcine MHC antigens. We have shown that one of these genes can be introduced into mouse L cells by DNA mediated gene transfer and subsequently expressed on the surface of these cells. DNA sequence analysis is progressing on this clone in order to determine whether the organization and fine structure

of MHC genes is conserved among different species. Hybridization studies using probes generated from regions surrounding this gene have identified repetitive sequence elements which appear to be specifically linked to MHC genes. These elements are currently being studied in an attempt to determine whether they might be involved in the regulation of expression of this particular gene family.

Publications:

Rudikoff, S.: Immunoglobulin structure-function correlates: antigen binding and idiotypes. In Inman, F.P. and Kindt, T.J. (Eds.): Contemporary Topics in Molecular Immunology, Vol. 9, New York, Plenum Publishing Corp. 1983, pp. 169-209.

Clarke, S.H., Claflin, J.L., Potter, M. and Rudikoff, S.: Polymorphisms of anti-phosphocholine antibodies reflecting evolution of immunoglobulin families. J. Exp. Med. 157: 98-113, 1983.

Osborne, B.A. and Rudikoff, S.: Murine thymocyte and splenocyte Ia antigens are indistinguishable by two-dimensional gel electrophoresis. J. Immunol. in press, 1983.

Osborne, B.A., Lunney, J.K., Pennington, L., Sachs, D.H., and Rudikoff, S.: Two dimensional gel analysis of swine histocompatibility antigens. J. Immunol. in press, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08726-06 LGN

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biochemistry of Tumor Cell Surface Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
M. Rogers, Research Chemist, Laboratory of Genetics, NCI

COOPERATING UNITS *(if any)*

Emory University, Department of Immunology

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

The purpose of this work is to examine the biological and chemical properties of histocompatibility antigens (H-2), tumor associated transplantation antigens (TATA) and other tumor associated proteins with the aim of determining their potential roles in tumorigenesis and the antitumor immune response. The approach is to purify the molecules by conventional biochemical techniques and/or prepare polyclonal and monoclonal antibodies against them. The purified molecules and antibodies are then used to study the biological and chemical properties of these molecules including 1) their ability to specifically influence antitumor immunity both in vivo and in vitro, 2) their subcellular distribution, association with the plasma membrane, and post-translational modification 3) their tissue distribution, 4) their distribution in neoplastic and preneoplastic tissue. Ultimately suitable DNA probes will be prepared and used to study the organization and expression of the genes encoding these molecules.

PHS 6040 (Rev. 1/83) GPO 895-100

A. Histocompatibility Antigens

The project begun last year to induce H-2 restricted CTL reactive with cytochrome b_5 was abandoned, because no CTL could be obtained that were capable of killing cells with membrane bound cytochrome b_5, even after secondary stimulation in vitro.

The project involving SV40 transformed C3H fibroblasts with mutated H-$2K^k$ gene was completed. C3H fibroblasts transformed in vitro with SV40 and adapted to in vivo growth no longer expressed H-$2K^k$. Using a DNA probe from the 5' end of the H-$2L^d$ gene and the technique of Southern blot hybridization, a mutation was discovered in the H-2 genes of these tumors. A 5.5 kb fragment present in Sst I digests of normal C3H tissue, was missing in DNA from the variant tumors and replaced with a 7.0 kb fragment. By using congenic mice it was possible to map this mutation to the H-$2K^k$ region of the MHC. No evidence was found to support the notion that SV40 integration had somehow induced this mutation. Work is continuing on this project in two ways. First DNAse sensitivity studies are being performed to see if the 7 kb mutant band is being expressed. Second, possible mutant H-2 molecules are being searched for by immunoprecipitating with rabbit anti H-2^k serum.

In previous work from this laboratory, we found that a methylcholanthrene induced tumor of BALB/c (H-2^d) origin, C-1, expressed alien H-2^k antigens. This suggested that genetic information for more than one H-2 haplotype might be present in a given inbred strain of mice, and the expression of a particular gene might be under control of a regulator gene. Recent work on H-2 genes has shown that such a model is probably incorrect. We are reexamining the H-2 genes of the C-1 tumor at the genetic level to try to explain our original observation. The C-1 tumor has been cloned and found to be a mixture of two types of cells, one expressing alien antigens and one that does not. Southern blot analysis of the H-2 gene has revealed that the clones expressing H-2^k molecules appear to be a mixture of H-2^k and H-2^d haplotype while clones not expressing H-2^k are H-2^d only. All clones express the original C-1 TATA. This suggests that at the time of tumor induction, C-1 was an H-$2K^k$/H-2^d F_1 like cell, at least at chomosome 17, and subsequently H-2^d only variants arose. Earlier karyotype and isoenzyme analysis had indicated that C-1 could not be contaminated but the more powerful techniques of molecular biology clearly show that this is the case.

B. Tumor Associated Transplantation Antigen.

The TATA from detergent extracts of RBL-5 lymphoma cells has been purified to apparent homogeneity. It is a 75 kd glycoprotein. As little as 50 ng of this protein provides complete protection against a challenge with RBL-5 sufficient to cause tumors in 90-100% of control animals. When a rabbit antiserum was raised against the 75 kd protein, it precipitated a single protein from metabolically labeled RBL-5 cells with a molecular weight of 175 kd. By using an immunoabsorbent prepared with the rabbit antiserum it was possible to purify a mixture of 175 kd and 75 kd proteins from detergent extracts of RBL-5 cells. The 175 kd protein was separated from the 75 kd protein and found to express the

RBL-5 TATA. Mapping experiments using partial proteolysis with V8 protease and serum proteases revealed that the 75 kd protein was a breakdown product of the 175 kd protein. Apparently the 175 kd protein is subject to degradation during purification, in spite of the large amounts of protease inhibitors present at all times, and the 75 kd protein is the most stable degradation product.

Preliminary results indicate that the 175 kd protein is expressed on all cells from diverse normal tissues and on all tumors regardless of etiology. Clearly, however, the 175 kd protein on RBL-5 cells is somehow different because it alone is immunogenic in the in vivo assay. Thus, the critical question now becomes how the 175 kd protein from RBL-5 differs from the same protein from other sources. Three experiments are underway to answer this question: 1) Tunicamycin is being employed to influence the glycosylation of 175 kd protein to see if the immunogenic determinant resides on the sugar or protein portion of the molecule. 2) Monoclonal antibodies are being prepared in syngeneic mice and in rats. Such antibodies might distinguish the TATA epitope (recognized by syngeneic mice) from other portions of the molecule (detected by rat antibodies). 3) 175 kd protein will be purified from RBL-5, the cross-reactive tumor LSTRA, and the non-cross reactive tumor mKSA. The purified proteins will be examined by peptide mapping procedures in the hopes of finding differences.

The 175 kd protein from RBL-5 is also being sequenced with the idea of preparing a synthetic DNA probe which will allow isolation of the gene encoding this protein. Analysis of this gene should provide information on the alteration of this gene unique to RBL-5 and on the relationship of the alteration to the virally induced tumorigenic process.

C. Other Tumor Proteins

Recent progress in tumor biology has implicated alterations and rearrangements in so-called oncogenes as events of potentially great importance to oncogenesis. Knowledge about the proteins coded for by these genes and their biological functions is essential for elucidating their role in oncogenesis and normal cellular development. Dr. Potter has developed a number of tumors induced by a combination of pristane and Abelson virus which have rearranged myc and myb genes and contain mRNA coding for the products of these genes. In collaboration with Dr. Potter, we are attempting to immunize syngeneic mice and rats with some of these tumor cells with the hope of raising antisera against the products of the oncogenes being expressed by these cells.

At the same time, we are using these cells to immunize against challenge with RBL-5 (see section B). Since Abelson virus is a variant of Moloney virus it could express the FMR specific TATA found in RBL-5. If so, the expression of this antigen in cells with well characterized oncogene rearrangements could provide a fruitful approach to understanding the mechanism of induction of TATA during viral oncogenesis.

Publications:

Rogers, M.J., Gooding, L.R., Margulies, D.H., and Evans, G.A.: Analysis of a defect on the H-2 genes of SV40 transformed C3H fibroblasts that do not express H-2K^k. In press, Journal of Immunology.

Rogers, M.J., Hearing, V.J., and Law, L.W.: Purification of the tumor specific transplantation antigen from RBL-5 leukemia cells. Submitted.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB05552-14 LGN

PERIOD COVERED
October 1, 1982 through September 30, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
H.G. Coon, Research Biologist, Laboratory of Genetics, NCI

COOPERATING UNITS *(if any)* Dr. F. Saverio Ambesi-Impiombato, Istituto di Patologia Generale, Naples, Italy; Dr. William Topp, Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.; Dr. Eugene Bell, Dept. of Biology, MIT, Cambridge, MA

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	2.8	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.)*

It is the purpose of this project to analyze and develop new and difficult cell systems in culture. We have developed and are attempting to exploit applications of normal rat thyroid cell cultures. These cells are hormone dependent. They synthesize and secrete a very large protein product, thyroglobulin. They concentrate iodide 100-fold from the medium. They offer a unique opportunity to study secretion, ion uptake and cAMP response. These are being studied in our lab and in other labs, however, our approach is primarily to use electrophysiological techniques. We are attempting to study long term regulation of membrane potential and its relationship to secretion and hormone levels. We are also studying neurons and neuroblasts in cell culture. There are too few mammalian cell systems where "blast" cells can be observed in transition to mature, differentiated cells. We have tried this in nerve cells using cellular hybridization and cellular transformation (with ts SV40 viruses) and by using little known cell systems in which blast cells persist througout life (olfactory epithelium). We are especially interested in the development of electrophysiological competence and in the development of chemical identity (specificity).

PHS 6040 (Rev. 1/83) GPO 895-100

Introduction

The cellular genetics unit continues to pursue studies on differentiated cell strains in vitro. The emphasis of our work is, however, shifting from maintenance of differentiated cells in vitro toward the mechanisms and events of the acquisition of differentiation as it can be studied in vitro. The emphasis of our work in the past has been on maintaining the (normal?) differentiated state of cells in vitro, preserving it for studies using the methods of somatic cell genetics. A prime example of this has been our highly differentiated rat thyroid cell strains called FRTL (Fischer Rat Thyroid Low Serum). These cells share with normal thyroid gland follicle epithelia the ability to synthesize and secrete thyroglobulin (TG), the ability to concentrate iodide, to synthesize T3 and T4 as well as a dual response to thyrotropin (TSH).

I perceive a change in the emphasis of our work more toward study of the transition of cells from undifferentiated precursor (or embryonic cells) that have not yet expressed differentiated function(s) to cells that have acquired characteristic arrays of specialized synthesis defined as differentiated cells. We also wish to follow these processes in vitro so that studies using the techniques of somatic cell genetics, molecular biology, and electrophysiology can be used here as well. Current examples of cell strains in this category are CBSV1A, an SV40 transformed rat neuroblast cell line that differentiates into a neuron when grown in crowded cell culture, and an SV40 transformed rat proadipocyte, REC-A-16, studied by Dr. Yasumoto that differentiates in vitro to typical multilocular depot fat cells after being grown to confluence in the presence of insulin and hydrocortisone.

Both CBSV1A and REC-A-16 acquire specialized differentiated functions under specific culture conditions. Both of these systems appear to be "blast cells", that is formative cells that have been made useful as cell culture systems only after viral transformation. In the future we hope to learn (by lessons taught by these and similar cell lines) how to grow normal (non-transformed) cell strains that differentiate in vitro. We have learned, for example, that CBSV1A cells don't differentiate in media low in Ca++. Since one major difficulty with growing neuroblasts has been that they differentiate instead of dividing when they are put in culture, we anticipate that normal neuroblasts might be maintained in low Ca++ medium and then induced to differentiate by shifting them to the customary higher Ca++ levels. Similarly, we would like to study "thyroblasts" taken from very young rat embryos.

We have also initiated some studies of the ultimate paradigm for differentiation in vitro, the murine embryonal carcinoma (EC). The problem with these cell lines is not that they don't differentiate in vitro, they do so wonderfully well. The problem is that no one has really found how best to exploit the system using in vitro techniques such as those of somatic cell genetics.

1. FRTL cells

a) FRTL cell mutants

We are continuing studies of mutagenesis of FRTL cells trying to find mutants that result in loss of TG synthesis/secretion. Our screening system is an agarose-medium-anti-rat-TG assay patterned after that introduced by M. Scharff in studying heavy and light chain synthesis or secretion in plasmacytomas. Our cells are diploid and we must use a two step procedure of mutagenesis and selection, first for "underproducers" and secondly for non-producers. So far we have 15 strains of underproducers. They appear to be of varying stability - yielding subclones that synthesize differing amounts of TG. When these lines are better understood they will be subjected to a second round of mutagenization and null or true non-producers will be selected.

Non-producers will be tested for complementation by cell fusion and/or hybrid cell formation. They will be subjected to further analysis by members of the Naples group who are collaborating with us. Mutants are of interest in their ongoing efforts to understand glycosylation, mRNA production, as well as marker rescue experiments in which cloned portions of the rat TG gene will be transfected into the non-producing mutants and the cells then screened for normal TG synthesis.

b) FRTL cell hybrids

We are attempting to analyze hybrids between FRTL cells that do synthesize TG and cells that do not (fibroblasts, liver cells, etc.). We expect from previous experiments that hybrids of this kind will fail to synthesize TG, a well studied phenomenon called "extinction" of differentiated function in hybrid cells. We are asking a new question: is methylation of DNA involved in the differentiation process such that if the amount of methylation is altered can the hybrid cell "extinction" phenomenon be reversed? That is, if the TG negative cell is capable of negative regulation because of a DNA methylation state, can demethylation reverse the state? If we pretreat the extinguishing cell (fibroblast) with 5-aza-cytidine [thereby causing (at least) hypomethylation] will the treated fibroblast still extinguish TG synthesis after fusion to FRTL? Perhaps both cells and/or the early hybrid cells must be treated. The rationale for this experiment is that it may test whether either the production of a negative regulator (from the fibroblast) or the receptivity of the fibroblast (TG gene?) to a positive regulator (from the FRTL cell) is influenced by the state of DNA methylation. Our initial results indicate that it is indeed influenced: some of the hybrids form positive and some form sectored colonies in our TG assay.

c) In vivo functioning of FRTL cells

FRTL cells are diploid - even euploid as far as we can tell with quinacrine banding. They synthesize and secrete TG in a form not distinguishable from follicular TG. They concentrate iodide and respond to TSH in the same way that freshly isolated thyroid cells do. But they have doubled more than 400

times in culture. Are they still "normal" or are they, as the people who believe in in vitro cellular aging claim, 'transformed by definition' (>50 population doublings)? We don't know, and furthermore we are not sure how to find out. One test, the only one I can think of, is to see if FRTL cells can still participate normally in the operations of the thyroid gland in vivo. Alternatively, FRTL cells may be able to function in place of an extirpated thyroid gland (TX). Eugene Bell (MIT) and I are attempting to find out by grafting FRTL cells into young TX rats after the cells have been enmeshed in a loose collagen gel. Bell has shown that in such a gel skin cells and other cells "take" as grafts (even as allografts) and the cells differentiate normally. FRTL cells in this system "take" and (after a month as subcutaneous grafts) make good follicle structures that surround lakes of TG (judged by staining of frozen sections with FITC-anti-TG). They are now being tested to see whether such FRTL subQ grafts can reinitiate weight gain in TX animals that had stopped weight gain. It will be very interesting to see what these grafts do. They may require thyroid fibroblasts as well as FRTL cells to restore function efficiently. There may be other implications for thyroid and other prosthesis grafting.

d) Physiology of single ion channels in FRTL (Dr. C.N. Sinback)

Dr. Sinback is studying the properties of single ion channels in the FRTL membrane which control the movement of Na+, K+, Ca++, Cl-, and I-. He is determining the voltage sensitivity, chemosensitivity, channel open time, opening frequency, and ion conductances of the single channels. He can record the current through channels by sealing the tips of blunt electrodes (1μ in diameter) to the external surface of the FRTL cell membrane. By making a high resistance seal between the electrode and membrane (> 10E9 Ohms) the electrode will respond to current going through single channels surrounded by the tip circumference. This technique allows us to measure ion flow directly and not be forced to infer it from changes in the membrane voltage. It also allows us to record current and voltage from very small cells which are difficult to impale with micro electrodes (see 2 below). Since these patches of membrane can be excised from the cell membrane without breaking the seal, he can also study how intracellular Ca++ acts on the internal surface of the cell membrane to control Na+ and I- channels following stimulation of the cells with noradrenaline.

Increased intracellular Na+ concentration is necessary to trigger cell division which is initiated by a number of external signals. He will determine if the conductance characteristics of Na+ channels are different in stationary, growing, and transformed cells. For instance, he will characterize channels in FRTL cells growing in medium containing TSH and channels in stationary (non-growing) FRTL cells in medium without TSH.

2.) Lymphocyte Growth Control and Single Ion Channel Physiology (Dr. C. N. Sinback)

Dr. Sinback will also be looking at normal lymphocytes, cells which are too small ever to have had ion flux studies done with intracellular microelectrodes. By using external patch electrodes we could determine if a particular ion channel is opened when the lymphocytes are exposed to mitogens. We also will try to observe what change the ion channels undergo as the primary lymphocytoblasts stop dividing. Information about the ion fluxes in these cells may well show us how to alter the ionic environment so as to maintain continuously dividing cultures of untransformed lymphocytoblasts.

3.) CBSV1A - rat neuroblasts that divide and differentiate in vitro.

These cells that we have been studying for the past two years underwent a spontaneous change which left them heteroploid and unable to differentiate. Frozen stocks were not properly frozen or were made from non-dividing populations. The situation appeared hopeless until, quite recently and by accident, an unindexed cane was found in the freezer containing cells capable of differentiating. We are back in business and are planning to continue efforts to direct the neuron differentiation and to describe the appearance of tetanus toxin binding sites and other indeces of differentiation. These studies are continuing in collaboration with Dr. Suzanne Beckner.

4.) Transfection of Embryonal Carcinoma Cells

By analogy with the successful screen for oncogenes, i.e., purify DNA from a cell that expresses the oncogene and transfect into an indicator cell (3T3), we propose to try to screen for genes that initiate differentiation. By extracting DNA from embryos (where initiator genes are certainly active) and transfecting potential indicator cells (embryonal carcinoma - EC) we hope to see if there are single genes (or closely linked genes) that can initiate a cascade of complex gene expression (differentiation) in EC cells. The existence of such genes which might be called "master genes", can be inferred from the integrated (or coordinated) expression of complex phenotypes when pluripotent cells are caused to differentiate. This phenomenon is seen when 5-aza-cytidine treatment of 10T1/2 cells (a relative of 3T3) causes the appearance of colonies of differentiated muscle, or cartilage, or adipocyte. It also occurs during "transdetermination" of Drosophila imaginal disc cells passaged in larvae. The main question is: can complex differentiated cellular phenotypes like nerve cells or muscle cells be produced by activation of a single gene in a pluripotent embryonic cell? The answer is - very probably yes. But it is likely that not all phenotypes can be started by a single gene acting in the same stage embryonic cell. We may expect to find a limited range of primary transfectants that are perhaps not dramatically differentiated. If we isolate these and re-transfect, then the crucial issues are: do we get strain specific phenotypes among secondary transfectants? and, are any of these recognizable complex cellular differentiations? These experiments, if successful, can then be

repeated with DNA ligated to sequences that can be probed, and ultimately master genes may be isolated and studied further. In spite of the long shot nature of this experiment, it will be fun and probably instructive to look anyway. A modest effort will be made in the rest of FY '83.

5.) Transformed Cell Lines of Preadipocytes that Differentiate in vitro (Dr. Shigeru Yasumoto)

Cellular transformation of differentiating cells by tumor viruses is one alternative experimental system for analyzing the early events of transformation. This approach provides an opportunity to study the biological processes of differentiation as well as those of transformation. Among many cell types, preadipose cells have several advantages for the study of cellular transformation.

We have isolated preadipose cells and transformed them to cell lines by SV40 tsA58 del 2009 which is a conditional mutant for gene A and does not encode a functional small t antigen.

The clonal cell lines established have shown most of the transformed phenotypes. They still retain hormone dependent properties that have been described for adipose differentiation: induction occurs after glucocorticoids and insulin have been added. It has been found that the effect of glucocorticoids was apparently accompanied by a restoration of the loss of growth control in the transformed cell lines.

More recently, secondary transformants (or supertransformants) have been isolated which do not exhibit the hormone responsiveness for either growth regulation or adipose differentiation. We have further analyzed different transformed states of SV40-transformed preadipocytes and found that:

a) The integrated SV40 genomes were markedly amplified in secondary transformants.

b) Accompanying the amplification of SV40 genomes, alterations of gene expression were detected by in vitro translation of isolated poly A+ mRNAs.

c) Significant amounts of mRNA homologuos to v-myc gene sequences were detected in the secondary transformants as revealed by northern hybridization method (in collaboration with Drs. Mushinski and Steve Bauer).

By using this system we shall continue to study the mechanisms of cellular transformation and differentiation.

6.) Continuing our long-term collaboration with Dr. Jay Robbins (D, DCBD) and his colleagues, we have fused the skin fibroblast from a second xeroderma pigmentosum (XP) patient who also demonstrated Cockayne Syndrome with cells from representatives of other XP groups, and those of the only other hitherto known XP/Cockayne Syndrome patient (PoCo). The question was, would the new patient turn out to have the same defect as seen before or would a new complementation group be found. The two XP/Cockayne cell strains showed complementation as did all previously known XP cells complementation groups. Therefore, the cells from the new patient form a new complementation group, termed group H.

7.) In collaboration with Drs. Louis Miller and John Barnwell (I,LPD) we have used the techniques of micromanipulation and cell culture to clone strains of Plasmodium knowlesi malaria. One goal of this work is to attempt to understand how these malarial parasites are able to vary the erythrocyte membrane antigens that the parasites produce in infected cells. Because infections are ordinarily established by injection (by mosquito or by syringe) of large numbers of individual parasites, it could not be known whether variation in erythrocyte cell surface antigens represented successive waves of genetically different parasites growing up or variation of a single genotype to produce multiple phenotypes. What was needed was to establish an infection from a single individual parasite; in short, to clone the organism. This was accomplished by adapting an intracellular microinjection aparatus to remove an erythrocyte infected by a single parasite (under high power phase optics) and ejecting the lone erythrocyte under visual confirmation into a multiwell chamber containing culture medium. By withdrawing the loaded pipette through a silicone oil layer the possibility of adherent (not aspirated) erythrocytes being transmitted was minimized. Immediately after transfer of the single infected erythrocyte an excess of red cells were added that were freshly prepared from the blood of the monkey chosen to host the cloned parasite. The infected erythrocytes were taken at a stage just prior to their maturation. Thus, when the parasites emerged from the transferred erythrocyte they encountered and infected the putative host monkey's erythrocytes. After sufficient time for this first round of reinfection to be established the contents of the well (washed several times with host monkey erythrocytes) were injected (i.v.) into the host monkey. We got 100% success in infection using this technique and no successes without the intervening culture step. Subclonings were performed and the technique now seems firmly established.

Publications:

Moshell, A.N., Ganges, M.B., Lutzner, M.A., Coon, H.G., Barrett, S.F., Dupuy, J.-M. and Robbins, J.H.: A new patient with both xeroderma pigmentosum and Cockayne Syndrome establishes the new XP complementation group H. In UCLA Symposia on Molecular and Cellular Biology, New Series, Vol., 11, Cellular Responses to DNA Damage, New York, Alan R. Liss, 1983.

Barnwell, J.W., Russel, J.H., Coon, H.G., and Miller, L.H.: Splenic requirement for antigenic variation and expression of the variant antigen on the erythrocyte membrane in the cloned Plasmodium knowlesi malaria. Infection and Immunity, in press, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB08950-01 LGN

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Immunochemistry and Genetics of Protein-binding Immunoglobulins

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Sandra Smith-Gill, Expert, Laboratory of Genetics, NCI

COOPERATING UNITS *(if any)*

W. Drohan, Molecular Genetics Group, Meloy Laboratories, VA

LAB/BRANCH

Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Monoclonal antibodies directed against protein antigens are used as probes to study antibody-protein interactions and to study developmentally regulated antigens in normal and neoplastic development. In order to define the complementary structure of an antibody and a protein epitope as precisely as possible, antigenic regions and specific epitopes recognized by monoclonal antibodies to avian lysozyme are mapped by comparing antibody reactivity with related lysozymes, and the antibodies are analyzed structurally by sequencing, crystallography and computer modelling. Structurally and functionally related antibodies are compared to determine genetic mechanisms underlying anti-protein specificity. Experiments are in progress to generate monoclonal antibodies to specific tumor associated antigens, and to onc gene protein products; these antibodies will be used to study these proteins in normal development and in plasmacytoma cells.

PHS 6040 (Rev. 1/83)

GPO 895-100

Anti-lysozyme Monoclonal Antibodies

In order to develop a model system for the study of antibody-protein interactions, a total of 13 monoclonal antibodies have been prepared to hen egg white lysozyme (HEL) by hybridoma technology. Of these, 7 have been characterized in detail, and the others have been partially characterized, with respect to binding specificity and structural characteristics

1. Antigenic regions on lysozyme. The epitope recognized by each monoclonal antibody has been mapped by comparing the reactivity of each antibody with a battery of sequences lysozymes from various avian species, and by comparing the patterns of competition among the antibodies for binding to lysozyme. Each antibody characterized has had a unique pattern of specificity for lysozyme, suggesting that the surface of a globular protein such as lysozyme is recognized as multiple, overlapping epitopes. One region below the catalytic cleft may be immunodominant in BALB/c mice, forming an antigenic region which is recognized as at least 5 distinct but partially overlapping epitopes. Within this antigenic region, two antibodies, HyHEL-8 and HyHEL-10 (each derived independently in two separate fusions) recognize very similar, if not identical, overlapping epitopes.

2. Antibody diversity in the anti-lysozyme response. In collaboration with Dr. S. Rudikoff, N-terminal sequences have been obtained for the heavy and light chains of the characterized hybridomas. These N-terminal sequences have been used to group the heavy and light chains into V-region isotypes. The V_K-V_H pairs used in the anti-lysozyme response are extremely diverse, with a different V_K-V_H pair associated with each different epitope. Only 2 antibodies shared heavy and light chain isotypes, HyHEL-8 and HyHEL-10. This suggests that V_L-V_H restriction may be associated with specific epitopes, as it is for non-protein hapten specificity, such that antibodies binding the same epitope will share structural characteristics as defined by the V_L-V_H pairing, while antibodies recognizing different epitopes, even within the same antigenic region, would have different V_L-V_H isotypes.

3. Structure of antibody-protein interaction. In order to define the complementary structure of an antibody and a protein epitope as precisely as possible, the primary structure of the V regions of the heavy and light chains of selected antibodies are being determined by a combination of protein sequencing, in collaboration with Dr. S. Rudikoff, and be cloning and sequencing the c-DNA from anti-lysozyme hybridomas in collaboration with Dr. W. Drohan, Meloy Laboratories. The complete heavy chain V-regions of HyHEL-8 and HyHEL-10 have been determined, and two-thirds of each of the respective light chains. The complete light chain of HyHEL-5, which binds an entirely independent (non-overlapping) epitope, has also been completed, and the heavy chain V-region sequencing is in progress. The three-dimensional secondary and tertiary structures will be defined, both through compouter modelling and energy minimization in collaboration with M. Potter and C. Mainhart, and through crystallography. In collaboration with Drs. D. Davies and E. Silverton (LMB, NIADKO), the Fab portions of both HyHEL-8 and -10 have been prepared and crystallized individually and in complex with the antigen, lysozyme. The HyHEL-10 complex is currently under study at 4Å resolution. With the three-dimensional model of the antibody determined, and the three dimensional structure of the protein epitope similarly constructed from known coordinates of HEL, modeling of the interaction of the

antibody with the specific epitope can be accomplished.

4. Genetic mechanisms underlying anti-protein specificity. The survey of N-terminal sequences has suggested that binding specificity for specific haptens may be restricted to families of closely related genes coding for both the heavy and light chain V regions. Although HyHEL-8 and HyHEL-10 share both heavy and light chain V isotypes, they each have distinct fine specificities, such that their patterns of reactivities with various variants of lysozyme and of competition with other anti-HEL antibodies are not identical. Furthermore, their isotypes, V_K23-V_H9, are shared by XRPC-25, a DNP-binding myeloma protein which does not bind HEL. The differences in fine-specificity between HyHEL-8 and -10, and in overall specificity among the 3 antibodies must lie in somatic sources of diversity, namely combinational and recombinational diversity resulting from V-J and V-D-J joining, and in somatic mutation. Comparative sequence data on the 3 heavy chains shows that HyHEL-8 and -10 share the same D and J, and have the same length of CDR-3, while XRPC-25 heavy chain has a different D and J, and the length of CDR-3 differs from that of the anti-HEL proteins. The data to date is consistent with the hypothesis that the overall specificity for hapten versus protein is determined by the D and J in CDR-3, while fine specificity may be a function of somatic mutation. This would be the first actual demonstration of functional significance for either of these sources of structural diversity.

These hypotheses are currently being tested by several approaches. First, anti-idiotypic antibodies specific for the V_K23-V_H9 pair are being generated to be used to enhance the expression of this idiotype by low-dose immunization, thereby increasing the sample size of available antibodies for structure-function studies. In addition, chain recombination experiments among XRPC-25, HyHEL-8, HyHEL-10 and other anti-HEL antibodies are in progress in our laboratory and in collaboration with Dr. K. Dorrington, Toronto University, to determine functional correlates of the various structural variations among closely related heavy and light chains, as well as the kinetics of heavy and light chain pairing. Finally, the rearranged V_K23 and V_H9 genes are being cloned from HyHEL-10 in collaboration with T. Lavoie and with Drs. W. Drohan and G. Ricca, Meloy Laboratories. These will be sequenced to allow comparison of the sequences of the expressed genes with the germline genes in order to assess the amount of somatic mutation. In addition, attempts will be made to use these genes for in vitro expression and selective modification by genetic splicing mechanisms to directly test hypotheses concerning structure-function relationships.

5. Use of monoclonal antibodies to probe lysozyme function. The monoclonal antibodies to HEL whose epitopes have been mapped are being used to probe function of lysozyme, in collaborative studies with M. Pincus, Columbia College of Surgeons and Physicians, H.A. Sheraga, Cornell University, and J. Rupley, University of Arizona. Competition experiments with oligosaccharide substrates have helped elucidate details of kinetics of substrate binding. Monoclonal antibodies are also being used to examine the kinetics of protein refolding. Both sets of experiments are expected to provide additional insight into the details of protein-antibody interactions, as well as into the function of lysozyme as an enzyme.

Monoclonal Antibodies to Ovomucin

In collaboration with J. Markley, Purdue University, studies have been initiated to develop ovomucin as a model protein antigen to study antibody-protein interactions in parallel studies to those on lysozyme. Ovomucin has been extensively characterized as a serine protease inhibitor, but less so as an inhibitor. It is known to be a primary component in the allergic response to egg white proteins, however, and as such has been demonstrated to experimentally induce an IgE response in mice. We have immunized BALB/c mice with the intact ovomucin protein from turkey egg white, and have detected a significant primary and secondary antibody response to both the entire protein and to the isolated third domain of the protein. Spleens from these mice are being used to produce hybridomas which will be screened against the third domain of turkey ovomucin, and then characterized using 34 sequenced evolutionary variants of the third domain. Because ovomucin differs considerably in its physiochemical properties from lysozyme, this additional protein antigen will provide an important parallel model system in which to test hypotheses generated from the anti-lysozyme system concerning structure and function of anti-protein antibodies.

Monoclonal Antibodies to Tumor Antigens

Experiments are in progress to generate monoclonal antibodies which will be utilized as probes to study the role of tumor-associated protein antigens in normal and neoplastic development. Synthetic peptides made from the sequence of the mouse myc oncogene have been obtained from P. Reddy. These peptides have been conjugated to KLH and have been used to immunize BALB/c mice in anticipation of producing anti-myc hybridomas. These monoclonal antibodies will be used as probes to attempt to identify and purify the mouse myc protein product from plasmacytoma cells known to express c-myc mRNA. In addition, the antibodies will be used to examine potential expression of the normal myc product in normal morphogenesis using immunohistochemical methods.

Production and Characterization of a Monoclonal Antibody to Bovine β-Crystallin

In collaboration with D. Carper, NEI, a monoclonal antibody to a bovine lens 27K β-crystallin polypeptidde has been produced from a rat x mouse hybridoma. The antibody reacts predominantly with the 27K polypeptide, and cross-reacts somewhat with 23K and 20K polypeptides in bovine, mouse, rat, monkey, and human lenses. The antibody does not react with any of the other major bovine β-crystallin polypeptides but does recognize a large number of native β-crystallin proteins. This antibody is being utilized as a probe to study the role of this crystallin in normal lens development and in cataractogenesis.

Publications

Smith-Gill, S.J.: Developmental plasticity: Developmental plasticity versus phenotypic modulation. Amer. Zool. 23: 47-56, 1983.

Smith-Gill, S.J.: Ontogeny and phylogeny of vertebrate gut associated lymphoid tissue. Accepted for publication, Develop. Compar. Immunol.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB08951-01 LGN

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Proteins Associated with the Biological Effects of Murine Leukemia Viruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
S.K. Ruscetti, Senior Staff Fellow, Laboratory of Genetics, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Genetics

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	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 spleen focus-forming virus (SFFV) and Friend mink cell focus-inducing virus (Fr-MCF) both induce eythroleukemia in susceptible strains of mice. Studies are being carried out to determine how these highly related viruses specifically interfere with erythropoiesis. Nucleotide sequencing of the SFFV envelope gene and comparison with the envelope genes of various ecotropic and MCF viruses has revealed a large deletion and two insertions that may account for some of the unusual properties of its product. Certain strains of mice are resistant to erythroleukemia induced by Fr-MCF virus. Since these mice endogenously express a novel MCF-virus-related glycoprotein, we have proposed that it confers resistance by preventing the replication and spread of MCF viruses. Genetic mapping studies are in progress to determine the number and location of the gene(s) conferring this resistance. Erythroleukemia cell lines derived from mice infected with either Friend MuLV or SFFV were found to express high levels of a transformation-related protein, p53, and monoclonal antibody to p53 was found to co-precipitate a 70K protein in these cells. Studies are currently being carried out with these cells to determine if increased levels of any onc genes can be detected and to characterize the 70K co-precipitating protein.

Project Description

Objectives:

(1) To localize the regions of the spleen focus-forming and Friend MCF viruses involved in the induction of erythroleukemia.

(2) To determine the mechanisms by which the spleen focus-forming and Friend MCF viruses alter erythropoiesis.

(3) To determine the etiology of the second stage diseases induced by the spleen focus-forming and Friend MCF viruses.

(4) To further understand the mechanisms of resistance of mice to diseases induced by murine leukemia viruses.

Methods Employed:

(1) Preparation of specific antisera to viral-encoded proteins, particularly the preparation of monoclonal antibodies.

(2) Use of specific antisera to precipitate proteins from cytoplasmic extracts of metabolically labeled virus-infected cells and tissues and analysis of immune precipitates by SDS-polyacrylamide gel electrophoresis and autoradiography.

(3) Analysis of viral protein expression in individual cells by immunofluorescence.

(4) Development of *in vitro* assays to study erythropoiesis.

(5) Injection of various strains and genetic crosses of mice with murine leukemia viruses and analysis for development of leukemia.

(6) Molecular techniques, including molecular cloning and nucleotide sequencing.

Major Findings:

(1) Normal DBA/2 mouse cells synthesize a glycoprotein which interferes with MCF virus infection.

We previously demonstrated that MCF viruses play a crucial role in the generation of erythroleukemia after injection of certain strains of newborn mice with ecotropic Friend MuLV and that resistance to this disease is mediated through the endogenous expression of an MCF/xeno-gp70-related protein that interferes with the replication and spread of MCF viruses. In order to test the possibility that this novel envelope protein is responsible for resistance to F-MuLV-induced disease by a mechanism analogous to viral interference, studies were carried out in collaboration with Dr. R. Bassin, NCI. It was shown that DBA/2 embryo

fibroblast lines are resistant to infection with MCF viruses in vitro. Treatment of these cells with inhibitors of glycosylation, such as 2-deoxy-D-glucose and tunicamycin, which have recently been shown to drastically reduce viral interference in MuLV-producing fibroblasts, rendered these cells significantly more susceptible to infection with several MCF isolates. These drugs were shown by pulse-labeling and immune precipitation studies to effect the addition of sugar moieties to the MCF/xeno-related envelope glycoprotein expressed in the DBA/2 fibroblasts, and cell surface labeling followed by immune precipitation indicated that the expression of this protein at the cell surface was altered. These data indicate that the endogenous expression of MCF/xeno-related envelope glycoproteins in DBA/2 cells can confer on these cell resistance to MCF infection by a mechanism analogous to viral interference. This mechanism may be the basis for the resistance not only to Friend MCF-induced disease but also to other leukemias that involve the generation and spread of MCF viruses.

(2) Genetic mapping of F-MuLV resistance gene(s)

In collaboration with Dr. M. Potter of this laboratory, an attempt has been made to map the gene(s) responsible for resistance of DBA/2 mice to F-MuLV-induced erythroleukemia. Newborn BALB/c mice congenic for various DBA/2 chromosomes were injected with F-MuLV and observed for the development of disease. Of 11 different congenic strains studied to date, 10 were found to be susceptible to disease. We are further investigating the genetic background of the one resistant strain as well as testing other congenic strains. Since earlier studies were inconclusive in determining the precise number of genes involved in the disease resistance, a large number of newborn (BALB/c x DBA/2)F_1 x DBA/2 backcross mice have been injected with F-MuLV and are currently being observed for development of disease.

(3) Localization of the pathogenic functions of F-MuLV and Friend MCF viruses to the env gene.

Studies carried out in collaboration of Dr. A. Oliff, Memorial Sloan Kettering Cancer Center, New York, N.Y., have attempted to localize the pathogenic functions of F-MuLV and Friend MCF virus to the env gene. This was done by molecularly cloning these viruses and generating novel recombinant viruses by co-transfection with the DNA from molecularly cloned amphotropic virus 4070. Viral proteins encoded by these hybrid viruses were characterized by immune precipitation with specific antisera and could be classified as being encoded by either F-MuLV or Friend MCF virus versus amphotropic virus. Animal studies with the F-MuLV/amphotropic virus recombinants have indicated that it is the F-MuLV env gene which contains the sequences required for disease. Animal studies are in progress to assess the pathogenicity of the Friend MCF/amphotropic virus recombinants and thus identify which Friend MCF env sequences are required for disease.

(4) Erythroleukemia cell lines derived from mice infected with F-MuLV or SFFV express high levels of a transformation-related protein.

It has been suggested that the erythroleukemias induced either by SFFV or Friend MuLV can be divided into two stages. Stage 1 is characterized by the rapid hyperplasia of erythroid precursor cells induced by the virus

infection. These cells have a limited proliferative capacity and are not transplantable. Stage 2 is characterized by the appearance of transplantable malignant erythroblasts that can be grown as permanent cell lines in culture. In an effort to determine whether biochemical differences could be found in cells obtained from these different stages, they were examined for the expression of a transformation-related protein, p53. This protein has been detected in a range of neoplastic cell types that were sponaneously transformed or transformed by viruses, chemicals or X-rays. When stage 2 cell lines derived from newborn mice infected with F-MuLV or adult mice infected with SFFV were pulse-labeled and immune precipitated with a monoclonal antibody to p53, high levels of this protein were detected. No p53 could be detected in stage 1 spleens from mice infected 2-4 weeks previously with F-MuLV or SFFV or in erythroid cell lines derived by in vitro infection of bone marrow cells with SFFV. The p53 in the erythroleukemia cell lines is phosphorylated and is expressed in cells in both stationary and logarithmic phases of the growth cycle. When these lines are induced to differentiate into mature red blood cells, p53 levels are unchanged. Monoclonal antibody to p53 was found to co-precipitate a 70,000 dalton protein in these erythroleukemia cell lines. These results indicate that p53 may be a marker for transformed erythroblasts present in the second stage diseases induced by F-MuLV or SFFV.

(5) Nucleotide sequencing of the SFFV envelope gene

A nucleotide sequence was determined for the envelope gene of the polycythemia strain of the Friend spleen focus-forming virus and from this the amino acid sequence of its gene product, gp52, was deduced. All major elements of the gene were found to be related to genes of other retroviruses that code for functional glycoproteins. Although the carboxy-terminus of gp52 is encoded by sequences highly related to sequences in its putative parent, ecotropic Friend MuLV, the majority of the protein (65%), including the amino-terminus, is encoded by MCF-like viral sequences. Nucleotide sequence comparisons suggest that the non-ecotropic region may be more closely related to the 5' substitution in MCF viruses than it is to the 5' end of xenotropic virus envelope genes. A large deletion and two unique insertions have been located in the $SFFV_P$ envelope gene and may account for some of the unusual structural characteristics, aberrant processing, and/or pathogenic properties of gp52. As a consequence of the deletion, amino-terminal gp70 and carboxy-terminal p15E coding sequences are juxaposed and it appears that translation from the p15E region, 3' to the deletion, continues in the standard reading frame used by other retroviruses. Insertions of 6 base pairs and 1 base pair at the very 3' end of the gp52 coding region result in a SFFV-unique sequence including 10 amino acids and a premature termination codon.

(6) Further biochemical analysis of the SFFV gp52

Based upon nucleotide sequences data, which demonstrated that sequences encoding the p15E region of the MuLV envelope gene were present in SFFV, we have made an attempt to detect p15E determinants on gp52. Several polyclonal antibody preparations as well as a number of monoclonal antibodies to p15E failed to precipitate gp52. However, gp52 was found by tryptic peptide analysis to contain a single spot which corresponds to one of the two spots detected in p15E, but not gp70. This data indicates that gp52 contains p15E peptides which are either non-immunogenic or are not available, due to folding of the protein, for antibody

binding. In addition, gp52 failed to be precipitated by a polyclonal antibody preparation to the R peptide, a peptide encoded by sequences present at the C-terminus of the envelope gene. This data is consistent with nucleotide sequencing data which indicated that the p15E sequence in the SFFV envelope gene terminates before the sequence encoding the R peptide.

(7) Identification of an SFFV in erythroleukemic mice infected with a wild mouse ecotropic MuLV.

In collaboration with Drs. W. Langdon and S. Morse, NIAID, and Dr. P. Hoffman, U. of Md., Baltimore, a new virus was isolated from the spleens of erythroleukemic NFS/N mice that had been inoculated at birth with an ecotropic MuLV obtained from wild mice. This virus was shown to be a replication defective virus which encodes a 50,000 dalton glycoprotein that is highly related to the gp52 encoded by the Friend strain of SFFV. The defective virus has been biologically cloned free of other viruses in the original stock and induces the same disease as the complex when pseudotyped with any helper virus. Additional studies carried out in collaboration with others at NIAID and NCI have shown that this new virus is highly related to SFFV at the RNA level, by its in vitro effects on erythroid burst formation, and by its inability to cause disease in Fv-$2^{r/r}$ mice, which are resistant to SFFV. It is, however, biochemicaally distinct from all Friend SFFVs and appears to represent the fourth independent isolate of an MuLV with spleen focus-forming activity.

(8) Identification of viral proteins in pristane-induced mouse plasmacytomas.

In collaboration with Dr. M. Potter of this laboratory, studies were undertaken to determine if MuLV's, particularly MCF viruses, are involved in the development of pristane-induced mouse plasmacytomas. 12 individual plasmacytomas were directly pulse-labeled and compared with normal peritoneal exudate cells for the expression of ecotropic and MCF viral envelope glycoproteins. 9 out of 12 plasmacytomas were found to express ecotropic envelope glycoproteins, and 10 out of 12 were found to express MCF-like envelope glycoproteins. No viral proteins could be detected in normal peritoneal exudate cells. Ecotropic virus has been isolated from several of the plasmacytomas, but after a number of attempts, no MCF viruses were isolated, suggesting that the latter viruses may be defective.

Significance to Biomedical Research and the Program of the Institute:

Understanding the proteins that are responsible for the biological effects of RNA tumor viruses is of great importance. The erythroleukemias induced in mice by the spleen focus-forming virus and Friend MuLV are associated with the expression of specific viral proteins which are products of the spleen focus-forming virus and a generated recombinant MCF virus, respectively. It is hoped that continued study of the products of mouse RNA leukemia viruses will help us to better understand the development of leukemia and give us insights into how to control it. Using reagents and information gained from studying these model systems may ultimately help us to develop immunoassays which would

detect cross-reacting leukemia-specific proteins in primate species such as man. In addition, the study of the mechanisms of resistance to virus-induced leukemia in the mouse will also have relevance for the treatment of human leukemia.

Proposed Course:

(A) Studies to determine the mechanisms by which the spleen focus-forming and Friend MCF viruses alter erythropoiesis:

Data to date have indicated that the spleen focus-forming virus alters the sensitivity of erythroid precursor cells to the hormone erythropoietin (EPO). Since our molecular studies have indicated that the envelope protein of this virus, gp52, is the crucial element for biological activity, studies are being planned to understand how this protein modifies erythropoiesis. We propose that the protein, which can be detected in the cytoplasm and the plasma membrane, mimics the receptor for EPO. Thus, cells infected with SFFV will express more "EPO-receptors" and will thus be more effective than uninfected hematopoietic cells in binding small amounts of EPO present in serum. It has been observed that the polycythemia-inducing and anemia-inducing strains of SFFV, which differ in their sensitivity to EPO, differ in the processing of their envelope glycoproteins and consequently the amount that can be detected at the plasma membrane. Since an equal amount of gp52 can be detected in the cytoplasm, this data suggests that, if our hypothesis is correct, the receptor for EPO is at the cell surface. Using monoclonal antibodies to gp52 and its cross-reacting protein, MCF gp70, as well as purified SFFV gp52, we plan to test the hypothesis that a product of a murine leukemia virus, SFFV gp52, can alter the response of a cell, an erythroid precursor, to its hormone, erythropoietin. Using a protocol including the rat myeloma line Y3-Ag1.2.3 and spleen cells from rats immunized with Fr-MCF-infected cells, we have been successful in preparing a number of monoclonal antibodies to MCF gp70 which will be useful for these purposes.

Studies carried out at the molecular level comparing the anemia and polycythemia-inducing strains of SFFV may also give clues into the mechanisms by which these viruses interfere with erythropoiesis. Both of these viruses have been molecularly cloned and experiments have been initiated to engineer viruses that contain portions of each envelope gene and to obtain mutant viruses which will help us to localize the areas within the envelope gene that are involved in altering hormone sensitivity. Sequencing of the envelope gene of the polycythemia strain of SFFV has been carried out and we have begun to sequence the envelope gene of the anemia-inducing strain of SFFV.

No in vitro assays have been developed to date which indicate how Friend MCF virus, a virus very closely related to SFFV, interferes with erythropoiesis. The fact that the animals infected with this virus, or its ecotropic parent, F-MuLV, become profoundly anemic suggests that Friend MCF virus may in some way block erythropoiesis. As with SFFV, the envelope glycoprotein of this virus, gp70, has been proposed as the crucial element for the biological activity of this virus. We propose that MCF gp70, which cross-reacts with SFFV gp52, also mimics the receptor for EPO. However, since MCF gp70, unlike SFFV gp52, can leave the cell as part of the virion, we propose that it binds to EPO before it can reach the EPO receptors on the cell surface. Thus, hematopoietic cells infected with Friend MCF virus would be "blocked" from differentiating into mature red blood cells due to neutralization of EPO by the viral envelope glyco-

protein. This hypothesis will be tested first by developing a good in vitro assay for studying erythropoiesis (the technique described recently by Dexter et. al. has been working in our laboratory) and showing how Friend MCF virus alters erythropoiesis in this assay. We also plan to test this hypothesis using monoclonal antibodies to MCF gp70 (and cross-reacting SFFV gp52) and purified MCF gp70 to determine if EPO can be neutralized by the product of a murine leukemia virus.

Our studies can be extended to other murine leukemia viruses, particularly those that have MCF virus intermediates. For example, AKR MCF virus may lead to thymomas by altering the response of thymocyes to T-cell growth factor (TCGF); viruses which cause myeloid leukemias may modify the response of a cell to any of the many factors involved in proliferation and differentiation along the myeloid pathway.

Also along these lines, we are interested in the target cell specificity of MCF viruses. Preliminary data from our laboratory indicate that a variety of MCF viruses (Friend, Moloney, AKR) have the ability to infect erythroid precursor cells, yet only Friend MCF virus can interfere with normal erythropoiesis. We plan similar experiments to determine if the various MCF viruses can infect T-cells and B-cells. If so, the data would suggest that target cell specificity is not due to types of hematopoietic cells. Instead, the data suggest that target cell specificity resides in the cell itself, i.e. a substrate with which the viral protein must interact in order to modify the proliferation and growth of that particular cell.

(B) Studies on the etiology of the second stage diseases induced by the spleen focus-forming and Friend MCF viruses:

Our results indicate that p53 may be a marker for transformed erythroblasts present in the second stage of diseases induced by F-MuLV or FVP. Since high levels of p53 are associated with transformation by a variety of oncogenes, this result could indicate that a cellular oncogene has been activated in hematopoietic cells of mice infected with F-MuLV or FVP and is responsible for the transformed phenotype of the stage 2 spleen cells. It has been proposed that lymphomas induced in birds by the avian leukosis viruses are the result of the promotion of the transcription of a particular cellular oncogene, and such a model may explain the stage 2 transplantable cell lines from F-MuLV or FVP-infected mice. In studies carried out in collaboration with Dr. A. Oliff, Memorial Sloan-Kettering Cancer Center, N.Y., none of twelve known onc genes were found to be expressed in cell lines derived from stage 2 spleens nor were any new RNA species detected using a probe for the viral LTR. Additional studies along these lines are planned to further investigate this model, including transfection of high molecular weight DNA into NIH 3T3 as well as hematopoietic cells. Also, non-erythroid cell lines derived from mice infected with F-MuLV, transplantable cell lines derived from leukemias induced by other murine leukemia viruses, and human leukemia cell lines will be examined for expression of p53.

Monoclonal antibody to p53 was found to co-precipitate a 70K protein from stage 2 erythroleukemia cell lines. Efforts are being made to determine if this protein is specifically complexed with p53 and to further characterize it. If such a protein plays an important role in erythroid cell differentiation, bind-

ing of p53 to it could modify its biological function and have profound effects on erythroid differentiation. Alternatively, binding of the 70K protein to p53 could stabilize p53 and extend its half life, altering the effects of p53 on the cell.

(C) Studies on the mechanisms of resistance of mice to diseases induced by murine leukemia viruses:

(1) Resistance of certain strains of mice to erythroleukemia induced by F-MuLV:

Data accumulated in this laboratory are consistent with the hypothesis that MCF viruses play an important role in the generation of erythroleukemia after injection of certain strains of newborn mice with ecotropic Friend MuLV and that resistance to this disease is mediated through the endogenous expression of an MCF/xeno-gp70-related protein that interferes with the replication and spread of MCF viruses. Additional studies are being planned in order to further understand the basis for this resistance. This will include (a) genetic studies to further document the association between resistance and the expression of endogenous viral proteins as well as to determine the number and location of genes involved in this resistance; (b) studies to determine if this resistance gene can be activated in susceptible cells by agents such as azacytidine and IudR; (c) studies to determine how this resistance can be overcome; (d) studies to molecularly clone this endogenous envelope gene and the adjacent cellular sequences and determine its biological effects; and (e) studies to biochemically compare the endogenous MCF-related protein involved in resistance with the envelope protein present in Friend MCF virus to determine why the later protein is pathogenic while the former protein can block MCF viral receptors on the cell but is not pathogenic.

(2) Studies on the resistance to leukemia of susceptible strains of mice with age:

Data generated in this laboratory have indicated that the increasing resistance with age of NIH Swiss mice to F-MuLV-induced disease is due to the failure to replicate MCF viruses due to either a lack of a suitable number of target cells into which the virus can integrate or an effective immune response against the MCF viral envelope. These studies will be extended to determine if differences in the number of target cells can be found in newborn versus adult mice and whether soluble factors, such as those recently described in Fv-2^r mice, effecting the cycling of the target cells in the adult mouse can be found. Also, the role of natural killer cells in the resistance of adult mice to F-MuLV-induced disease will be investigated.

(D) Studies to localize the regions of the SFFV and Friend MCF envelope genes involved in the induction of erythroleukemia.

Studies will be continued at the molecular level to precisely localize the areas of the envelope gene of SFFV and Friend MCF virus that are involved in pathogenicity, phenotype and target cell specificity. This will be done by preparing recombinant viruses, by inserting specific mutations and by comparing wild type viruses with variants of these viruses that may or may not differ in

pathogenicity.

(E) Studies of mouse plasmacytomas.

Studies will be continued to determine the role of ecotropic and/or MCF viruses in the development of pristane-induced mouse plasmacytomas. Further attempts will be made to isolate MCF viruses from these plasmacytomas, including defective MCF viruses, and the oncogenicity of these MCF viruses, along with the ecotropic viruses isolated from plasmacytomas and combinations of the two, will be tested in BALB/c mice.

Publications:

Wolff, L., Koller, R. and Ruscetti, S.: Monoclonal antibody to spleen focus-forming virus-encoded gp52 provides a probe for the amino-terminal region of retroviral envelope proteins that confers dual/xenotropism. J. Virol. 43: 472-481, 1982.

Anderson, S.J., Furth, M., Wolff, L., Ruscetti, S.K. and Sherr, C.J.: Monoclonal antibodies to the transformation-specific glycoprotein encoded by the feline retroviral oncogene v-fms. J. Virol. 44: 696-702, 1982.

Anderson, S.J., Furth, M., Wolff, L., Ruscetti, S.K. and Sherr, C.J.: Prepartion of rat monoclonal antibodies to epitopes encoded by the viral oncogene (v-fms) of McDonough feline sarcoma virus. J. Cell. Biochem. 19: 275-280, 1982.

Bassin, R.H., Ruscetti, S., Ali, I., Haapala, D.K. and Rein, A.: Normal DBA/2 mouse cells synthesize a glycoprotein which interferes with MCF virus infection. Virology 123: 139-151, 1982.

Kaminchik, J., Hankins, W.D., Ruscetti, S.K., Linemeyer, D.L. and Scolnick, E.M.: Molecular cloning of biologically active proviral DNA of the anemia-inducing strain of spleen focus-forming virus ($SFFV_A$). J. Virol. 44: 922-931, 1982.

Langdon, W.Y., Hoffman, P.M., Silver, J.E., Buckler, C.E., Hartley, J.W., Ruscetti, S.K. and Morse, H.C: Identification of a spleen focus-forming virus in erythroleukemic mice infected with a wild-mouse ecotropic murine leukemia virus. J. Virol. 46: 230-238, 1983.

Vedbrat, S.S., Rasheed, S., Lutz, H., Gonda, M.A., Ruscetti, S., Gardner, M.B. and Prensky, W.: Feline oncornavirus-associated cell membrane antigen: a viral and not a cellularly coded transformation-specific antigen of cat lymphomas. Virology 124: 445-461, 1983.

Ruscetti, S.K. and Scolnick, E.M.: Expression of a transformation-related protein (p53) in the malignant stage of Friend virus-induced disease. J. Virol., in press.

Oliff, A. and Ruscetti, S.: A 2.4 kbp fragment of the F-MuLV genome contains the sequences responsible for F-MuLV-induced erythroleukemia. J. Virol., in press.

ANNUAL REPORT OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1982 through September 30, 1983

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 and a high incidence of neoplasia as well as in patients with malignancy especially those with 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 immunoglobulin genes and their rearrangements and deletions that are involved in the control of immunoglobulin synthesis, 2) the genetic control of the immune response especially as related to immune response genes associated with the major histocompatibility complex, 3) identification of unique cell surface determinants especially receptors for growth factors on subpopulations of lymphoid cells with different functional capabilities using antibodies developed with hybridoma technology, 4) 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 in patients with immune dysfunction. 5) studies of the immune response including the generation of specific antibodies and cytotoxic cells to viruses, 6) the isolation and characterization of biological modifiers that suppress the human immune response that are produced in vivo or by T cell lines and T-T cell hybridomas. The second major goal of the Metabolism Branch is to determine the physiologial and biochemical effects that a tumor produces on the Metabolism of the host. Both patients with neoplastic disease 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 special emphasis is laid on the biochemcial events accompanying normal growth and hormonal control of this growth as it relates to our understanding of malignant growth and on the regulatory role played by amino acids and on the metabolism of porphyrins.

THE ARRANGEMENT OF IMMUNOGLOBULIN GENES AND THEIR REARRANGEMENT THAT CONTROL IMMUNOGLOBULIN SYNTHESIS

The human immunoglobulin light chain genes in their embryonic or germline state are organized in a discontinuous system of multiple germline variable (V_L) regions, alternative joining (J_L) segments and a single or even multiple constant (C_L) regions. Heavy chains are similarly organized but have an additional diversity (D_H) segment incorporated between the V_H and J_H regions. During the process of differentiation of a stem cell into a mature B cell there is a rearrangement of the cellular genome to combine a single (V_H) and a single (D_H) and a single (J_H) to form an active heavy

chain gene and the combination of a single light chain variable region (V_K or V_L) with with an appropriate J_K or J_L region to activate a light chain gene. ^{32}P-labelled clones of human constant, joining, diversity and variable region genes were used as probes to study the gene arrangements in B cell, T cell and non-T, non-B forms of lymphocytic leukemias or cell lines. All B cell leukemias have rearranged the immunoglobulin genes of at least one set of heavy chain genes and one set of light chain genes. In addition, in many cases aberrant rearrangements of other light and heavy chain genes may be present. In contrast, human hematopoietic cells and T cells pursing non-B cell pathways of development ususally retained their immunoglobulin genes in the germline configuration. Thus immunoglobulin gene rearrangements would place cells in the B cell lineage. A series of leukemias of controversial origin were analyzed with the immunoglobulin gene probes to define their origin in order to accurately categorize these malignancies. All eight cases of Hairy cell leukemia examined had heavy chain gene rearrangements when examined with the J_H probe probe. All these cells also had a kappa or lambda chain gene rearrangement. The appropriate light chain RNA was demonstrable in these cells dispalying surface kappa or lambda chains. Thus Hairy cell leukemia is a malignancy is a malignancy of mature B cells committed to immunoglobulin production at the immunoglobulin gene level. Immunoglobulin genes were also examined in the cells of patients with the chronic myelogenous leukemia and chronic myelogenous leukemia in blast crises. In the chronic granulocytic phase or in myloid blast crisis the immunoglobulin genes remained in the germline configuration. In contrast in virtually all cases of lymphoid blast crisis there was a rearrangement of the heavy chain genes and in three of eight cases of the light chain genes as well. Thus in most cases lymphoid blast crises in patients with chronic myelogenous leukemia reflects a B cell precursor form of leukemia. In certain cases serial examination of a single patient revealed lymphoid blast crises with different clonal patterns of immunoglobulin gene rearrangement showing that the stem cell which represents the site of the malignant transformation in chronic myelogenous leukemia has the capacity to mature along different pathways including different lineages of B cell development. Since all of the immunoglobulin gene reorganization and regulatory events appear to be completed by the time B cells are synthesized we have examined acute non-T, non-B lymphocytic leukemia, a leukemia that consists of cells at early stages of differentiation. Twelve of the 37 patients with acute lymphocytic leukemia displayed T cell associated antigens and had germline light chain genes and heavy chain genes. In contrast, all 25 cases of "non-T, non-B" ALL, which lacked definitive T cell markers and surface immunoglobulin had rearranged heavy chain genes and in 11 cases had progressed to light chain gene rearrangements. The observations of patients with heavy chain gene rearrangements without light chain gene rearrangements but the failure to observe any patients with light chain gene rearrangements who did not also have heavy chain gene rearrangements supports the view that heavy chain gene rearrangements preceed those of light chains. Furthermore, the obsevation that seven leukemias had aberrant rearrangements of the kappa light chain genes or deletions of the kappa light chain genes with the lambda genes remaining in the germline configuration whereas there were no cases where there were aberrant lambda rearrangements in a kappa producing cell supports our view that kappa gene rearrangements and deletions preceed those of lambda light genes. That is imunoglobulin gene rearrangements preceed in hierarchical order with heavy chain genes rearrangements preceeding light chain and

with kappa gene rearrangements or deletions preceeding those of lambda light chain genes. Despite the fact that all 25 cases of common acute lymphocytic leukemia had heavy chain gene rearrangments and that 11 of these 25 had light chain gene rearrangements as well, only 5 produced mu chains, one produced gamma chains and one produced lambda light chains in the cytoplams. In some of the 19 cases that did not produce immunoglobulin heavy chains there had been rearrangements of both sets of heavy chain genes. Those cells that have had an aberrant V, D, J aberrant on both of the 14th chromosomes would have deleted the residual D regions as well as the signal flanking hepta- and nonanucleotides required for immunoglobulin gene reorganization. Thus, these cells may not have the residual genetic D-region material nor the important flanking signals for gene reorganization that are required to reproduce an immunoglobulin molecule and may thus, be frozen in the B cell precursor stage of maturation as a result of these aberrant rearrangements. This would explain the failure of maturation of B cell precursors in certain patients with acute B cell precursor lymphocytic leukemia.

In addition, studies by Korsmeyer and coworkers clonal populations of B cells populations of B cells, B cell precursors and plasma cells were shown to have specific individual immunoglobulin gene rearrangements. They have shown that the detection of such rearrangements by the identification of a new band on Southern hybridization blots with immunoglobulin gene probes provides a sensitive marker for clonality and B cell lineage within lymphoid tissue lacking expresssion of diffinitive surface phenotypes. He has utilized these genetic markers to: (1) establish a diagnosis of lymphoma in a malignancy of uncertain type, (2) show that some lymphomas previously classified as of T cell type in fact contain monoclonal B cells, (3) detect clonal B cell populations within lymphomatous tissues of undcertain immmunotype and with an atypical lymphoreticular hyperplasia having no other clonal surface markers. These sensitive and unique indicators of clonality located directly at the DNA level are capable of providing insights into the cellular origin and aid in the early detection of neoplasia.

GENETIC CONTROL OF THE IMMUNE RESPONSE

The critical importance of genetic factors in regulating the immune response, and especially of genes which appear to be specific for particular antigens (so-called immune response or Ir genes), has become increasingly apparent in the last 15 years. However, the mechanism of action of these genes and the nature of the product encoded by them remain unknown. Most of the Ir genes so far described are linked to the major transplantation or histocompatibility gene complex. The primary objective of this project has been to define the mechanism by which Ir genes linked to these major transplantation antigens (HLA in man and H-2 in mice) function with the hope of understanding the modes of recognition involved at this regulatory level, the cells which participate and the cell membrane receptors which mediate the regulation.

To this end, Dr. Berzofsky has been using myoglobin as a well-defined model protein antigen. He had previously shown that there are two Ir genes mapping in different subregions (I-A and I-C) of the H-2 complex which control the murine immune response to different determinants (antigenic sites or epitopes) on the same myoglobin molecule. Also, these same genes operate in

the antibody response both in vivo and in vitro, and control T cell interactions with both macrophages and B lymphocytes.

In the current studies Dr. Berzofsky has approached the genetic regulation and the receptors involved from several converging perspectives. First, he examined the specificity of B10-D2 high responder H-2^d T cells for a series of myoglobins and localized an immunodominant determinant, i.e. a site for which the majority of T cells are specific, around residue Glu 109 The response to the Glu 109 site mapped to the I-A subregion. The site recognized by T cells is different from the sites recognized by antibodies that have been described.

This observation is important in planning synthetic peptides as vaccines, as a peptide which elicits antibodies but does not immunize helper T cells which will recognize the native molecule may not lead to an anamnestic response when the individual is later challenged with the whole molecule, as during infection. To produce protective immunity, synthetic peptides should be chosen which include a major helper T cell recognition site as well as antibody recognition sites.

To further explore T cell recognition with homogenous populations of cells, we prepared and cloned long-term cultured T cell lines from the high responder H-2^d mice. One group of clones was specific for the immunodominant site around Glu 109, and all of these clones recognized myoglobin only when presented on macrophages bearing I-A^d. A second group of clones was isolated which were specific for Lys 140, and these required myoglobin presentation by cells bearing I-E^d. Thus there was a strong correlation between specificity for Glu 109 vs. Lys 140 and genetic restriction to I-A or I-E. This may reflect the regulatory role of these Ia molecules in determinant selection, i.e. selective presentation of different antigenic sites.

The Lys 140-specific T cell clones allowed Dr. Berzofsky to attempt to block T cell proliferation with antibody to the antigen, something which had previously failed in many laboratories. Dr. Berzofsky had previously described a monoclonal antibody to myoglobin with the identical specificity pattern implicating Lys 140, and thus was in the unique position of having a high affintiy monocloanl antibody and a homogeneous clone of T cells both specific for the same site. In this special situaiton, he achieved specific blocking with a slight molar excess of antibody over antigen. Thus, the complex hypotheses proposed to explain the wide-spread inability to obtain blocking are unnecessary. Rather, the problem may have been due to the phenomenon noted above, that most T cells were specific for sites distinct from those recognized by most serum antibodies.

Using these Lys 140-specific T cell clones also allowed Dr. Berzofsky to test the necessity for antigen cleavage during so-called "processing" for antigen presentation. These clones respond as well to the fragment (132-153) which contains Lys 140 as they do to the native myoglobin. However, he found that inhibitors of lysozomal proteases or of intracellular transport inhibited presentation only of native myoglobin, not of the fragment, to the same T cell clone. Thus, the requirements for "processing" a large protein are different from those for a small peptide. This result supports the notion that large proteins require fragmentation for T cell recognition. The mechanism of antigen handling by macrophages in presentation of the

antigen to T cells is becoming widely recognized as an important site of immune regulation.

Another hypothesis by Dr. Berzofsky and by Dr. Eli Sercarz is that helper T cells specific for one site on an antigen molecule will selectively help B cells specific for particular sites on that antigen and not others, and thus will selectively induce antibodies to only a subset of determinants on the molecule. To examine this issue Dr. Berzofsky has prepared T cells from mice immunized to fragments of myoglobin, and tested these for help for a population of B cells immunized and cultured with intact myoglobin. Preliminary results suggest that these T cells preferentially induce antibodies to the region of myoglobin which they recognize, even though B cells are present for all of the antigenic determinants. This result supports the above hypothesis, which may be an important way of regulating antibody specificity and which may explain how Ir genes which regulate T cell responses also can regulate antibody specificity, as Dr. Berzofsky previously observed.

All of these studies of Ir gene control of immune response to myoglobin are hoped to ultimately shed light on the mechanisms and role of genetic controls on immune responses which play a role in human disease, whether they be normal host defenses, excessive responses which escape control (in autoimmune disease), or deficient responses, as in malignancy or immunodeficiency diseases.

LYMPHOCYTE CELL SURFACE RECEPTORS FOR GROWTH FACTORS AND LYMPHOKINES

A major accomplishment of the Metabolism Branch over the past year by Drs. Greene, Waldmann and their co-workers has been the definition of T cell growth factor receptors on the cells of the immunoregulatory T cell circuit. T cell growth factor (TCGF or interleukin-II) is a 14,000 dalton glycoprotein hormone critical to the evolution of normal human immune responses that has been characterized and whose gene has been cloned. This factor is essential for the expansion and continued proliferation of cytotoxic, suppressor and some helper T cells. The normal pathway of T cell activation involves the interaction of antigen or lectin with macrophages which are induced to synthesize and secrete the monokine interleukin-I. In the presence of antigen or lectin and interleukin-I, inducer/ helper T lymphocytes are activated to synthesize and secrete TCGF. Resting T cells do not manifest receptors for T cell growth factor, however, as T cell are acivated, they are induced to express T cell growth factor receptors. In the presence of T cell growth facor, the cells with the newly induced receptors are then capable of proliferation and continued growth.

We have developed a monoclonal antibody using hybridoma technology that we term anti-Tac that appears to identify the T cell growth factor receptor. We have utilized this antibody to elucidate the role of the T cell growth factor receptor in the development of various lymphocyte functions. The antibody was selected on the basis of its ability to bind to activated T cells, but not to bind to resting T cells or various B cell and pre-B cell lines. This pattern of cellular reactivity was essentially identical to that of TCGF receptors. We therefore hypothesized that anti-Tac recognizes the human receptor for T cell growth factor. We, therefore, hypothesized that anti-Tac recognized the human receptor for T cell growth factor.

Our other data that we have developed in support of this hypothesis are as follows: 1) anti-Tac blocks 80% of the TCGF induced DNA synthesis of TCGF dependent continuous T cell lines but does not inhibit DNA synthesis of TCGF independent T cell lines, 2) anti-Tac but not control monoclonal antibodies block over 95% of the binding of ^{3}H-TCGF to Hut-102B2 cells and PHA lymphoblast that manifest T cell growth factor receptors; 3) TCGF in high concentration blocks the binding of ^{3}H-anti-Tac but not ^{125}I-anti OKT11 to PHA lymphocyte lymphoblasts; 4) after TCGF is covalently cross linked to Hut 102B2 cells both anti-Tac and anti-TCGF can immunoprecipitate a band that is 12-14,000 daltons larger than the receptor immunoprecipitated by anti-Tac in the absence of crosslinking to its receptor; 5) TCGF immobilized on aphagel beads and used as an affinity column will purify a receptor band from extracts from PHA activated normal lymphocytes that is identical to that identified by anti-Tac conjugated to Sepharose.

Utilizing anti-Tac monoclonal antibody, we have defined those reactions that require an interaction of T cell growth factor with its inducible receptor on activated T cells. We find that anti-Tac 1) blocks T cell proliferation induced by soluble antigens, autologous antigens and allogenic HLA antigens; 2) partially inhibits T cell proliferation induced by mitogenic lectins; 3) abrogates the generation of cytotoxic T lymphocytes in allogenic cell cultures and; 4) inhibits the immunoglobulin production by B cells activated by T cell dependent polyclonal activators such as pokeweed mitogen and wheat germ agglutinin. In contrast, anti-Tac does not inhibit the immunoglobulin synthesis by the T helper cell independent activator, the Epstein-Barr Virus. The first three actions discussed above can be interpreted as the prevention of the binding of TCGF by activated T cells. The inhibition of immunoglobulin synthesis by B cells stimulated by T helper cell dependent activators may either reflect an inhibition of the secretion of a B cell growth and differentiation factor by the helper T cells or a direct action on the B cells themselves. The issue as to whether B cells can be controlled directly by T cell growth factor is a controversial one. We have evidence that under certain circumstances B cells may manifest an antigen recognized by the anti-Tac monoclonal antibody. All Hairy Cell Leukemic cells that are clearly B cells as discussed above on the basis of their immunoglobulin gene reorganization are Tac (TCGF receptor) positive. Similarly, certain B cell lines from patients with Burkitt's lymphoma or with HTLV induced T cell leukemia or even B cell lines from normal individuals induced with Epstein-Barr Virus can be induced to manifest the Tac antigen by exposure to phorbol esters. On the basis of preliminary studies, it appears that the binding of the anti-Tac monoclonal to Tac positive B cells is inhibited by purified TCGF supporting the view that in these B cells the tac antigen represents a receptor for TCGF. In aggregate, these data provide additional support for the hypothesis that anti-Tac recognizes the human T-cell growth factor receptor and illustrate ways in which this antibody can be used to modulate the human immune response.

IMMUNOREGULATORY INTERACTIONS IN IMMUNE RESPONSE

A major effort of the Metabolism Branch over the past few years has been directed toward defining the events of cellular differentiation, cellular interaction involved in the specific circulating immune response. These studies have placed emphasis on two types of studies. The first involves the definition of the defects of B cell maturation and of regulatory T cell and

macrophage interaction with B lymphocytes and plasma cells that occur in patients with immunodeficiency diseases associated with a high incidence of malignancy, in patients with autoimmune disorders as well as in patients with malignancies. Overall this set of studies was directed at defining the factors in normal an abnormal states controlling the production of antibodies and the synthesis of immunoglobulin molecules. The second series of studies that have reflected a special area of emphasis this year has been on leukemias of the T lymphocyte system that retain immunoregulatory function. These studies were directed toward defining the elements of the regulatory T cell network, to the diagnosis of different forms of T cell leukemia, to the definition of the mechanisms leading to the malignant transformation of these cells and to the development of the scientific information base required to develop rational therapeutic approaches for these T cell leukemias.

The regulatory T cell network of normal individuals represents a complex array of different cells with different and at times opposing functions. It has, therefore, been quite difficult to analyze the function, phenotype and mode of action of inividual T cells with a unique specific function. In order to get a better understanding of this regulatory network, we have been studying clonal T cell leukemias with retained immunoregulatory function. Over the past year Dr. Waldmann and his coworkers have been examining leukemias of mature T cells. The first type, the Sezary syndrome, is characterized by erythroderma, generalized lymphadenopathy, and circulating pleomorphic malignant T lymphocytes with a propensity for epidermal infiltration. The second leukemia, the adult T cell leukemia (ATL), is also a malignancy of mature T cells that has a tendency to infiltrate the skin. However, certain clinical features aid in distinguishing these syndromes. First, ATL has a more aggressive course and is often complicated by hypercalcemia and pulmonary infiltrates. Second, cases of ATL are clustered geographically, occurring in the South west of Japan, the Carrabian basin and in certain areas of the Southeastern United States. Recently, a unique human type C retrovirus, human T cell leukemia/lymphoma virus (HTLV) has been isolated from the neoplastic T cells of patients with ATL from multiple areas of the world. In the present study we compared the immunoregulatory function and cell surface phenotype of leukemic cells from patients with the clinical diagnosis of adult T cell leukemia and from patients with the Sezary syndrome who had circulating antibodies to HTLV with the function and cell surface phenotype of leukemic cells from the majority of patients with the Sezary syndrome who lack serum antibodies to HTLV. Leukemic cells of both groups were of the T3, T4+, T8- phenotype. Despite the similar phenotype HTLV negative Sezary leukemic cells frequently functioned as helper T cells whereas most HTLV+ ATL cells functioned as suppressors of immunoglobulin biosynthesis. It appears from these studies as well as from more extensive analyses from other laboratories that the cells defined by the T4 and T8 antibodies are complex populations that are not solely helper/inducer and suppressor/cytoxic populations respectively. They support the view that cell populations that react with T4 and T8 monoclonal antibodies differ not in terms of their function per se, but in terms of the class of the antigen with which the T cell interacts; i.e., T8+ cells appear to be involved in those cellular interactions and functions that are controlled by class I HLA (i.e. HLA-A or B molecules) whereas, T4+ cells are involved in interactions that concern class molecules (i.e., HLA-DR or SB). In this scheme, T4+ cells would not only be helper cells in their interactions with other lymphoid cells but might also also act as cytotoxic or suppressor cells when HLA-Dr molecules are involved.

Since the T3, T4 and T8 monoclonal antibodies were not of value in differentiating HTLV negative Sezary leukemic cells from ATL cells we examined these leukemic cells populations using other monoclonal antibodies to a series of activation antigens on T cells. The monoclonal, anti-Tac, discussed above which was developed in our laboratory and which appears to react to the membrane receptor for T cell growth factor was of great value in differentiating cells from the patients with ATL and the two Sezary patients with antibodies to HTLV from cells of the remaining Sezary patients who were HTLV negative. The Sezary T cell leukemic populations from patients that did not have antibodies to HTLV were Tac antigen negative whereas all of the leukemic T cell populations from patients with antibodies to HTLV were Tac antigen positive. Thus the HTLV associated mature T cell leukemia cells were Tac antigen positive and thus manifest the receptor for T cell growth factor. The fact that all ATL cell poplations as well as HTLV induced T cell lines manifest the Tac antigen that is associated with the inducible receptor for T cell growth factor may have implications for our understanding of one of the pathogeneic factors associated with the leukemic transformation and the rapid proliferation of these malignant T cells. The infection of mature T cells with HTLV leads to the production of T cell growth factor by some of these cell poplations in culture and to the expression of TCGF receptors in all cases. This production of T cell growth factor in some cases and the induction of the receptor required for its action may play a role in the pathogenesis of the uncontrolled growth of these neoplastic cells. For those cells producing TCGF there may be a self stimulatory circuit in which the same cell produces and responds to this growth factor for T cells. More recently it has been shown that certain HTLV transformed cord lines do not release detectable T cell growth factor. It is thus possibile that in certain cases HTLV infection leads to a bipassing of the TCGF-TCGF receptor system causing altered growth by mechanisms that have not been defined. Alternatively HTLV may directly affect the receptor changing its form so that it behaves as if T cell growth factor were bound to it and thus directly stimulates the growth of the malignant T cell. In accord with this latter observation we have isolated the receptors from a variety of human T cell leukemia/lymphoma virus infected T cell lines. While all are glycoproteins with intra-chain dilsulfide bonds we have found significant size hetrogeneity. The most abberant receptor (found on Hut 102B2 cells) is 5,000 daltons smaller and has a slightly more basic PI than the normal receptor on PHA activated lymphoblasts. Pulse chase and tunacamycin experiments demonstrate that both the Hut 102B2 and PHA lymphoblasts receptors have ostensively the identical precursor peptide of 33,000 daltons and that differences in post translational processing resulting in differences in the size of the receptors are present. It is conceivable that the constant presence of receptors on these cells and/or the abberancy of these receptors may contribute to the uncontrolled growth of these malignant cells. We have initiated a study to evaluate the effect of anti-Tac in the therapy of patients with adult T cell leukemia whose leukemic T cells are universally Tac antigen positive but whose normal resting cells do not bear this cell surface antigen.

In a distinct series of studies the response of human peripheral blood lymphocytes to viruses either the Epstein-Barr virus or influenza viruses was evaluated to define the normal factors regulating immunoglobulin and antibody synthesis as well as cytotoxic systems in normal individuals and to define the disorders that occur in patients with immunodeficiency disease that are associated with neoplasia.

Studies of the immunobiology of the Epstein-Barr virus (EBV) have been an active subject of research by Dr. Blases and coworkers on the Metabolism Branch for the past five years. EBV is a virus of the herpes group which infects B lymphocytes specifically and as a consequence these infected B cells are induced to proliferate, differentiate for immunoglobulin production, and eventually transform in culuture into permanently growing B cell lines. The virus has been implicated in the etiology of Burkitt's lymphoma, nasal pharyngeal carcinoma and the polyconal B cell malignancies occuring in patients receiving immunosuppressive drug therapy. Patients with a form of acquired immunodeficiency develop hypogammaglobulinemia following EBV infection. This propensity to develop immunodeficiency following EBV infection is inherited in an x-linked fashion. Since this virus persists in the genome of some B cells for the life of the host following primary infection, immune mechanisms which prevent B cell transformation by this virus are critically important to survival.

To determine the frequency of endogenously EBV-infected B cells capable to transforming into continuously growing cell lines and to further characterize the mechanisms which control these cells, Dr. Blaese has developed sensitive techniques for determining the rate of spontaneous B cell transformation in the human peripheral blood lymphocyte population. Using limiting dilution cultures of purified B cells grown on a feeder layer of autologous irradiated T cells, the frequency of endogenously EBV infected B cells in sero-positive normal subjects capable of spontaneous transformation was found to range form 1 to 10 cells per million B cells. In EBV sero-negative adults and children, as expected, no spontaneously transforming B cells were found. In patients with acute EBV infection, i.e., infectious mononucleosis, 500 to 5000 cells per million B cells spontaneously transformed. When unirradiated autologous T cells were added to these cultures, essentially no B cells transformed from the normal EBV sero-positive donors, demonstrating the effectiveness of these immune T cells in controlling virus induced B cell transformation. By contrast autologous T cells from patients with acute infectious mononucleosis were totally without effect in preventing endogenous EBV infected B cells from transforming, despite the fact that these T cells have profound suppressor activity for both immunoglobulin production and transformation when exogenous EBV was added to the cultures. These observations indicate that suppressor T cells activated in acute infectious mononucleosis, while capable of preventing previously uninfected B cells from being activated by EBV, are unable to control cell growth by B cells which acquired EBV infection before this suppressor mechanism became activated. Clearly then, additional defense mechanisms must be present to control these B cells which were infected by EBV early in the disease.

In addition to its association with certain B cell lymphomas and acute infectious mononucleosis, EBV also has an as yet undefined association with the common form of the adult rheumatoid arthritis. For example, patients with rheumatoid arthritis have high titers of antibody to EBV associated antigens. In studies by Drs. Blaese and Tosato when cultures of peripheral blood lymphocytes from normal EBV sero-positive donors were infected with EBV, initially the virus activates B cells to produce immunoglobulin which peaks at 8 to 10 days of culture and then this production rapidly falls as a consequence of the EBV immune suppressor T cells present in these patients. By day 14 of culture, the presence of EBV immune suppressor T cells inhibits

immunoglobulin production in these cultures by 90% compared with EBV infected B cells cultured alone. By striking contrast, when lymphocytes from EBV sero-postive rheumatoid arthritis were stimulated with EBV in vitro, immunoglobulin production progressively increases throughout the 14 day culture period indicating a defect in EBV specific suppressor T cell activity in these patients. When the number of endogenously EBV infected B cells in these rheumatoid arthritis patients was determined by precursor frequency analysis they were found to have an average of 10 times as many spontaneously transforming B cells as the normal subjects. Thus, the in vitro demonstration of defective suppressor T cell activity in these patients correlates well with the presence of an increased burden of EBV infected B cells in vivo and again suggests that many of the immune abnormalities in patients with rheumatoid arthritis are related to defective T cell control of this virus and the consequent effects of virus induced B cell activation.

During the past year Dr. Nelson had continued to focus his interest on antigen-specific human immune responses in vitro to hemophilous influenza antigens. These studies centered on the generation of immune cytotoxic T-cells and the production of specific antibody production by B-cells.

Immune cytotoxic T-cells recognize antigen in the context of major histocompatibility (MHC) antigens expressed on the cells (targets) undergoing cytolytic destruction. Such cells are generated in vitro by incubating peripheral blood mononuclear cells with chemical haptens or viral antigens. Immune T-cells may also recognize MHC antigens which differ among individuals of the same species (allogeneic MHC antigens). Cytotoxic T-cells of the former type are termed "self" MHC restricted cytotoxic T-cells while those of the latter type are termed alloimmune cytotoxic T-cells.

Immune cytotoxic T-cells probably play major roles in the recovery from certain viral infections and in host defense against neoplasia. Previous studies from this Dr. Nelson's laboratory have demonstrated profound defects in the generation of "self"-MHC restricted cytotoxic T-cells for chemical and viral antigens in patients with two immunodeficiency states; ataxia-telangiectasia and the Wiskott-Aldrich syndrome. Both diseases are associated with recurrent infections and a high incidence of cancer. These studies were extended by examining alloimmune phenomenon and cytotoxic responses in these diseases. The cells from each of the Wiskott-Aldrich syndrome patients exhibited a weak proliferative response and generated no cytotoxic response. Of the five ataxia-telangiectasia patients studied, all mounted weak proliferative responses and only a single patient generated alloimmune cytotoxic T-cells. These studies confirm the existence of profound defects in immune cytotoxic T-cell production among these two patient groups and suggest that these defect(s) are not limited to a few antigenic specificities. Such inabilities to produce immune cytolytic T-cells may contribute to the recurrent infections and high incidence of cancer in these patients.

In parallel studies the ability of peripheral blood mononuclear cells from normal individuals and those with immunodeficiency disease were studied in terms of their capacity to produce antigen specific antibody responses in vitro when stimulated with influenza virus antigens in the absence of any polyclonal activator. Utilizing this system, Dr. Nelson has shown that antigen-induced antibody production by human B cells in vitro was: 1) antigen-specific with a fine specificity that differentiated one

influenza type from another (influenza A versus influenza B), 2) required de novo protein synthesis, 3) depended on the cooperative interaction of macrophages and a subset of T-cells which served an "amplifier" function and bore the specificity recognized by the murine monoclonal antibody OKT4. B cells stimulates with whole virus were shown to produce antibody to viral hemagglutinin, one of the two virion surface glycoproteins and the one responsible for viral infectivity. demonstrated that these in vitro responses faithfully recapitulate the in vivo response to the same antigens and that the B-cell repertoire for influenza viral antigens differs from the T-cell repetoire as defined in this laboratory which was cross-reactive among virus subtypes.

Antigen-induced B-cells from normal individuals secrete antibodies of IgM, IgG, and IgA classes. Using a B-cell limiting dilution technique and Poisson analysis to calculate the precursor frequencies of antibody producing cells, the mean precursor frequencies of B-cells producing IgM, IgG, and IgA antibody were 1:183,000, 1:148,000 and 1:1,534,000 respectively. In cultures containing < 1 B-cell per culture the production of IgG, IgM and IgA were independent-that is antigen-activated human B-cells only make one isotype of antibody.

The ability to study antigen-induced specific antibody production in vitro has furthered our understanding of human immunodeficiency diseases. Among patients with hypogammaglobulinemia, no patients with x-linked hypogammaglobulinemia lacking B-cells produced specific antibody in vitro. but not in vivo. In addition, patients with ataxia-telangiectasia failed to produce specific antibody in vitro manifesting defects in both helper T-cells and/or B-cells in these patients. Patients with selective IgA deficiency who possess normal serum IgG and IgM levels produced specific antibody in vitro of IgM and IgG classes but not those of the IgA class.

THE ISOLATION AND CHARACTERIZATION OF BIOLOGICAL MODIFIERS THAT REGULATE THE HUMAN IMMUNE RESPONSE

Studies on the production and characterization of SISS-B an inhibitor of immunoglobulin synthesis has been continued. In previous years this lymphokine defined on our branch were shown to be produced following stimulation of polyclonal mononuclear cells with the mitogenic lectin Concanavalin A as well as by continuous cultures of human T cells grown in T cell growth factor. Over the past year we have demonstrated that the SISS-B is produced by human T-T cell hybridomas, by cultured T cell lines from patients with common variable hypogammaglobulinemia and excessive suppressor T cell numbers as well as by lines derived from patients from the malignant T cells of patients with the adult T cell leukemia associated with human T cell leukemia lymphoma virus. This humoral suppressor produced 40-80% inhibition of the polyclonal immunoglobulin synthesis stimulated in vitro by pokeweed mitogen, wheat germ agglutinin or the Epstein-Barr virus. This factor was: 1) of molecular weight 70-90,000 daltons, 2) non-cytotoxic, 3) reversed by the monosaccharide L-rhamnose but not a variety of other simple sugars including alpha-methyl-D-mannoside, 4) produced by T cell populations, 5) acts on non-T cells since it is effective on T cell depleted B cells and macrophages stimulated by Epstein-Barr virus.

Over the past few years Dr. Muchmore and his coworker have provided evidence that cellular cooperation, recognition and regulation may in part

be mediated by endogenous lectins and complex carbohydrate receptors. These early studies examined the potential role of carbohydrate recognition in assays of spontaneous monocyte mediated cytotoxicity, antigen specific T cell proliferation, and the recognition of the DR antigen of the major histocompatibility complex. By blocking various in vitro immunological reactions with simple sugars, Drs. Muchmore, Blaese, and their coworkers could begin to disect the role of carbohydrate recognition. These studies, however, were limited by the simple (but defined) nature of these blocking sugars. Dr. Muchmore has, therefore, undertaken a major project aimed at purificaiton of complex carbohydrates from pregnancy urine in an effort to characterize more complex sugars with immunoregulatory capacity. It is felt that some of these purified complex sugars will in fact represent specific receptors for endogenous lectins. Pregnancy urine was chosen as starting material since previous work from Dr. Muchmore's laboratory showed that it contained immunoregulatory glycoproteins and that such glycoproteins might play an important role in maintenance of the fetal allograft. Utilizing a multistep purification procedure, Dr. Muchmore has found two immunosuppressive sugar-containing materials in pregnancy urine. The smaller suppressive material purified on thin layer chromatography was shown by mass spectrometry to be manose 1-6 manose. The larger molecular weight material was a 55,000 kilodalton molecule. The manose dimer reversibly suppresses antigen specific proliferation at concentrations of 1-5 micrograms/milliliter. This materials had no effect on the generation of spontaneous cell-mediated cytotoxicity. The larger molecular weight material inhibits both antigen specific proliferation and spontaneous cell mediated cytotoxicity at concentrations as low as 50 nanograms/milliliter. The material is heat labile and only active if added at the initiated of the culture.

MECHANISM OF ACTION OF INSULIN-LIKE GROWTH FACTORS

Several years ago Dr. Nissley's laboratory provided evidence that MSA or rat insulin like growth factor-II (IGF-II) may be a fetal growth factor in rats since levels of IGF-II are high in fetal rat blood and decline postnatally to reach low levels by day 20 of extrauterine life. Dr. Nissley recently reported that rat embryo fibroblast synthesize IGF-II, have cell surface receptors for IGF-II and respond to IGF-II with increased DNA synthesis, thus, constituting an autocrine or paracrine system. In addition, he showed that placental lactogen stimulates the production of IGF-II by rat embryo fibroblasts. Placental lactogen has extensive amino acid sequence with growth hormone and has been proposed as a fetal counterpart to growth hormone in regaulating the production of a fetal somatomedin or insulin-like growth factor. Furthermore, the transition from fetal IGF-II to adult IGF-I noted in the circulation was duplicated in cultured fibroblasts from rat fetuses and postnatal animals: fetal fibroblasts produced primarily IGF-II whereas postnatally fibroblasts produced IGF-I.

Dr. Nissley has now begun to investigate whether or not there are parallels to the rat model in human fetal fibroblasts. Fetal fibroblast strains obtained from skin or lung were cultured under serum-free conditions and the conditioned media collected and gel filtered under acid conditions to dissociate and separate IGFs from a binding protein. Column fractions were then analyzed for IGF by bioassay, radioreceptor assay, and radioimmunoassay. Dr. Nissley has shown that there are two different size classes of IGFs as well as IGF binding protein produced by the human fetal fibroblasts.

Human IGFs purified from human plasma are small polypeptides (MR-7500). Because of harsh purification conditions, there is a possibility that the circulating form of IGF may be larger than Mr=7500. Dr. Nissley previously identified larger forms of rat IGF-II in medium conditioned by the BRL-3A rat liver cell line (Mr=16,300 and 8700 in addtion to Mr=7400). The Mr=16, 300 species is now bing purified to homogeneity using high pressure liquid chromatography in order to establish a precursor;product relationship between the Mr=16,300 species and the Mr=8700 and 7400 forms and to determine whether the specific biologic activity of the Mr=16,300 species is higher than the specific activity of the smaller species.

Results of competitive binding and affinity crosslinking studies demonstrate that there are two types of IGF receptors. Type I which preferentially binds IGF-I over IGF-II, interacts weakly with insulin and has a binding subunit which is the same size as the alpha subunit of the insulin receptor. By contrast, the type II receptor prefers IGF-II over IGF-I, does not recognize insulin, and has a binding subunit with a Mr=260,000. Dr. Nissley has purified the type II receptor from Swarm rat chondrosarcoma cells. In agreement with the results of affinity labeling studies performed on intact cells, the size of the major component in the purified receptor preparation is 250,000. In addition, a Mr<68,000 species was also seen. Recent studies indicate that the purified receptor is fully active with a binding stochiometry for rat IGF-II of approximately 1:1.

REGULATORY FUNCTIONS OF AMINO ACIDS

Dr. Phang has shown that proline and its metabolite pyrroline-5-Carboxylate, regulates major metabolic pathways. Pyrroline-5-Carboxylate, the obligate intermediate in the direct interconversion of proline, ornathine, and glutamate can initiate a redox-dependent metabolic cascade. This cascade includes 1) the activation of the pentose phosphate pathway, 2) increased formation of phosphoribosil pyrrophosphate (PRPP) and 3) increased production of nucleotides. Furthermore, he has shown that the interconversions of proline and pyrroline-5-Carboxylate constitute a metabolic cycle in which oxidizing potential in the form of pyrrroline-5-Carboxylate, can be generated and transferred between cellular compartments as well as between different cells with asymmetric enzyme capacities. Thus, the proline-P5C cycle by catalytically generating oxidizing potential can open metabolic gates necessary for cellular activation and for nucleotide synthesis. Based on these findings, Dr. Phang has proposed that proline-P5C functions as a system for transferring metabolic information, i.e., the system operates as a "second messenger" within cells and as a simple but generalized "hormone system" between cells. Two lines of evidence support this hypothesis. First, Dr. Phang has shown that early events accompanying mitogenic activation of quiescent cells e.g., PRPP and nucleotide synthesis, can be reproduced by P5C. Furthermore, the effect of P5C is synergistic to that of serum suggesting that serum factors alter the compartmentation and/or regulatory functions of P5C. The second line of evidence is that P5C has been identified and quantitated as an extracellular moiety. It is present in several biologic fluids (plasma, saliva, etc.). Furthermore, P5C is released by cultured cells and the release is regulated. Thus, the previous in vitro demonstration of redox transfer by the release of P5C by one tissue and uptake by a target tissue can now be considered as a physiologic paradigm. Additional studies are necessary to prove Dr. Phang's hypothesis.

Nevertheless, Dr. Phang defined a new conceptual framework for cellular regulation which may be important in defining pathogenetic mechanisms in human diseases such as type 2 hyperprolinemia and gyrate atrophy of the choroid and retina, in hepatic fibrosis with increased callagen production and in oncogenesis.

PROPHYRIN METABOLISM

In previous studies, Dr. Tschudy has shown that succinylacetone (SA) is now both a tumor inhibitory and immunosuppressive activity. When rats were treated with SA and immunized with sheep erythrocytes, antibody production to the erythrocytes was blocked. When the same animals were given erythrocytes at a later time but without SA, antibody production occurs but was less than the original primary response. The possiblity of partial clonal deletion by repeated antigen challenge with SA treatment is raised by these data. In other studies utilizing human peripheral blood cells in in vitro cultures succinylacetone inhibited lymphocyte transformation to antigens as assessed by thymidine incorporation and prevented the development of cytotoxic cells during the mixed lymphocyte reaction. Growth curves of viable human lymphocytes during pokeweed mitogen stimulation with and without succyinylacetone present indicates that this agent does not have an acute cytoxic action on these cells. Other investigators have discovered an unexplained immunodeficiency in minks with hereditary tyrosinemia. This observation may be an example of the immunosupressive effect of succyinylacetone which is known to be produced in some forms of human hereditary tyrosinemia.

Three pyrroles have been synthesized and tested for their ability to inhibit growth of L1210 cells in culture by Dr. Tschudy. These are: 1) 2-methyl-4carboxyethyl pyrrole, 2) 2-succinyl-3-carboxyethyl-5-methyl pyrrole, and 3) 2-methyl-3-acetyl-5-(1,2,3,4 tetrahydroxybutyl) pyrrole. They were compared with SA for growth inhibitory activity. Pyrroles 1) and 2) were less effective than SA at equal concentrations, but pyrrole 3) was much more effective than SA and the other pyrroles.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB04022-01 MET

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Studies of the Human Receptor for T-Cell Growth Factor

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Warner C. Greene, M.D., Expert, Metabolism Branch, NCI, NIH

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3 1/2	3	1/2

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.)*

Using monoclonal anti-Tac antibody, the human receptor for T-cell growth factor (TCGF, interleukin-2) has been characterized and purified to homogeneity. This receptor is a 55,000 dalton glycoprotein with internal disulfide bonds and an isoelectric point of 5.4 - 5.7. The receptor is composed of 33,000 daltons of peptide backbone and is post-translationally modified by introduction of N-and O-linked carbohydrate as well as sialic acid yielding mature receptor. The receptor does not appear to be phosphorylated, sulfated, nor covalently linked to palmitic acid. TCGF receptor expression has been evaluated in varying lymphoid malignancies. The adult T cell leukemia which is etiologically associated with type C retrovirus infection (human T cell leukemia/lymphoma virus, HTLV) consistently displays large numbers of TCGF receptors which may be aberrant in size. In contrast, Sezary leukemic T cells and acute lymphocytic T leukemic cells do not contain these receptors. Select acute lymphocytic leukemias however can be induced to differentiate and exhibit TCGF receptors using phorbol esters. The monoclonal antibody is now being used to aid in the diagnosis of these leukemias as well as being evaluated for potential therapeutic effects in patients with adult T cell leukemia. Hairy cell leukemia, and select Burkitt lymphomas also express TCGF receptors as defined by anti-Tac. These leukemias are of B cell origin, yet unexpectedly exhibit small numbers of receptors recognized by anti-Tac. Monoclonal anti-Tac has also been used to modulate the normal human immune response. Anti-Tac markedly inhibits 1) in vitro T cell proliferation induced by soluble, allogeneic and autologous antigens, 2) the maturation of cytotoxic T cells, and 3) T cell dependent B cell immunoglobulin production.

Other Professional Personnel:

Joel Depper, M.D.	IPA	MET	NCI
Warren J. Leonard, M.D.	Senior Staff Fellow	MET	NCI
Thomas A. Waldmann, M.D.	Chief	MET	NCI
Stanley J. Korsmeyer, M.D.	Senior Investigator	MET	NCI

Project Description:

Objectives: The principle objectives of these studies were 1) to purify and characterize the membrane receptor recognized by monoclonal anti-Tac, 2) to study expression of these receptors in varying lymphoid neoplasms, 3) to evaluate potential use of anti-Tac as a biological modifier of the human immune response, and 4) to initiate studies attempting to clone the gene encoding this receptor.

Methods Employed: As a source of human cells expressing TCGF receptors short and long term human T cell lines were established in conditioned medium containing TCGF. HTLV infected T cell lines were also established and carried in long term culture. Purification and characterization of the TCGF receptor involved biosynthetic labeling of activated T cells with varying isotopes (^{35}S-methionine, ^{3}H-D-glucosamine, ^{32}P-orthophosphoric acid), or surface iodination with lactoperoxidase, immunoprecipitation of labeled receptors with anti-Tac, and analysis by one and two dimensional SDS-PAGE. Covalent crosslinking of purified TCGF to its radiolabeled membrane receptor was performed using disuccinimidyl suberate (DSS). Purified receptor has also subjected to acid hydrolysis and amino acid analysis, as well as partially sequenced by automated Edman degradation. Post-translational modification of the receptor has been studied using pulse-chase labeling, tunicamycin blockade of N-linked glycosylation, digestion with endoglycosidase F and neuraminidase. Radioreceptor binding assays with ^{3}H-anti-Tac (prepared by reductive methylation) have been established to quantitate receptor number in multiple cell lines. Messenger RNA has been prepared from cell lines expressing receptors for use in cell free translation systems (wheat germ lysate, rabbit reticulocyte lysate) as well as for use as templates in cDNA library construction in bacteriophage gt10. High molecular weight DNA has been prepared from HUT 102B2 cells for use in gene transfer (transfection) studies with thymidine kinase deficient mouse L cells.

Major Findings: TCGF is a 14,000 dalton glycoprotein critical to the evolution of a normal human immune response. This ligand is not elaborated by resting T cells; however, following T cell activation with antigen and interleukin-1 (lymphocyte activating factor), TCGF is synthesized and secreted. TCGF has been biochemically characterized, purified to homogeneity and its amino terminus amino acid sequence determined. Recently, the gene encoding TCGF has been cloned and expressed in a eucaryotic vector. Like other polypeptide hormones, TCGF acts through binding to high affinity specific membrane receptors. These receptors, however, are

not present on resting T cells but like TCGF are induced by antigen and interleukin-1. Unlike TCGF, the TCGF receptor has not been purified nor extensively characterized. We have identified a monoclonal antibody, anti-Tac, which appears to recognize the human TCGF receptor. We have used this antibody to purify and characterize the receptor in normal and neoplastic states, to modulate the human immune response in vitro, and to begin studies designed to clone the gene encoding this receptor.

Evidence that anti-Tac recognizes the human TCGF receptor. We have established several lines of evidence indicating that anti-Tac is an anti-activated human T cells and TCGF dependent T cell lines but not to resting T cells, B cells, thymocytes, monocytes, or TCGF independent T cell lines. (2) Anti-Tac inhibits >95% of the binding of radiolabeled TCGF to activated human T cells and purified TCGF blocks >90% of the binding of ^{3}H-anti-Tac to these cells. (3) Following DSS mediated covalent cross-linking of purified TCGF to radiolabeled HUT 102B2 cells, both monoclonal anti-Tac and anti-TCGF immunoprecipitate a protein band 12-14,000 daltons larger than the free receptor. The protein size and immunoprecipitability by both anti-Tac and anti-TCGF suggests that it is a receptor-ligand complex. This band is not identified in cells similarly-treated but not covalently crosslinked with DSS. (4) Following ^{35}S-methionine or ^{3}H-D-glucosamine labeling of cells and extraction of membrane proteins in nonionic detergent, purified TCGF coupled to affigel beads binds a protein identical in m.w. as recognized by anti-Tac bound to sepharose beads. Further, when these radiolableled cell extracts are sequentially cleared with either TCGF or anti-Tac affinity supports, then subsequent precipitation with the other reagent fails to precipitate additional radioactivity. These data indicate that TCGF and anti-Tac recognize the same membrane protein. (5) Anti-Tac but not anti-Ia antibody inhibits 70-85% of TCGF induced proliferation in TCGF dependent human T cell lines. Taken together, these data strongly suggest that anti-Tac is an anti-TCGF receptor antibody. Unlike TCGF, anti-Tac has no agonistic properties when added either alone or with a second cross-linking antibody. These data suggest that anti-Tac, while binding to the receptor and inhibiting TCGF binding, does not react precisely with the same site as recognized by TCGF.

Characterization of the human TCGF receptor. Following ^{35}S-methionine biosynthetic labeling of PHA activated lymphoblasts, extraction in nonionic detergent, immunoprecipitation, and SDS-PAGE analysis; anti-Tac identified 1 major and 2 minor protein bands with apparent molecular weights 55,000 (p55), 113,000 (p113) and 180,000 (p180) daltons. In contrast, anti-Tac immunoprecipitations of cells externally labeled with ^{125}I revealed only p55. These data indicate that neither p113 or p180 display tyrosine residue on the external plasma membrane. p55 was also identified in cells labeled with ^{3}H-D-glucosamine indicating that it is a glycoprotein. Analysis of p55 under nonreducing conditions revealed a smaller apparent m.w. of 50,000 consistent with intrachain disulfide bonding. Isoelectric focusing of p55 indicated a pI of 5.4 5.7. Using ^{32}P-orthophosphoric acid, $H_2{}^{35}SO_4$, and ^{3}H-palmitate, p55 was not labeled suggesting that it is

neither phosphorylated, sulfated nor covalently associated with palmitate. To investigate whether p113 and/or p180 represented larger precursor forms of p55, pulse-chase labeling studies were performed. Both p113 and p180 labeled after p55 was identified indicating that these proteins were not precursors of p55. However, in these studies we identified two smaller proteins of m.w. 35,000 and 37,000 daltons which labeled prior to p55 and disappeared as p55 was synthesized. These data suggested that p55 represented a processed form of these smaller precursors. To investigate this possibility further, cells were labeled with ^{35}S-methionine in the presence of tunicamycin which inhibits N-linked glycosylation. Following this treatment, p35 and p37 migrated as a single band of m.w. 33,000. Similarily p55 was reduced in size by 2,000-4,000 daltons. Treatment of pulse labeled cells with endoglycosidase F which selectively digests N-linked sugars recapitulated the findings with tunicamycin. p35 and 37 migrated as p33 while p55 migrated as a slightly smaller protein. These data suggest that the TCGF receptor is composed of 2,000 - 4,000 daltons of N-linked sugar attached to a protein backbone of 33,000 daltons. Further studies of the large saltatory increase in apparent m.w. from 35-37 kd to 55 kd indicated that O-linked glycosylation and addition of sialic acid was involved. Neuraminidase digestion of p55 revealed a decrease in m.w. of 6 kd, but no change in the migration of p35 and p37. Sequential digestion with endoglycosidase F and neuraminidase provided evidence that sialic acid residues were present on non N-linked carbohydrate structures. These data indirectly confirmed the presence of O-linked sugar.

Studies with the carboxylic ionophore monensin, which blocks Golgi associated post-translational protein processing (including glycosylation), revealed blockade of the transition in m.w. of p37 to p55. Interestingly, p35 was not present and p37 was more intense suggesting that p35 was processed to p37 prior to further modification to p55. These data suggested that the large saltatory increase in receptor molecular weight occurring 60-120 minutes after synthesis of the peptide backbone took place in the Golgi apparatus. Though as yet undetected, we cannot completely exclude covalent bonding of a smaller peptide via transglutamination producing at least part of the large m.w. increase from p37 to p55.

Studies of TCGF receptor expression by human lymphoid neoplasms. We have recently investigated a leukemia of mature T cells termed adult T cell leukemia (ATL). This leukemia has been etiologically associated with human T cell leukemia/lymphoma virus (HTLV) infection. Thus far every case of ATL studied has displayed receptors for TCGF as have continuous T cell lines established from these patients. We have found that some, but not all, HTLV infected cell lines display aberrantly sized TCGF receptors. The receptor in HTLV infected HUT 102B2 cells is approximately 5,000 daltons smaller than that present in PHA lymphoblasts. Using pulse chase, tunicamycin, endoglycosidase F, and neuraminidase analyses, we have demonstrated that this aberrancy is related to altered O-linked glycosylation. We are currently investigating whether the presence of these aberrant receptors

is involved in the uncontrolled proliferation of these leukemic cells. In studies with Dr. Thomas Waldmann, we have also used anti-Tac diagnostically to distinguish ATL from a second leukemia of mature T cells,the Sezary Syndrome. Both of these leukemias may produce a similar clinical presentation with leukemic infiltration of the skin. Further, both leukemias display a similar cell surface phenotype (T3+, T4+, T8-) despite often manifesting contrasting immunoregulatory function. ATL cells may function as suppressor cells while Sezary leukemic cells may exert helper cell activity. These leukemic cells can be distinguished by the presence of TCGF receptors on ATL cells. We are presently exploring potential therapeutic effects of anti-Tac in the aggressive usually fatal adult T cell leukemia. These studies will evaluate unmodified anti-Tac as well as anti-Tac conjugated to varying toxins (e.g., ricin A chain) and radionuclides with high linear energy transfer (e.g., alpha-emitters).

We have also studied TCGF receptor expression by varying acute lymphocytic leukemic T cells. Thus far we have identified no acute T cell leukemia which constitutively displays these receptors. This was not an unexpected finding in view of the generally immature stage of differentiation of these cells. However, we have demonstrated that select acute lymphocytic leukemic T cells (JURKAT, HSB-2) can be induced to express TCGF receptors by stimulation with phorbol esters. Expression of receptors in these induced cells involves new messenger RNA activation synthesis as indicated by studies with actinomycin D. Of interest, the JURKAT TCGF receptor is 2-3 kd smaller than the normal TCGF receptor present on PHA lymphoblasts.

In collaborative studies with Dr. Stanley Korsmeyer, we have investigated TCGF receptor expression in Hairy Cell Leukemia (HCL). We have detected anti-Tac reactivity with every HCL case thus far analyzed. Receptors displayed by these cells bind small amounts of TCGF and are of normal size. Expression of TCGF receptors in this leukemia is unexpected since these leukemic cells are of the B cell lineage. All cases studied have displayed rearranged heavy and light chain Ig genes, mRNA encoding Ig, and appropriate surface Ig in select cases. It is unclear whether TCGF receptors are expressed during a discrete stage of normal B cell differentiation. We have; however, detected expression of small numbers of receptors on select Burkitt and EBV transformed B cell lines. Additional studies are underway to investigate whether TCGF receptors are of physiologic importance in the growth and maturation on normal human B cells.

Quantitation of TCGF receptor expression in normal and neoplastic lymphocytes. We have developed a radioreceptor binding assay for measurement of TCGF receptor number in varying cell populations. Anti-Tac and its Fab fragment were tritiated to high specific activity by reductive methylation. Scatchard analysis of ^{3}H-anti-Tac binding has revealed 50,000-70,000 receptors in PHA activated lymphoblasts. In contrast, HTLV infected leukemic T cell lines express 2-10 fold more receptors. Induced JURKAT leukemic T cells express 5-10,000 receptors. These studies suggest these receptors possess a single affinity through some T cells display slightly curvilinear

plots. The monoclonal antibody reacts with these receptors with an average k_d of 10^{-9} moles/liter. Following activation with PHA, normal T cells express TCGF receptors within 4-8 hours. Receptor expression in these cells requires RNA and protein synthesis but not DNA synthesis. We have also detected alterations in receptor expression dependent upon culture conditions. HTLV infected HUT 102B2 cells in early logrithmic growth express 2-4 times as many receptors as present in stationary growth. Similarly, TCGF dependent activated T cells display progressively fewer receptors with prolonged culture. Failure of receptor expression may be involved in the "crisis" period commonly encountered in cultures of normal activated T cells. Thus far we have been unable to detect marked receptor recycling in either PHA activated lymphoblasts or HTLV infected leukemic T cells.

Use of anti-Tac to modulate the human immune response. We have evaluated the capacity of anti-Tac to inhibit a variety of in vitro immune reactions with human lymphocytes. Anti-Tac blocks proliferation of human T cells stimulated with soluble, autologous, and allogeneic antigens. Anti-Tac partially inhibits T cell proliferation induced by mitogenic lectins and the degree of inhibition inversely correlates with the potency of the mitogenic stimulus. Anti-Tac inhibition of antigen induced T cell proliferation can be reversed by the addition of purified TCGF. Anti-Tac also completely abrogates the maturation of cytotoxic effector T cells in allogenic cell cocultures, however, once formed, the effector function of these cells is not inhibited by anti-Tac. Further, anti-Tac inhibits T cell dependent B cell immunoglobulin (Ig) production. The site of anti-Tac action in this system is not yet defined. Anti-Tac may inhibit helper T cell function, however, a direct action on human B cells is not excluded. Anti-Tac does not inhibit EBV induced proliferation of purified B cells.

Significance to Biomedical Research:

The identification of a monoclonal antibody recognizing the human TCGF receptor may permit effective therapeutic manipulation of the immune response in various pathologic states including autoimmunity, graft versus host disease, and acute or chronic transplant rejection. Further, anti-Tac, either unmodified or complexed with select toxins or radionuclides may provide an alternative therapy for certain lymphoid neoplasms including the adult T cell leukemia. Complete biochemical characterization of the TCGF receptor will be required to define the precise mechanisms through which clonal populations of T cells are stimulated to proliferate. Little or no information exists regarding the critical signals which occur in T cells following TCGF binding to its receptor. With further identification and study of these biochemical events, T cell responses may be inhibited or enhanced by the introduction of appropriate drugs or biologic modifiers. Finally, the TCGF receptor provides a model for the study of inducible gene expression. Resting T cells do not display receptors; however, within

hours following antigen exposure receptors are detectable. Thus studies of this gene system may yield additional information on the regulation of eucaryotic gene activation and expression.

Proposed Course:

We plan further biochemical studies of the TCGF receptor including partial determination of the primary amino acid sequence. Other studies will focus on cloning the gene encoding this receptor. We have initated gene transfer experiments involving cotransfection of high molecular weight HUT 102 cellular DNA into TK$^-$ mouse L cells in the presence of the pTK plasma. Thus far several stable transformants have been isolated which express T cell associated antigens; however, none have been identified which react with anti-Tac. In other studies, we have constructed cDNA libraries in gt 10 bacteriophage using mRNA isolated from human T cells activated with PHA and phorbol ester. We plan to screen this library with a synthetic oligonucleotide probe prepared based on the amino acid sequence of this receptor. Alternatively, we plan to screen colonies using radiolabeled cDNA's prepared from induced and uninduced JURKAT leukemic cells. As noted above, induced JURKAT leukemic cells express TCGF receptors while uninduced cells do not. Colonies hybridizing with induced but not uninduced JURKAT probes will be further analyzed by selective hybridization or hybrid arrest of translation. Finally we plan to continue study of potential therapeutic and diagnostic applications of anti-Tac in various disease states.

Publications:

Greene, W.C., Fleisher, T.A., Depper, J.M., Leonard, W.J., Stanton, G.J., and T.A. Waldmann.: Characterization of a soluble suppressor of human B cell function produced by a continuous human suppressor T cell line. II. Evidence for suppression through a direct action of CTC-SISS-B on human B cells. J. Immunol. 129:1120, 1982.

Fleisher, T.A., Attallah, A., Tosato, G., R.M. Blaese, and W.C. Greene.: Interferon mediated inhibition of human polyclonal immunoglobulin synthesis. J. Immunol. 129: 1099, 1982.

Greene, W.C., Fleisher, T.A., Nelson, D.L., and T.A. Waldmann.: Production of human suppressor T cell hybridomas. J. Immunol. 129:1986-1992, 1982.

Leonard, W.J., Depper, J.M., Uchiyama, T., Smith, K.A., Waldmann, T.A., and W.C. Greene.: A monoclonal antibody that appears to recognize the receptor for human T cell growth factor: partial characterization of the receptor. Nature. 300:267-269, 1982.

Greene, W.C., Depper, J.M., Leonard, W.J. Fleisher, T.A., and T.A. Waldmann.: Construction of human suppressor T cell hybridomas. Lymphokine Research. 2:2:53, 1983.

Cossman, J., Neckers, L.M., Leonard, W.J., and W.C. Greene.: Polymorphonuclear neutrophils express the common acute lymphoblastic leukemia antigen (CALLA). J. Exp. Med. 157:1064, 1983

Depper, J.M., Leonard, W.J., Smith, K.A., Waldmann, T.A., and W.C. Greene.: Monoclonal anti-Tac blocks the action of human interleukin-2. in Interleukins, Lymphokines, and Cytokines. Edited by J.J. Oppenheim and S. Cohen. In press.

Koretzky, G.A., Daniele, R.P., Greene, W.C., and Peter C. Nowell.: Evidence for an interleukin-independent pathway for human lymphocyte activation. Proc. Nat. Acad. Sci. USA. In press.

Greene, W.C., Cossman, J., Leonard, W.J., Bakshi, A., Waldmann, T.A., and Korsmeyer, S.J.: Hairy Cell Leukemia: a malignant proliferation of B cells which express Tac antigen. Normal and Neoplastic Hematopoiesis. UCLA Symposia on Molecular and Cellular Biology. Vol 9. Edited by D.W. Golde, and P.A. Marks. Alan R. Liss, Inc. N.Y., N.Y. In press.

Greene, W.C., Leonard, W.J., Depper, J.M., and T.A. Waldmann.: Studies of the human TCGF receptor with monoclonal anti-Tac. J. Clin. Immunol. Immunopath. In press.

Blaese, R.M., Tosato, G., Greene, W.C., Fleisher, T.A., and A.V. Muchmore.: The role of cell surface lectin-carbohydrate interactions in cellular recognition, cooperation, and regulation. Am. J. Ped. Hematol. Oncol. In press.

Depper, J.M., Leonard, W.J., Waldmann, T.A., and W.C. Greene.: Blockade of the interleukin-2 receptor by anti-Tac antibody: Inhibition of human lymphocyte activation. J. Immunol. In press.

Korsmeyer, S.J., Greene, W.C., J. Cossman, S. Hsu, L.M. Neckers, Marshall, S.L., Jensen, J.P., Bakshi, A., Leonard, W.J., Jaffe, E.S., and T.A. Waldmann.: Rearrangement and expression of immunoglobulin genes and expression of Tac antigen in Hairy Cell Leukemia. Proc. Nat. Acad. Sci. USA. In press.

Depper, J.M., Leonard, W.J., Waldmann, T.A., and W.C. Greene. A monoclonal antibody which recognizes the human receptor for T cell growth factor: functional effects and use as a probe to investigate receptor regulation. Lymphokines. In press.

Leonard, W.J., Depper, J.M., Waldmann, T.A., and W.C. Greene. A monoclonal antibody to the human receptor for T cell growth factor. Monoclonal Antibodies to Growth Factors and their Receptors. Edited by M. Greaves. In press.

Waldmann, T.A., Broder, S., Greene, W.C., Sarin, P., Goldman, C.K., Frost, K., Sharrow, S., Depper, J., Leonard, W.J., Uchiyama, T., and R. C. Gallo. A comparison of the function and phenotype of Sezary T cells with human T-cell leukemia-lymphoma virus associated adult T-cell leukemia cells. (submitted for publication, N. Engl. J. Med.)

Littman, B., Steinberg, A., Muchmore, A., and W.C. Greene. Production of human B cell hybridomas secreting autoantibodies. (submitted for publication, J. Clin. Invest.)

Koretzky, G.A., Greene, W.C., and Peter C. Nowell. Human T lymphocytes require two signals for expression of an IL-2 receptor. (submitted for publication, J. Immunol.)

Leonard, W.J., Depper, J.M., Robb, R.J., Waldmann, T.A., and W.C. Greene. Characterization of the human receptor for T cell growth factor. (submitted for publication, Proc. Nat. Acad. Sci.)

Lipkowitz, S., Greene, W.C., Rubin, A.L., Stenzel, K.H., and A. Novogrodsky. IL-2 receptor expression and commitment of T lymphocytes to proliferate. (submitted for publication, J. Exp. Med.)

Leonard, W,J., Depper, J.M., Waldmann, T.A. and W.C. Greene. Expression of aberrant receptors for T cell growth factor by T cells infected with human T cell leukemia-lymphoma virus (submitted for publication, Proc. Nat. Acad. Sci. USA)

Robb, R.J. and W.C. Greene. Direct evidence that TCGF and anti-Tac recognize the same T cell membrane receptor (submitted for publication, J. Exp. Med.)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB04021-01 MET

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Genetic Mechanisms in Human Lymphoid Neoplasms.

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Stanley J. Korsmeyer, M.D., Senior Investigator, Metabolism Branch, NCI, NIH

COOPERATING UNITS *(if any)*
Philip Leder, M.D. - Department of Genetics, Harvard Medical, Boston, Mass.
Elaine S. Jaffe, M.D. - Lab of Pathology, NCI
David G. Poplack, M.D. - Pediatric Oncology Branch, NCI

LAB/BRANCH
Metabolism Branch, DCBD, NCI

SECTION

INSTITUTE AND LOCATION
NIH, NCI, 10/4N104, Bethesda, Maryland, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	4	1

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

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

The assemblage of the human heavy chain Immunoglobulin variable region from $V_H/D_H/J_H$ subsegments and the light chain variables from V /J and V /J subsets was examined within human lymphoid neoplasms representing multiple phases of differentation. Mature B cells displayed the appropriate recombinations of heavy and light chain genes, whereas, most cells pursuing non-B cell pathways of development retained germline genes. An extensive study of the controversial "non-T, non-B" form of acute lymphocytic leukemias indicated that they represented a developmental series of B-cell precursors. They further revealed a hiearchy of immunoglobulin gene rearrangement in which heavy chain genes preceded light and rearranged before . This order was confirmed within normal -bearing B cells. Similarly lymphoid blast crises of chronic myelogenious leukemia correspond to B cell precursors and the clonally affected B cells in this disease are capable of sequential heavy and light chain rearrangements. Clonally unique immunoglobulin rearrangements served as tumor-specific markers not only allowing assignment of cellular origin but also providing a tool for early detection and serial examination of clonal B cells. Hairy Cell Leukemia was shown to be a committed B cell at the gene level, but unexpectedly displayed the Tac antigen which serves as a membrane receptor for T cell growth factor. This molecular genetic approach is thus providing insight into the origins of controversial malignancies as well as their capacity for differentiation and mechanisms of transformation.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:

Ajay Bakhshi, M.B., B.S.	Medical Staff Fellow	MET	NCI
Andrew Arnold, M.D.	Medical Staff Fellow	MET	NCI
Katherine A. Siminovitch, M.D.	Guest Worker	MET	NCI
Thomas A. Waldmann, M.D.	Branch Chief	MET	NCI
Warner C. Greene, M.D.	Senior Investigator	MET	NCI
Warren Leonard, M.D.	Senior Staff Fellow	MET	NCI
Joel M. Depper, M.D.	Expert	MET	NCI

Project Description:

Objectives: The purpose of these investigations was to determine the patterns of immunoglobulin (Ig) gene rearrangement and expression within various human lymphoid malignancies and normal cellular populations. This attempt was focused upon defining the process by which these genes are utilized during the differentiation of a B cell. By establishing the patterns of their rearrangement and expression within cells of well known lineage and stage of development, it would then be possible to examine controversial lymphoid malignancies. Such studies would provide definitive insights into the cellular origin and stage of differentiation based on a molecular genetic marker. Since various leukemias and lymphomas represent stages of development they might be expected to reveal the sequential process by which the Ig genes are utilized. Events related to normal development and those unique to transformation could be assessed by examining normal as well as malignant cells. As Ig gene rearrangements proved to be unique to separate clones of B cells, these recombinations could be utilized as tumor-specific markers which would provide a clinically useful tool for following these cells and their progeny. Once the respective stages of differentiation for individual neoplasms were defined attempts at inducing their further maturation could be undertaken. Such insights would then provide the necessary background to search for stage-specific oncogenesis and differentiation genes. Overall, such studies are aimed at improving our understanding of the molecular genetic mechanisms participating in these disorders.

Methods Employed: Genomic DNA was extracted by a variety of procedures tailored to a particular type of cellular tissue examined which were designed to yield high molecular weight DNA. This DNA was then digested to completion with the appropriate restriction endonuclease, size fractionated over agarose gels, transferred to solid phase paper, and was then hybridized with nick-translated DNA probes. The probes utilized were predominately purified fragments prepared from subclones of genomic, germ line clones of the coding segments of various immunoglobulin or cellular oncogenes. Identified genes of interest within such genomic DNA were then cloned from either gene machine enriched size fractions of digested DNA or from phage libraries. Standard screening isolated phage with the desired inserts and routine procedures of restriction endonuclease mapping and DNA sequence analysis followed.

RNA for analysis was prepared from fresh viable cells. A guanidine thiocyanate procedure was used to prepare total RNA, and nuclear and cytoplasmic fractions were also prepared at times. Poly (A) selected RNA was selected over oligo-dT-cellulose columns. Such RNAs were analyzed by Northern analysis using formaldehyde gels or were assessed by dot blot quantitation.

Analytic flow microfluorometry with a fluorecence activated cell sorter was used to assess the presence of various monoclonal and hetero-antibody defined cell surface antigens. This included at times dual fluoresence studies utilizing both Texas Red and fluorescein to assess the simultaneous presence of cell surface antigens. The cell sorter was also used in a positive, selective fashion to preparatively select a population of purified λ B cells.

Surface antigens were also characterized by techniques of immunoprecipitation using either protein A or a second antibody following initial preclearing. The final washed pellets were reduced and analyzed on SDS polyacrylamide gels. Cytoplasmic and secreted Igs were measured by a double antibody radioimmunoassay sensitive to the picogram range. Radiolabeled binding of purified anti-Tac and TCGF were performed to characterize receptor number and extent of factor binding to the TCGF receptor. Routine procedures of cell separation including sheep RBC rosette separation antigen-antibody complex plates, anti-Ig columns, and monoclonal antibodies plus complement were used.

Major Findings: The human immunoglobulin (Ig) genes within their germline (embryonic form) have been shown to be a discontinuous set of gene subsegments which encode the variable and constant portions of the antibody molecule. The human κ gene locus located on chromosome 2 at band 2p13 is comprised of multiple germline variable regions (V_κ) an alternate strip of joining segments (J_κ) and but one constant region (C_κ) per allele. The lambda light chain genes on chromosome 22 at band 22q11 also consist of multiple Vλs but have multiple sets of duplicated $J\lambda C\lambda$ units as well. While, the heavy chain genes in addition to possessing multiple V_H regions and six functional J_H segments have an additional set of diversity elements (D_H) which comprise an internal portion of a rearranged $V_H/D_H/J_H$ heavy chain gene. At some point during differentiation a B cell must assemble the respective gene subsegments which determine a final κ, λ, or heavy chain variable region protein. We have investigated this process of DNA rearrangement by utilizing a variety of human lymphoid malignancies as representatives of various stages of differentiation. Their patterns of Ig gene rearrangement have not only provided definitive insights into their cellular origins, but have also revealed an ordered, yet error prone, sequence to Ig gene rearrangements during B cell development.

When the κ genes were initially examined in non B cell sources of tissue all individuals had their J_κ and C_κ genes on a single 12.0 Kb Bam HI

fragment and thus no genetic polymorphism was demonstrated within the human population. In marked contrast, however, the λ gene locus has proven to be highly polymorphic within normal individuals. The allelic variation in the $C\lambda$ gene inheritance is depicted by a series of EcoRI generated restriction fragment length polymorphisms. While all individuals have a 5' located 14kb, and 3' located 16kb EcoRI fragment which contain one and three $C\lambda$ regions respectively; there is considerable variation in the length of the center EcoRI fragment in this locus. The multiple allelic forms of this center fragment can be either 8 kb, 13 kb, or 23 kb in size. The incremental enlargement of this fragment is a duplicated 5kb segment which itself bears a single $C\lambda$ region. This variable amplification of $C\lambda$ genes means that the actual number of $C\lambda$ genes in man can vary etween 6 to 9 per haploid genome depending upon which center allele is inherited. This EcoRI defined restriction fragment length polymorphism also serves as an allelic marker located directly at the DNA level of chromosome 22. Such markers may facilitate the mapping of inherited diseases to specific chromosomes in humans.

Humans utilize both light chain classes appreciably expressing κ approximately 60% of the time and λ, 40%. Despite this, our prior examination of mature human B cell lines and leukemias, revealed that producers retained germline λ genes whereas λ producing B cells had no germline κ genes remaining, having either rearranged or deleted them. Whether these unexpected recombinations of κ genes within such malignant λ-producing B cells reflected a normal developmental process or were secondary to transformation was uncertain. In fact, chromosomal translocation within certain human B cell malignancies can involve the very bands bearing the Ig genes. Thus, it was of paramount importance to examine the configuration of light chain genes in normal, non-transformed λ-bearing B cells. To do this, we enriched circulating λ-bearing B cells from a normal individual to 97% purity by using a series of negative selection steps and a final positive selection on a cell sorter. Over 95% of the collective κ genes in these λ B cells were no longer in their germline form, with the majority (60%) deleted and the remainder present but in a rearranged state. When examined, the incidence of κ gene deletions was higher in long term than in freshly transformed, short-term λ B cell lines. This implies that the deletion of κ genes may operate as a second event which may serve to eliminate aberrantly rearranged genes and their products. Thus, despite the nearly equal usage of κ and λ light chain genes in man, there appears to be a sequential order to their expression during normal B cell ontogeny in which κ gene rearrangements precede those of λ.

In order to examine the earlier events of Ig gene assemblage we examined 25 cases of the so-called "non-T, non-B" form of acute lymphocytic leukemia. All these cells proved to represent monoclonal expansions of a series of human B cell precursors. All 25 cases had rearrangments of their heavy chain genes, while 23 of 25 T cell malignancies examined to date have retained germline heavy chain genes. This provides strong

evidence that cells of this leukemia are committed to the B cell lineage at the gene level. Approximately, half of the cases had rearranged heavy chain genes, but retained germline light chain genes indicating that heavy chain gene rearrangements appear to precede light chain gene rearrangements. Such rearangements could be either D_H/J_H intermediate forms of rearrangements or attempts at $V_H/D_H/J_H$ recombinations. Of note, there were no instances in which D_H gene segments were recombined and J_H regions remained germline. This is consistent with a proposed ordered in which D_H/J_H recombinations would in general precede V_H/D_H joining. Also within the B cell precursor leukemias were cells that had progressed to light chain gene recombinations. All patterns of light chain rearrangements predicted by a model which proceeds from κ to λ genes were observed. Specifically, each of the 7 B cell precursors in which κ genes had been recombined retained germline λ genes, whereas, the four with λ recombinations had no remaining germline κ genes. Despite the uniform presence of rearranged Ig genes, only 5 cases produced cytoplasmic μ-chain, one exceptional case produced γ-chain, and another produced only λ-chain. The cases of B-cell precursor type that do not produce Ig may frequently be cells which possess only ineffectively rearranged Ig genes. Included in this group may be a set of cells trapped within the B cell precursor series because their ineffective, aberrant rearrangements have eliminated necessary gene subsegments required for the assemblage of an effective heavy chain gene. We have identified one such potential example which has rearranged both heavy chain genes but fails to make cytoplasmic μ-chain. This cell has deleted all known germline D_H gene subsegments and may well be incapable of forming any further $V_H/D_H/J_H$ rearrangements due to loss of the necessary middle element (D_H). Both rearranged H chain genes have been identified and cloned and sequence analysis should reveal the precise defects in these apparently aberrant forms of rearrangement. Thus, somatic recombinatorial gene joining not only provides a dynamic system to generate antibody diversity; but also appears to be error prone, creating cells which are at times incapable of further maturation. Identifying cells which are genetically trapped is thus providing a molecular explanation for some of the cell wastage which also occurs during differentiation.

Examination of other cases within these B cell precursor leukemias has revealed cells which can be induced to mature into an Ig synthesizing B cell. For example, a B-cell precursor lacking surface and cytoplasmic Ig was induced with a phorbol ester (TPA) to develop to an early B cell stage. De-novo, this cell had rearranged μ and κ genes and after induction of differentiation expressed both of these genes and displayed surface IgM κ. Thus, not all of the B cell precursor leukemias are trapped at this stage of development. Such induced gene expression and differentiation may provide valuable insights into the actual mechanisms of maturational arrest that retain these cells at a B cell precursor stage of development in-vivo.

Rather striking correlations were noted when the cell surface phenotype and the Ig gene patterns of B-cell precursor ALLs were compared. A coordinate sequence of cell surface antigen expression and Ig gene rearrangement was evident. By this developmental scheme, heavy chain gene rearrangements and cell surface HLA-DR and the antigen identified by the B_4 monoclonal would precede all other events. Sequential expression of the B cell associated antigens recognized by the antibodies BA1, J_5 recognizing CALLA, and B_1 would then occur. The final stages of B cell precursor maturation would include light chain gene rearrangements which would proceed in a κ before λ hierarchy. Attempts to advance cells along this pathway include not only the TPA stimulation noted above, but also the exposure of such targets to a variety of cell product factors. Certain cells within this series have been drug marked to allow fusing them with another B cell precursor in experiments designed to examine the capacity of these cells to complement one another and subsequently advance maturationally. This detailed heirarchy of antigen expression and Ig gene rearrangement that were described provides the necessary yardstick to measure such gene complementation.

Induction of differentiation in the B cell lineage is not limited to the B cell precursor leukemias. Cossman and his coworkers have shown that most chronic lymphocytic leukemias (CLL) when exposed to the phorbol ester, TPA, show marked secretion of IgM in vitro. When such cells were examined at several time points following TPA exposure we have noted an antecedent increase in μ RNA that is predominately the μ-secreted (μ_s) as opposed to the μ-membrane (μ_m) form. As ratios of μ_s/μ_m are much higher in plasma cells than early B cells, the increment in μ_s RNA in the CLL cells suggests that they are capable of undergoing plasmacytoid differentiation. As part of this process, the increment in IgM secretion appears to be mediated at least in part by a pretranslational increase in μ_s RNA.

The capacity to differentiate following phorbol ester induction is also seen in some secretory B cell stage malignancies including some Burkitts, and non-Burkitts lymphomas bearing the t(8:14) translocation. One such cell line, JD-38, was shown by Benjamin and his coworkers to augment its IgM secretion approximately 17 fold following exposure to TPA. Once again, there was a small, but antecedent increase in the transcription of Cμ that was predominately the μ_s as compared to the μ_m form. The t(8:14) translocation has been shown to introduce a cellular oncogene, c-Myc, normally located at chromosone $8q_{24}$, into the 14q+ chromosome near the aberrantly rearranged Ig gene that resides on that chromosome. In fact, in JD-38 this translocation has actually resulted in a detetable rearrangement of the c-Myc gene when it has moved near the Ig heavy chain gene locus. In this regard, we questioned what would happen to the expression of this c-Myc gene when JD-38 was induced to differentiate towards a plasma cell. If c-Myc were pivotally responsible for maintaining the arrested state of maturation, one would envisage that the transcription of c-Myc might decrease as the cell underwent the process of differen-

tiation. In fact; however, c-Myc transcription was minimally increased as was Ig gene transcription during this differentiation suggesting the possibility that these genes might be coordinately regulated in this setting of translocation. In this regard, Gillies and his coworkers have discovered an apparent enhancer sequence located within the heavy chain gene locus between the J_H and μ switch regions. This area is capable of augmenting the production of extraneous genes linked to it when they are transfected into a plasmacytoma target cell. It will be important to determine if this Ig gene enhancer is playing a role in augmenting the production of an associated, translocated c-Myc gene.

Not all attempts at the assemblage of a heavy chain $V_H/D_H/J_H$ rearrangement are fully successful. Intermediate recombinations of D_H/J_H and aberrant rearrangements may occur frequently in B-cell precursor leukemias accounting for their lack of Ig production. Defective or incomplete heavy chain proteins have been observed within the more mature B-cell malignancies referred to as heavy chain disease. Two such cases are currently under analysis. One of these is a mu heavy chain disease which produces a truncated mu chain and no apparent light chain protein. Two rearranged heavy chain genes have been identified and cloned from this cell. One of these appears to represent the non-responsible allele and displays two intermediate rearrangements, a V_H/D_H and a separate D_H/J_H. The allele apparently responsible for the mu chain produced has a rearrangement at the J_{H4} joining region that is currently being sequenced to pinpoint the reason for this abbreviated heavy chain. The other heavy chain disease we have recently initiated an examination of, produces on IgG_1 subclass Ig that is missing the C_{H1} domain information in its protein. This rearranged and switched heavy chain allele has been identified and an enriched fraction of digested DNA containing this entire Ig gene will be cloned into a phage vector for analysis. These studies should expand our knowledge of the errant processes responsible for these abnormal Ig products.

Cytogenetic and isoenzyme studies have established that chronic myelogenous leukemia (CML) is a clonal proliferative disorder arising from a progenitor cell with rather pleuri-potential capacity. Due to this multipotential capacity of the clonally derived cell in CML, the exact cellular lineage and indeed the state of differentiation of the cells comprising the blast crisis phases of this disorder have remained uncertain. Most cases of lymphoid blast crisis lack both detectable cytoplasmic and surface Ig as well as definitive T-cell antigens that would permit their clear assignment to either B or T-cell lineage. When Ig genes were examined in these instances 8 of 9 episodes of lymphoid blast crisis had rearranged heavy chain genes and 3 also displayed light chain gene rearrangements; whereas, cells from chronic myeloid, myeloid blast, and erythroid-like phases retained germline Ig genes. These findings provide strong evidence for placing the cells of CML lymphoid blast crisis in a B-cell precursor category. In addition, serial examination of a single patient during multiple phases of his disease revealed that two separate

lymphoid blast crisis episodes represented monoclonal expansions of malignant cells at distinctly different stages of genetic maturation. Both crises displayed the same heavy chain gene rearrangements, but one had progressed to lambda light chain gene recombination while the other had totally germline light chain genes. Thus, both of these episodes can ultimately be traced to a common lymphoid progenitor cell which had undergone heavy but not light chain gene rearrangments. However, the immediate precursor cells that gave rise to the separate lymphoid crises were at different discrete steps of genetic maturation in which one had undergone light chain rearrangements and the other had not. Thus, the clonally affected B cells in CML are capable of differentiation and sequential Ig gene rearrangements.

A polyclonal population of normal B-cells possesses numerous different Ig gene rearrangments, and collectively none of its Ig gene rearrangements is detectable because they are below the threshold of sensitivity for a Southern blot. In contrast, a monoclonal expansion represents the progeny of a single cell that will display a unique, identifiable DNA rearrangement. This serves as a sensitive and specific marker capable of identifying even minority populations (5%) of clonal B-cells within tissues of mixed cellularity. This genetic marker of Ig rearrangement has enabled us to assign a diagnosis of lymphoma to a malignancy which could not be classified as a lymphoma or an undifferentiated carcinoma based on histology, surface marker, and electron microscopy examination. We have also demonstrated the presence of monoclonal B-cells in several malignant lymph nodes in which T cells actually predominated and a mistaken diagnoses of T cell lymphomas might have been made. In addition, we have have detected the presence of a clonal B-cell subpopulation within the progressively enlarging lymph nodes of an immunodeficient patient. The histopathologic impression of this lymph node was that of an atypical hyperplasia and not a follicular lymphoma. Whether the clonal B-cell subpopulation present in this lymph node is benign and still under regulation or represents the early detection of a malignancy, will require serial examinations to resolve. The detected DNA rearrangments do however, provide the necessary genetic marker unique to thse clonal cells that will enable us to follow their natural history. Furthermore, it is frequently, impossible to detect a small percentage of malignant cells within a given tissue because of the lack of sensitive, truly tumor-specific markers. DNA rearrangements within B cell and B cell precursor malginancies serve as just such a sensitive tumor-specific marker that should enhance the ability to identify persistent tumor following therapy and facilitate the early detection of recurrences within a given tissue.

No fully equivalent cell within normal cellular differentiation has ever been found which closely resembles the neoplastic cells comprising Hairy Cell Leukemia (HCL).. These mononuclear cells characteristically have a ruffled cell surface and contain acid phosphatase resistant to tataric acid. Several past investigations had found the presence of B-cell associated surface antigens whereas others have reported T-cell

associated surface antigens upon these leukemic cells. All 8 cases of HCL that we examined demonstrated heavy and light chain gene rearrangements and two cases examined contained the corresponding mRNA for heavy and light chain Ig. Furthermore, the observed patterns of heavy and light chain gene rearrangments suggest a rather mature stage of B-cell differentiation. Consistant with this B-cell genotype all cases displayed cell surface HLA-DR and B-cell associated antigens, but lacked routine T-cell associated antigens. Unexpectedly, all cases demonstrated cell surface Tac antigen which previously had been predominantly restricted to select T-cell malignancies and activated T-cells. This no doubt reflects the fact that the anti-Tac monoclonal antibody recognizes the gp 53-57,000 cell surface receptor for interleukin-2 (IL-2), T cell growth factor (TCGF). The Tac antigen was definitively shown to be on the actual malginant B cells of HCL by a dual fluorescence study in which the same cells expressing cell surface Ig also expressed Tac. Immunoprecipitations with anti-Tac and SDS-PAGE revealed an antigen on leukemic hairy cells with an Mr of 53-57,000, identical in size to the receptor on activated T-cells. Further studies have demonstrated the binding of radiolabeled TCGF to purified leukemic hairy cells. In addition the binding of anti-Tac to HCL cells can be inhibited by preincubation with purified TCGF. This finding has been shown both by radiolabeled binding experiments as well as by flow microfluorometry determinations. Therefore, the Tac antigen on these malignant B-cells is a cell surface receptor capable of binding TCGF. The presence of Tac antigen, a TCGF receptor, upon cells that by all other criteria are malignant B-cells raises several interesting possibilities. First, the simultaneous expression of B-cell and T-cell associated genes might be interpreted as evidence for a biphenotypic leukemia. It is conceivable that such a status might occaionally result from a transformation associated activation of a gene, in this particular instance, the gene encoding Tac antigen. Alternatively, HCL may be a malignancy of a unique state of normal B-cell differentiation in which a TCGF receptor is expressed and utilized in some B-cell expansions. However, the short term proliferation of purified HCL cells in the presence of TCGF was only 1.5-3.0 fold increased. While this might mean that the receptor on these malignant B-cells may not be fully capable of stimulating proliferation; alternatively additional growth factor may also be required for successful expansion of these cells. As there may be a normal equivalent cell of Hairy Cell Leukemia which expresses Tac antigen, this leukemia is a useful model in further examining the role of this antigen in the B-cell lineage.

Proposed Course:

Continued and future efforts will be focused upon the molecular genetic basis for defects within a variaty of human lymphoid malignancies and immunodeficiency states. Specifically we will be concentrating upon Ig genes, cellular oncogenes, and other differentiation related gene expression. We will be attempting to define reasons for genetic entrapment within the B-cell precursor stage of development based on sequence analysis

of aberrantly rearranged heavy chain genes. Two other abnormal heavy chain genes in the setting of Human Heavy Chain Disease are also being pursued. We will be defining the number of unique species of RNA at various stages of B-cell precursor maturation which should enable further differentiation related genes to the cloned and identified. In addition we will be searching for oncogenesis events that are developmental stage specific that might prove to be characteristic of malignancies of different states of maturation. This will include trying to identify clonally committed cells that may exist prior to the translocation process within certain malignancies with karyotypic abnormalities. The purpose of such attempts would be to potentially define multiple steps in the pathogenesis of malignancies. We will further utilize the unique clonal, tumor-specific markers that are generated by changes in restriction endonuclease sites when Ig genes or oncogenes rearrange. This technique will be used to search for clonally committed cells within so-called "polyclonal B cell neoplasms" and to follow their natural history. These DNA level markers unique to established tumors will also be helpful in searching for residual disease and the early detection of recurrences. Further characterization of the TCGF receptor (Tac antigen) upon the malignant B-cells of Hairy Cell leukemia is underway. The receptor number and their affinity upon these B-cells as compared to activated T-cells will be assessed. Furthermore, the unique clonal marker of Ig gene rearrangements should provide an opportunity to produce a long-term Hairy Cell Leukemia cell line that is of certain identity with the original leukemia. These approaches should provide a better understanding of the molecualr genetic basis of these human disorders.

Publications:

Korsmeyer, S.J., Hieter, P.A., Sharrow, S.O., Goldman, C.K., Leder, P., and Waldmann, T.A.: Normal human B-cells display ordered light-chain gene rearrangements and deletions. J. Exp. Med. 156:975-985, 1982.

Korsmeyer, S.J., and Waldmann, T.A.: Immunoglobulin gene organization and assembly. In Stites, D.P., Fudenberg, H.H., Stobo, J.D., and Wells, J.V. (Eds.): Basic and Clinical Immunology, 4th Edition. Los Altos, California, Lange Medical Publications, 1982, pp. 43-51.

Waldmann, T.A., Korsmeyer, S.J., Hieter, P.A., Ravetch, J.V., Broder, S., and Leder, P.: Regulation of the human immune response from immunoglobulin genes to regulatory T-cell networks. Fed. Proc. 42: 2498-2503, 1983.

Kirsch, I.R., Ravetch, J.V., Hieter, P.A., Korsmeyer, S.J., Waldmann, T.A., and Leder, P.: Genetic rearrangements in the humoral immune system. In Serrou, B., et al. (Eds.): Current Concepts in Human Immunology and Cancer Immunomodulation. Elsevier, Biomedical Press B.V., 1982, pp. 113-124.

Cossman,J., Neckers, L.M., Arnold, A., Korsmeyer, S.J.: Induction of differentiation in the primitive B-cells of common, acute lymphoblastic leukemia. N. Engl. J. Med. 307: 1251-1254, 1982.

Waldmann, T.A., Broder, S., Goldman, C.K., Frost, K., Korsmeyer, S.J., and Medici, M.A.: Disorders of B-cells and helper T-cells in the pathogenesis of the immunoglobulin deficiency of patients with ataxia telangiectasia. J. Clin. Invest. 71: 282-295, 1983.

Korsmeyer, S.J., Arnold, A., Bakhshi, A., Ravetch, J.V., Siebenlist, U., Hieter, P.A., Sharrow, S.O., LeBien, T.W., Kersey, J.H., Poplack, D.G., Leder, P., Waldmann, T.A.: Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T-cell and B-cell precursor origins. J. Clin. Invest. 71: 301-313, 1983.

Cossman, J., Neckers, L., Braziel, R.M., Bakhshi, A., Arnold, A., and Korsmeyer, S.J.: Induction of differentiation in B-cell leukemia. In Bernard, A., et al. (Eds.): Human Leucocyte Markers Detected by Monoclonal Antibodies. New York, Springer-Verlag, 1983. In Press.

Korsmeyer, S.J., Greene, W.C., Cossman, J., Hsu, Su-M., Neckers, L. M., Marshall, S.L., Jensen, J.P., Bakhshi, A., Leonard, W.J., Jaffe, E.S., and Waldmann, T.A.: Rearrangement and expression of immunoglobulin genes and expression of tac antigen in hairy cell leukemia. Proc. Natl. Acad. Sci. USA. 1983. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB04020-07 MET

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Genetic Control of the Immune Response to Natural Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Jay A. Berzofsky, M.D, Ph.D. Senior Investigator, Metabolism Branch, DCBD

COOPERATING UNITS *(if any)*

VA-Oncology Branch, NCI
Department of Chemistry, Indiana University

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.8	4.4	1.4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors B

☐ (a2) Interviews

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

The mechanisms of determinant-specific Ir gene control and of antigen recognition by T and B lymphocytes were explored at several levels in the response to myoglobin. An immunodominant epitope of myoglobin centering on Glu 109 was identified for high responder T cells restricted to I-A^d, under Ir gene control. Cloned T cell lines specific for this epitope were produced, along with clones specific for a minor epitope at Lys 140. All 109-specific clones were I-A restricted, whereas all I-E restricted clones were specific for Lys 140. Monoclonal antibodies to the Lys 140 site could block the latter clones, a novel result presumably possible because of T cell-antibody shared specificity. One clone helped B cells to make antibody in vitro. Fragment specific T cells selectively helped certain B cells, resulting in antibody only to related sites. Inhibitors of processing inhibited presentation of native myoglobin, not fragment, to the same T cell clone, implying a necessity for cleavage of the whole molecule before presentation. Anti-idiotype in vitro induced specific antibody without antigen. Syngeneic monoclonal anti-idiotypes to two monoclonal anti-myoglobins were produced. One monoclonal antibody has been partially sequenced. Alterations at the N-terminus affect antibody binding. Monoclonal antibodies can act as allosteric effectors to alter the spin state of the myoglobin heme.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel

Ira Berkower, M.D., Ph.D.	Expert	MET	NCI
Hajime Kawamura, M.D.	Visiting Fellow	MET	NCI
Howard Streicher, M.D.	Medical Staff Fellow	MET	NCI
John Minna, M.D.	Chief, VA-Oncology Branch		NCI
Frank Cuttitta, Ph.D.	Investigator, VA-Oncology Branch		NCI
Frank R.N. Gurd, Ph.D.	Distinguished Professor	Indiana Univ.	
Heather B. Simmerman, Ph.D.	Postdoctural Fellow	Indiana Univ.	
Mark Busch	Graduate Student	Indiana Univ.	

Project Description:

Objectives: The critical importance of genetic factors in regulating the immune response, and especially of genes which appear to be specific for particular antigens (so-called immune response or Ir genes), has become increasingly apparent in the last 15 years. However, the mechanism of action of these genes and the nature of the product encoded by them remain unknown. Most of the Ir genes so far described are linked to the major transplantation or histocompatibility gene complex. The primary objective of this project has been to define the mechanism by which Ir genes linked to these major transplantation antigens (HLA in man and H-2 in the mouse) control the immune response to natural protein antigens, and especially the control of the specificity of this response, with the hope of understanding the modes of recognition involved at this regulatory level, the cells which participate and the cell membrane receptors which mediate the regulation.

Methods Employed: Myoglobins from various species were purified by the method of Hapner et al., (J. Biol. Chem. 243:683, 1968). Fragments of myoglobin were prepared by CNBr cleavage and chromatographic purification. For antibody responses, mice were immunized i.p. with 200 micrograms in complete Freund's adjuvant and boosted twice with 100 micrograms in saline, as described previously (Berzofsky, J. Immunol. 120:360, 1978). Myoglobin and its fragments were radiolabeled at the N-terminal alpha-amino group with $K^{14}CNO$ or N-succinimidyl-[2,3-^{3}H] propionate. Antibodies to these were measured using the polyethylene glycol precipitation direct binding assay described previously (Berzofsky, J.A., J. Immunol. 120:360, 1978). In competition studies, other unlabeled myoglobins were added at various concentrations.

T-lymphocyte proliferation was studied by a method based on that of Corradin, Etlinger and Chiller (J. Immunol. 119:1048, 1977). Briefly, nylon-wool passed T-cells from draining lymph nodes of mice immunized s.c. in the tail 8 days previously with myoglobin in Freund's adjuvant were cultured for 5 days in the presence of varying concentrations of antigen (myoglobin or fragments). Proliferation was assessed from the amount of methyl-^{3}H-thymidine incorporated into DNA during the final 4 hours of

culture. In some experiments, irradiated T-depleted spleen cells as a source of macrophages were pretreated with antigen in culture medium at 37° in polypropylene tubes at 10^6 cells/0.2 ml for one to 24 hrs, washed and then added to macrophage-depleted T cells without soluble antigen present during the 5-day culture, to assess stimulation of the T cells.

A culture system for assessment of in vitro antibody responses to myoglobin and its fragments was developed by a modification of that of Mishell and Dutton (J. Exp. Med. 126:423, 1967). Optimum conditions were determined to be as follows: 4 X 10^6 spleen cells from immunized mice were cultured with 0.1 to 1.0 ug myoglobin in 1.5 ml of supplemented RPMI-1640 medium with 10% fetal calf serum in flat-bottom wells for 9 days at 37°, 6% CO_2, on a rocking platform. On the fourth day one ml of supernatant was exchanged for fresh medium, and cultures were fed daily thereafter. On the 9th day, culture supernatants were taken to test for the presence of secreted antibody, or else cells were harvested on day 6 to count antibody-secreting cells by the hemolytic plaque method. Besides whole spleen, various cell mixtures could be cultured. For instance, spleen cells depleted of macrophages by passage over Sephadex G10 (Hodes and Singer, Eur. J. Immunol. 7:892, 1977) were cultured alone or with macrophages from syngeneic or semisyngeneic sources. Macrophages were irradiated, glass-adherent, splenic macrophages. T cells were eliminated, where necessary, by treatment with rabbit anti-mouse brain antiserum plus complement. T cells were purified, where necessary, by removal of B cells and macrophages on nylon wool columns.

Concentrations of antibodies in culture supernatants were measured by a solid phase radiobinding assay. Wells of polyvinyl chloride flexible microtiter plates were coated by incubation with 50 ul myoglobin, 100 ug/ml, for 1.5 hrs. Unoccupied sites on the well were saturated by a 15 minute incubation with 10% bovine serum albumin. Then 50 ul aliquots of culture supernatants were incubated in the wells for 1.5 hours and unbound material washed out. Finally, ^3H-labeled, affinity purified, goat antibody to the Fab fragment of mouse IgG was incubated in the wells for 1.5 hrs to bind to any antibody attached to the myoglobin on the plastic. All incubations were carried out at room temperature, and were followed by 3 washes with 1% bovine serum albumin in saline. Individual wells of the plate were the cut apart and radioactivity determined by scintillation counting. Controls for nonspecific binding were all close to machine background. A standard curve with known antimyoglobin antibody was used for quantitation.

Hybridoma monoclonal antibodies specific for sperm whale myoglobin were described in a previous annual report (1980-81). These had high affinities, between 0.2 X 10^9 and 2.2 X 10^9 M^{-1}. Their relative affinities for myoglobins of other species were determined by competitive radioimmunoassays, in which increasing concentrations of competitor were added to a constant concentration of labeled sperm whale myoglobin (7 nM)

and of monoclonal antibody (on the order of 4-6 nM), and polyethylene glycol (MW 6000, final concentration 10%) used to precipitate all immunoglobulin plus bound antigen. The concentration of competitor resulting in a 50% decrease in bound/free ratio for labeled myoglobin was taken as a measure of relative dissociation constant.

Antiidiotypic antibodies were raised in guinea pigs, tolerized one day previously with 5 mg of deaggregated mouse Ig given intravenously, by immunizing subcutaneously in the footpads with 20 micrograms of affinity purified monoclonal antibody emulsified in complete Freunds adjuvant. The animals were boosted intramuscularly three and five weeks later, first with 20 micrograms in complete adjuvant, and bled repeatedly from 10-18 days after the last immunization. The sera were absorbed extensively by repeated passage through columns of normal mouse Ig-Sepharose to remove antibodies to constant region determinants. They were studied using an enzyme-linked immunoassay (Elisa) as follows: Myoglobin was bound to polyvinyl chloride microtiter plates. Alkaline-phosphatase conjugated monoclonal antimyoglobin antibodies were preincubated with a putative antidiotypic antisera, and then incubated on the myoglobin-coated plastic. After washing to remove unbound material, the amount of enzyme bound was determined by optical absorbance change at 405 nm after addition of p-nitrophenyl phosphate as substrate.

To establish a long-term T cell line in culture, the procedure of Kimoto and Fathman (J. Exp. Med. 152:759, 1980) as modified my Matis, et al. (J. Immunol. 128:2439, 1982) was used. Six B10.D2 mice were immunized in the foot pads with 100 g of sperm whale myoglobin in complete Fruend's adjuvant. Seven days later, draining inguinal and popliteal lymph nodes were removed and single cell suspensions were prepared. There were passed through a 2 gram nylon wool column equilibrated with 10% fetal calf serum in RPMI 1640, and the purified T cells were incubated at 4 x 10^6 cells per well in large well plates (Costar No. 3524) with 2 x 10^5 irradiated syngeneic spleen cells and sperm whale myoglobin at 1 μM in long-term growth medium, consisting of a 1:1 mixture of RPMI 1640 and Eagle-Hanks' amino acid medium, supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 x 10^{-5} M-mercaptoethanol, 5 mM HEPES, and 10% decomplemented fetal calf serum. Four days later, responding T cell blasts were harvested from the wells and purified from dead cells by Ficoll denisity gradient centrifugation. The T cells were then put in resting culture at 2 x 10^5 cells/well with 4 x 10^6 irradiated spleen cells for 10 days. Then 5 x 10^6 irradiated spleen cells plus myoglobin were added to begin another round of stimulation and rest. After the first 3 or 4 rounds, most cells were antigen-specific (allo-reactivity, for example, was not detected) and the total cell population grew exponentially, about 5- to 10-fold per cycle.

The T cell line was cloned after the sixth round of stimulation and rest. The day before cloning 4 x 10^6 irradiated spleen cells and fresh antigen were added to resting T cells. The day of cloning, the stimulated T cells were counted and diluted to 4 cells per ml. Then 0.1 ml of this dilution of cells was added to each microtiter well (Costar No. 3596), along

Berzofsky, J.A.: T-B reciprocity: An Ia-restricted epitope-specific circuit regulating T cell-B cell interaction and antibody specificity. Survey of Immunol. Research. In press.

Berzofsky, J.A.: Monoclonal Antibodies as Probes of Antigenic Structure. In Venter, J.C., Fraser, C.M., and Lindstrom, J.M. (Eds.): Monoclonal Antibodies as Probes of Antigenic Structure, Receptor Biochemistry and Methodology, New York, Alan R. Liss, 1983, Vol. 4. In press.

Further studies of the H-2D linked Ir gene for equine myoglobin are planned as soon as the magnitude of the in vitro response to this antigen can be optimized. Studies of T cell proliferation in neonatally tolerized mice are planned to examine further the effect of H-2 tolerization on the repertoire of T cells specific for antigen, in the absence of the complications introduced by T-B interactions and B cell priming in the antibody response.

Publications:

Berzofsky, J.A., Buckenmeyer, G.K., Hicks, G., Killion, D.J., Berkower, I., Kohno, Y., Feldmann, R.J., Minna, J., and Gurd, F.R.N.: Topographic Antigenic Determinants Detected by Monoclonal Antibodies to Myoglobin. In Celada, F., Sercarz, E., and Shumaker, V. (Eds.): Protein Conformation as Immunological Signal, New York, Plenum Press. In press.

Berkower, I., Buckenmeyer, G.K., Gurd, F.R.N., and Berzofsky, J.A.: A Possible Immunodominant Domain of Myoglobin for Murine T Lymphocytes. In Celada, F., Sercarz, E., and Shumaker, V. (Eds.): Protein Conformation as Immunological Signal, New York, Plenum Press. In press.

Berzofsky, J.A., and Berkower, I.: Antigen-Antibody Interaction. In Paul, W.E. (Ed.): Fundamental Immunology. New York, Raven Press. In press.

Lando, G., Berozfsky, J.A., and Reichlin, M.: Antigenic structure of sperm whale myoglobin: I. Partition of specificities between antibodies reactive with peptides and native protein. J. Immunol. 129: 206-211, 1982.

Berkower, I., Buckenmeyer, G.K., Gurd, F.R.N., and Berzofsky, J.A.: A possible immunodominant epitope reocgnized by murine T lymphocytes immune to different myoglobins. Proc. Natl. Acad. Sci. USA. 79: 4723-4727, 1982.

Kohno, Y., and Berzofsky, J.A.: Genetic control of the immune response to myoglobins. Both low and high responder T cells tolerant to the other MHC help high but not low responder B cells. J. Exp. Med. 156: 791-809, 1982.

Simmerman, H.K.B., Wang, C.-C., Horwitz, E.M., Berzofsky, J.A., and Gurd, F.R.N.: Semisynthesis of sperm whale myoglobin by fragment condensation. Proc. Natl. Acad. Sci. USA. 79: 7739-7743, 1982.

Kohno, Y., and Berzofsky, J.A.: Genetic control of immune response to myoglobin. Ir gene function in genetic restriction between T and B lymphocytes. J. Exp. Med. 156: 1486-1501, 1982.

Berzofsky, J.A., Kohno, Y., and Kawamura, H.: Both Low and High Responder Myoglobin-Specific T Cells, Tolerant to F_1, Help High but Not Low Responder B cells. In Pierce, C.W., Cullen, S.E., Kapp, J.A., Schwartz, B.D., and Shreffler, D.C. (Eds.): Ir Genes: Past, Present, and Future. Clifton, N.J., Humana Press, 1983, pp. 269-275.

around the epitope. For instance, the fragment (132-153) which stimulates the Lys 140-specific T cell clones, can be cut at the two Glu residues at positions 136 and 148 with staph protease to try to produce a 12-residue peptide which can be tested for retention of activity. Byproducts may include the overlapping 17 residue peptides from partial cleavage as well as products of cleavage at Asp 141. These should be separable by high pressure liquid chromatography. We would like to define the minimum determinant necessary for full stimulation of the T cell clones. These smaller peptides would also be useful in studies of antigen processing and presentation, given our results so far with inhibitors of processing. We plan to use both the inhibitors of processing and also the monoclonal antibodies which block T cell proliferation to try to define the steps in antigen handling and presentation by macrophages. Also, we plan to prepare anti-idiotypic antibodies to the T cell clones to try to use these to isolate the T cell receptors.

The studies of T cell control of antibody specificity are promising, but will require elimination of "background" effects -- such as nonspecific T cell help, myoglobin carry-over on macrophages from immune animals, and B cell blasts which may produce antibody -- before definitive results can be obtained. These same problems plague the use of T cell clones as helpers, and also the induction of antibody by anti-idiotypes in vitro. Several approaches planned to try to circumvent these problems include the (1) use of $Lyb5^-$ B cells from $(CBA/N \times DBA/2)F_1$ male mice with an x-linked defect in development of $Lyb5^+$ B cells, (2) use of Percoll or BSA gradients to separate resting B cells from B cell blasts, (3) examination of IgG instead of total antibody, since IgM may be less dependent on T cell help, (4) removal of macrophages from immune spleen cells which might carry over antigen, and replacement with fresh macrophages. All of these approaches will be tried.

The monoclonal anti-idiotypes should be characterized for crossreactivity with various antimyoglobins and for reactivity with serum antimyoglobin of various strains, and possible allotype linkage. These should then be useful in studies of idiotype regulation both in vivo and in vitro.

We hope to make further progress on the sequencing of the monoclonal antibodies and computer modeling of the combining sites. Also, we would like to see whether Fab fragments of any of the monoclonals not yet tested will crystallize, for x-ray diffraction studies in collaboration with David R. Davies of NIH. A series of semisynthetic myoglobins with different amino acids replacing the N-terminal valine are being prepared by Dr. Gurd's laboratory at Indiana University to study the electrostatic effects on myoglobin structure. Given the proximity of the N-terminal to sites recognized by three of the monoclonal antibodies, this graded series of substitutions should also be useful for analyzing electrostatic effects on antibody binding.

artificially fragmented in advance. This finding may provide another handle on the mechanisms of antigen-presentation and processing, which have recently come to be recognized as central to a number of major regulatory pathways of the immune system.

Another level of regulation of the immune response is the selection by helper T cells of B cells with specificity for particular epitopes, a process which we have hypothesized and now have preliminary evidence to support. Thus, T cell regulation may be much finer than originally envisioned. This process may explain how Ir genes which control T cell specificity can also control antibody specificity. The T cell clones may also be extremely useful in these studies.

The anti-idiotype studies support the concept that the immune system may be regulated by internal idiotype networks that are independent of, but perturbed by, antigen. The use of syngeneic monoclonal anti-idiotype should allow the exploration of these networks without the introduction of foreign agents such as xenogeneic antibodies. The understanding of these internal networks may ultimately allow manipulation of the immune regulation in a specific way to eliminate tumors or to prolong the acceptance of organ transplants without broad immunosuppression.

The monoclonal antibodies are useful models of high affinity receptors for other proteins. Understanding the molecular mechanisms involved in this protein-protein interaction can lead to progress in protein biochemistry in general, and that of receptors in particular. Moreover, the use of monoclonal antibodies to probe protein structure is a new tool of widespread usefulness. The conformation specificity and the use of monoclonal antibodies as allosteric effectors will allow these to be used to study the function as well as the structure of such important but less well defined proteins as cell surface receptors.

The Ir gene controlling the response to equine myoglobin which maps in H-2D is unprecedented. It suggests either a new type of Ir gene, or a new type of genetic restriction element for presentation of soluble antigen. Finally, the studies of neonatal tolerance and its effect on the T cell repertoire for recognition of antigen in association with Ia will hopefully allow us to understand the mechanisms by which this repertoire and this genetic restriction of T cell recognition develop. All of these studies are hoped to ultimately shed light on the mechanisms and role of genetic controls on immune responses which play a role in human disease, whether they be normal host defenses, excessive responses which escape control (in autoimmune disease), or deficient responses, as in malignancy or immunodeficiency diseases.

Proposed Course:

The fine specificity of the T cell clones, which do not seem to require native antigen, will be studied using smaller and smaller peptide fragments

their ability to help high responder B cells. Fluorescent activated cell sorter analysis (by Susan O. Sharrow, Immunology Branch, NCI) demonstrated elimination of F_1 cells both at the time of adoptive transfer and again four weeks later when the recipient spleen was removed for culture. Therefore, we expected that no H-2^d antigen presenting cells were left to prime T cells specific for myoglobin plus H-2^d. To our surprise, in two of two such experiments, the homozygous low responder T cells helper H-2^d B cells anyway. Whether this unexpected priming is due to an unusual crossreaction (such as "aberrant recognition" of Bennink and Doherty's acute depletion studies) is currently under investigation. In any case, the neonatal tolerization model is more complex than originally envisioned, at least by us.

Significance to Biomedical Research:

The discovery of an immunodominant epitope (antigenic site) for T cell recognition of myoglobin, distinct from the epitope commonly recognized by antibodies, has several important implications. First, T cell fine-specificity seems to be more restricted to a limited number of sites on a molecule than antibody specificity. This may be due to regulatory controls on T cell specificity, such as Ir genes, some of which may be due to the constraints of antigen presentation on a cell surface in association with Ia. Second, the difference between T cell and B cell specificity implies that construction of synthetic peptide vaccines based on antibody responses may be limited by the lack of epitopes on the peptide which can be recognized by T cells. Such peptides may elicit antibodies which crossreact with the native protein, as Lerner has suggested, but if they do not prime helper T cells, then when the person or animal is exposed to the whole protein (such as a viral protein during infection), they may not develop an anamnestic response, i.e., will not have protective immunity. One solution is to determine the major sites of a protein which elicit T cell help, and design peptide vaccines which include these. This retains the safety feature that the vaccine can be synthetic, but no longer would allow one to arbitrarily use a peptide based on a DNA sequence without isolating the whole protein. Of course, the latter approach could still be used for preparing diagnostic or research reagents, just not protective vaccines. The isolation of T cell clones specific for the immunodominant and other sites should allow a better definition not only of these sites, but also of the mode of T cell recognition and the receptors involved. The strict correlation between genetic restriction for I-A vs I-E and epitope specificity may give us a handle on some of these mechanisms. The ability to block T cell clones with a monoclonal antibody to the same epitope also should be an extremely useful approach to defining these mechanisms. It suggests that the theories of antigen sequestration by presenting cells previously suggested to explain the widespread failure to achieve blocking are no longer necessary. Similarly, the observation that agents which inhibit lysozomal proteases or intracellular transport inhibit presentation of native myoglobin but not a small fragment, bearing the same epitope, to the same T cell clone suggests that transport and cleavage are necessary prerequisites for presentation of a large protein, but can be bypassed if the antigen is

hydroxide form low spin). Therefore, we performed optical spectral pH titrations of ferric myoglobin in the presence and absence of HAL 19 or HAL 39, and found, indeed, a decrease of about 0.08 units in the pK when antibody was saturating the myoglobin. The failure of a third antibody, HAL 43, to produce any spectral change demonstrates that this is not a nonspecific effect of the binding of any antibody. Thus, conformation-specific monoclonal antibodies may function as allosteric effectors and alter the function of active sites of proteins at a distance from the site of antibody binding.

The Unusual Ir Gene for Equine Myoglobin Mapping in or near the H-2D Locus: We have previously mapped one of the Ir genes controlling the response to equine myoglobin to the H-2D end of the H-2 conplex based on three independent criteria, a comparison of the strain pairs B10.A vs. B10.A(2R), B10.AKM vs. B10.BR, and B10.D2 vs. B10.D2(M504), the latter strain bearing a mutation involving H-2D and L. Since it was unexpected to find an H-2-linked Ir gene for the antibody response to a soluble protein antigen outside the I region, we have tried to explore the mechanism of its action. To do so required in vitro studies, and these have been hampered by the poor response we have been able to obtain with equine myoglobin in both T cell proliferation and the in vitro antibody response. In the in vitro antibody response, the difference between B10.A and B10.A(2R) and between B10.BR and B10.AKM have been confirmed, but in T cell proliferation, only a difference in B10.BR and B10.AKM has been consistently observed. The reason for this difference between antibody and T cell response is unclear, but may ultimately be a clue to the mechanisms involved. In neither system can low responsiveness yet be attributed to suppression. It is not relieved by treatment of the cells with anti-Lyt 2.2 plus complement, which would be expected to kill suppressor T cells, and in the antibody response, anti-I-J antibodies have no effect. Furthermore, monoclonal antibodies to the H-2D^{q} or ^{b} (low responder haplotypes) in the culture do not enhance the response. One monoclonal antibody to H-2D^{d}, the high responder haplotype, seems to inhibit the antibody response, but the specificity of this effect is not yet clear. Further progress in exploring the mechanism of this unusual Ir gene will depend on finding conditions to obtain a larger and more consistent in vitro response to equine myoglobin.

Mechanisms of Neonatal Tolerization: We had previously observed (Kohno and Berzofsky, J. Exp. Med. 156:791, 1982) that low resopnder mice (H-2^{k} or H-2^{b}) neonatally tolerized to the high responder haplotype (H-2^{d}) could help B cells of the high responder, allogeneic, haplotype. It was presumed that elicitation of these helper T cells during in vitro immunization required antigen presentation by the small population (2-4% of spleen cells) of F_1 origin which had survived from the tolerizing inoculum. To test this hypothesis, we had to eliminate these F_1 cells before primary immunization in vivo. Therefore, spleen cells were removed from nonimmune neonatally tolerized low responder mice and treated with anti-H-2^{d} and complement to kill F_1 cells before adoptive transfer of the tolerant low responder cells into lethally irradiated syngeneic low responder recipients for primary immunization. Four weeks later the T cells from these mice were tested in culture for

Monoclonal Antimyoglobin Antibodies: Several monoclonal antibodies to sperm whale myoglobin have been described is a previous report (see Berzofsky et al., J. Biol. Chem. 257:3189, 1982). These serve as models of myoglobin binding sites both for antibodies and possibly for T cell receptors. Since we have identified the antigenic determinants of myoglobin recognized by three of these, we would like to examine the structure of the antibody combining site to understand the protein-protein complementarity involved in this high affinity binding. To this end, we have begun to sequence two of the monoclonal antibodies. HAL 38 (clone 3.4) heavy chain had a blocked N-terminal, but we have been able to sequence the first 40 residues of HAL 43 (clone 5). This initial sequence places HAL 43 in the heavy chain subgroup II, which includes myeloma proteins MOPC 104E and MOPC 21, although it resembles the former much more than the latter. The sequences of the other hypervariable regions will be necessary before any computer modeling of the combining site can be done. We have also made Fab fragments of these two monoclonal antibodies, and prepared 1:1 complexes of these with myoglobin, but have been unsuccessful at crystallization.

Having noted that the amino terminal residue is centered in the middle of a triangle formed by the antigenic determinants recognized by the three monoclonal antibodies of clones 1, 3.4, and 5 we have begun, in collaboration with Prof. F.R.N. Gurd and Dr. H. Simmerman of Indiana University, to examine the effects of altering the N-terminal by antibody binding. If the affinity of myoglobin for clone 3.4 antibody (HAL 38) is lowered by acetimidylation of the lysine but not the N-terminal, acetimidylation of the N-terminal alpha amino group increases the affinity. This modification brings the positive charge of the N-terminal amino group closer to the carboxyl of Glu 4, which is part of the antigenic determinant, and may partly compensate for the weaker bond of Glu 4 with Lys 79 when the latter is acetimidylated (a modification which does not eliminate the charge of the Lys but moves it farther out on the side chain). Conversely, removing the charge of the N-terminal alpha amino group by carbamoylation lowered the affinity. These studies are hoped to ultimately map the electrostatic interactions involved in stabilizing antigen-antibody interactions and to explore the possible long-range effects of antibody binding on the protein (see proposed course below).

Such long-range effects become important when one considers the possible use of monoclonal antibodies as allosteric effectors. Since our monoclonal antibodies appear to be conformation-specific but do not bind near the heme, we asked whether they could alter the conformation sufficiently to perturb the spin state equilibrium of the heme, as judged by optical spectroscopy. The binding of HAL 19 or HAL 39 to sperm whale myoglobin at constant pH of 8.70, just below the pK of the heme, resulted in an increased proportion of heme in the low spin ferric state and a decrease in the high spin state. The degree of change was not large, but corresponded to a decrease of 0.07 units in the pK of water at the heme (the aquo form is high spin and the

culture. Although no increase in idiotype-positive antimyoglobin was achieved, in the course of the experiments we discovered an even more intriguing result. Spleen cells from A.SW mice immunized with anti-idiotype and myoglobin or even just with myoglobin, when cultured with anti-idiotype in the absence of myoglobin, produced specific anti-myoglobin antibodies, a large fraction of which (80-95%) were idiotype-positive (i.e., could be inhibited by antiidiotype from binding to myoglobin). The cells required in vivo immunization with myoglobin, but in the culture, anti-idiotype alone was sufficient to trigger anti-myoglobin production. We are now exploring the mechanism of this stimulation.

Guinea pig anti-idiotype has the problems that quantities are limited, purification is difficult, and different subclasses of anti-idiotype antibodies in the serum can have opposing effects. Also, if one wants to explore natural endogenous idiotype network regulation in the mouse, it would be useful to have syngeneic mouse anti-idiotypic antibodies which see the idiotypic sites (idiotopes) recognized by the mouse, not another species. Therefore, we set out to raise monoclonal syngeneic anti-idiotypic antibodies against two of our A.SW monoclonal anti-myoglobins (HAL 19 or clone 1, and HAL 43 or clone 5). Immunization of syngeneic A.SW mice with these antibodies in complete Freund's adjuvant failed to elicit anti-idiotypic antibodies. Therefore, we coupled HAL 19 and HAL 43 to keyhole limpet hemocyanin (KLH) as a carrier, and this elicited syngeneic anti-idiotype in A.SW mice. A second problem was a reliable, rapid assay for screening hybridoma clones for syngeneic anti-idiotype. Most plate binding assays (radioimmunoassay or enzyme-linked immunosorbent assays [ELISA]) for screening hybridoma culture supernatants require a labeled anti-immunoglobulin to detect antibody binding to antigen on the plate. In this case, however, the antigen is a syngeneic antibody, so labeled anti-immunoglobulin or anti-allotype would bind directly to the antigen-coated plate. To circumvent this problem, we took advantage of the fact that both monoclonal antimyoglobins were of the IgG_1 subclass. Therefore, we used an enzyme-labeled anti-IgG_2 antibody (which does not bind IgG_1) as detecting reagent. Although this limited the hybridoma monoclonal antibodies which could be detected to IgG_2, it worked well. In collaboration with Drs. Frank Cuttitta and John Minna of NCI, we fused one spleen immune to HAL 19 anti-myoglobin and one immune to HAL 43 anti-myoglobin. We obtained a number of single cell clones of each, of which we selected five to seven of each to grow in quantity for further study. Several of these inhibited myoglobin-binding by the corresponding monoclonal anti-myoglobin against which they were made, suggesting that they were specific for the myoglobin binding site. None of the anti-HAL 19 antibodies bound HAL 43 and vice versa, although such crossreactive antibodies had been detected in the serum of the mice whose spleens were used for fusion. These antibodies should prove useful in characterizing the endogenous indiotype network regulation of the myoglobin response.

specific Ir gene control, and therefore might not be expected to impose any restriction on antibody specificity. Approaches to circumvent this are considered below (see proposal course).

Mechanisms of Antigen Presentation and Processing: Since Ir gene control appears to depend heavily on presentation of antigen in association with Ia on another cell (such as macrophage or B cell), it is important to work out the biochemical events in this process which is as yet poorly understood. Also, it has been suggested, on the basis of the apparent inability of T cells to distinguish conformation as antibodies do, that T cells "never" see native antigen, but only antigen after it has been "processed" by macrophages (meaning, cleaved into fragments). So far, processing is largely suggested by phenomenology, not understood biochemically. Do processing and presentation require obligatory cleavage into fragments, chemical association with Ia, or some other active mechanism? As a first approach to this question, we took advantage of having T cell clones which responded to a single defined epitope on native myoglobin and on a 22-residue fragment (132-153) of myoglobin, to ask whether presentation of native myoglobin required more "processing" than presentation of the already smaller fragment. We used a series of inhibitors of various phases of possible steps in macrophage processing. Monensin inhibits intracellular transport, leupeptin inhibits lysozomal proteases, and chloroquine and ammonium chloride may inhibit both transport and lysozomal digestion. Macrophages which were preincubated with myoglobin or fragment for two hours and washed were able to present that antigen to the T cell clone without any additional antigen in the culture. However, if the macrophages were first treated with chloroquine or monensin before preincubation with antigen (either myoglobin or fragment), presentation of native myoglobin was almost completely inhibited at concentrations of these inhibitors which did not affect the ability to present the fragment (132-153) to the same T cell clone. (At very high concentrations, these inhibitors appear to be nonspecifically toxic.) Similarly, leupeptin or ammonium chloride added during the preincubation of macrophages with antigen inhibited their ability to present native myoglobin but did not affect the presentation of fragment. These results suggest that presentation of native myoglobin require active "processing" steps including internalization and cleavage into fragments, whereas presentation of the same epitope on a smaller fragment to the same T cell clone does not require these steps (although it still required recognition in association with Ia).

Idiotype Network Regulation of the Antibody Response to Myoglobin: The idiotypes described earlier on our monoclonal antibodies to myoglobin, as seen by guinea pig anti-idiotypic antibodies (Kohno et al., J. Immunol. 128:1742, 1982), were not very commonly represented among serum antimyoglobin antibodies from the same strain (A.SW). To try to increase the production of one of these (the idiotype of monoclonal antibody 1), we injected A.SW mice with guinea pig anti-idiotype before immunization with myoglobin for

The T Cell Control of Antibody Specificity: A large body of circumstantial evidence has suggested to us that helper T cells can influence antibody specificity by selectively helping B cells which bind antigenic determinants (epitopes) which bear some steric relationship to the site recognized by antibodies (Berzofsky, Survey of Immunologic Research, 1983, in press). For instance, in the cases of determinant-specific Ir gene control of T and B cell responses to staphylococcal nuclease and myoglobin, a given strain made antibodies to a particular region of the molecule if and only if it had T cells which proliferated in response to that part of the molecule. To test this hypothesis, we needed to ask experimentally whether T cells specific for a single epitope or a limited subset of epitopes on myoglobin would influence the spectrum of antibodies made by a mixed population of B cells immunized with whole myoglobin. One approach is to use cloned T cells specific for a single known epitope. However, until the problem with non-specific or "bystander" help described above is resolved, we cannot use the clones for these experiments.

A second approach has been to use T cells from mice immunized with nonoverlapping fragments of myoglobin, 1-55 and 132-153. In several experiments T cells immune to (1-55) cultured with myoglobin-immune B cells and intact myoglobin led to the production of an increased proportion of antibodies to myoglobin which bound fragment (1-55). In contrast, T cells immune to fragment (132-153) led to an increased proportion of antibodies specific for the region 132-153 of myoglobin. However, in other experiments, especially ones in which high background production of antimyoglobin was observed in the absence of added T cells, this effect was less clear. In these cases, it is possible that residual myoglobin-specific T cells left in the immune B cell population provided help for antibody production to other sites, or that a subpopulation of B cells already activated by priming and boosting immunizations in vivo, was autonomously producing antimyoglobin. Several possible approaches to circumvent this problem are being tested. (See proposed course).

A third approach which has been tested is to take advantage of the determinant-specific Ir gene control described above. The recombinant strain B10.A(5R) does not make antibodies to the 132-153 region of myoglobin and its T cells do not proliferate to this fragment. However, T cells from B10.A(5R) mice neonatally tolerized with $(H\text{-}2^b \times H\text{-}2^d)F_1$ spleen cells will help $(B10 \times B10.D2)F_1$ B cells, which can make anti-(132-153) with syngeneic F_1 T cells. When we used neonatally tolerant B10.A(5R) T cells to help these F_1 B cells, they still made anti-(132-153), i.e. no restriction of specificity was imposed. However, the population of tolerized B10.A(5R) T cells that helps the F_1 B cells appears to be one which is specific for myoglobin plus $H\text{-}2^d$, not one specific for myoglobin plus B10.A(5R) Ia antigens. Therefore, it is not the same population which displays the determinant-

The discovery of a group of T cell clones specific for Lys 140 offered a unique opportunity. One of the monoclonal antibodies to myoglobin which we had studied had been found to bind to a site involving Lys 140 (Berzofsky et al., J. Biol. Chem. 257:3189, 1982). Thus we were in a position to test whether a high affinity monoclonal antibody to a site identical to or at least overlapping with the site recognized by a T cell clone could block myoglobin-stimulation of the clone. Previous attempts by many laboratories to block T cell proliferation with antibody to the antigen (rather than to the Ia) had been unsuccessful, and these negative results have remained a standing enigma that has been difficult to explain. When we added a slight molar excess of monoclonal antibody HAL 43 over myoglobin in the culture of the T cell clone, we found we could inhibit proliferation. The inhibition appeared specific because another monoclonal antibody with comparable affinity but specific for a different site on myoglobin, HAL 39, did not block. Thus, the antibody had to bind to the same epitope that stimulated the T cell in order to inhibit stimulation. Since the HAL 43 antibody does not bind the non-native fragment (132-153) which does stimulate the T cell clone, no blocking was observed when the fragment was used for stimulation. This serves as a control for nonspecific inhibitory effects. We believe that the reason such antibody inhibition of T cell proliferation has been so difficult to demonstrate in the past is that the concentration of antibodies in antisera specific for the same site seen by the majority of T cells is very low. For instance, in the B10.D2 T cell response to myoglobin, we found the immunodominant site which stimulates the majority of T cells to be centered on Glu 109, a site for which no antibodies have yet been described. Thus a heterogeneous antiserum to myoglobin would be expected to have very little, if any, antibody which could block the site recognized by the bulk of the T cells in an immune population. Only by singling out a minor T cell clone and a homogeneous monoclonal antibody to the same site could we see any inhibition.

Finally, we have examined the helper activity of these T cell clones in the in vitro antibody response to myoglobin described earlier (Kohno and Berzofsky, J. Immunol. 128:2456, 1982). Only one of about 10 clones tested so far was found to provide help for a B cell response to myoglobins. Using 2 million primed B cells, as few as 1000 or even 100 cloned T cells will help. Higher numbers of cloned T cells are inhibitory, perhaps because they overgrow the culture. The optimum myoglobin concentration for help by the T cell clones is 20-30 μg/ml, in the range required for T cell proliferation but 10- to 100-fold higher than the range usually used in the antibody response. Under these conditions, although induction of help is specific for myoglobin, the efferent limb appears to include nonspecific helper factors. For instance, if a mixture of myoglobin-immune and fowl gammaglobulin (FGG)-immune B cells is present, both populations will be helped. We are currently exploring conditions to eliminate this nonspecific component of T cell help (see below).

gene complex using allotype congenic mice. This negative result does not support (but is certainly not sufficient to disprove) the notion that T cell receptor variable regions are encoded among the immunoglobulin heavy chain genes.

In order to study the function and the receptors of these T cells in more depth, we prepared cloned long-term proliferating lines of B10.D2 myoglobin-specific T cells. When the fine specificity of individual clones was analyzed, two patterns emerged. One group of clones displayed the immunodominant specificity pattern indicative of the Glu 109 epitope. A second set of clones manifested a new crossreactivity pattern which pointed unambiguously to Lys 140 as critical residue. This group of clones also responded as well to the peptide fragment 132-153, containing Lys 140, as to the native myoglobin, whereas the Glu 109-specific clones did not respond to this peptide at all.

Mapping of the genetic restriction specificity of these clones (the Ia antigens required on antigen presenting cells) was facilitated by their total lack of alloreactivity. None of the B10.D2 clones was stimulated by myoglobin on B10($\underline{H\text{-}2^b}$), B10.BR($\underline{H\text{-}2^k}$), B10.A($H\text{-}2^{k/d}$ recombinant), or B10.A(5R)($H\text{-}2^{b/d}$ recombinant) presenting cells. All of the Glu 109-specific clones responded to myoglobin on B10.GD($\underline{I\text{-}A^d}$,$I\text{-}E/C^b$) presenting cells. Thus, these clones appear to be restricted to $\underline{I\text{-}A^d}$, in concordance with the mapping of the immunodominance of the Glu 109 specificity to $\underline{I\text{-}A^d}$ in bulk T cell populations. However, none of the Lys 140-specific clones responded to myoglobin on the B10.GD presenting cells. This result mapped their restriction specificity to the right of $\underline{I\text{-}A}$ (i.e., to the right of the recombination event in B10.GD). Since these did not respond to myoglobin presented on B10.A or B10.A(5R), both expressing $\underline{I\text{-}C^d}$, we hypothesized that they might be specific for $\underline{I\text{-}E^d}$. This molecule requires a beta chain which maps to the $\underline{I\text{-}A^d}$ region and an alpha chain mapping in $\underline{I\text{-}E^d}$. However, the alpha chain is relatively non-polymorphic, and can usually be replaced by the alpha chain of $\underline{I\text{-}E^k}$. The B10.GD recombinant, although carrying the beta chain gene in $\underline{I\text{-}A^d}$, does not express this because no alpha chain is produced by the $\underline{I\text{-}E^b}$ gene. However, the F_1 hybrid between B10.GD and an $\underline{I\text{-}E^k}$ strain such as B10.MBR will express a hybrid molecule $E^d_\beta E^k_\alpha$ which closely resembles the $E^d_\beta E^d_\alpha$ molecule of B10.D2. Six of the Lys 140 specific clones responded to myoglobin on this F_1 hybrid. This complementation mapped the genetic restriction of these clones to $\underline{I\text{-}E^d_\beta E^{k \text{ or } d}_\alpha}$ (the so-called Ia.23 specificity, as was confirmed by blocking with a monoclonal antibody specific for I-E(14-4-4 of D.H. Sachs). However, two of the Lys 140-specific T cell clones did not respond to myoglobin on thie F_1 hybrid. These may prove to be very interesting as they may be the first T cells to detect the small difference found biochemically between the Eβ chain of B10.D2 and of B10.GD or to detect the difference between the $E\alpha^d$ and $E\alpha^k$ chains. It is striking, however, that all of the $\underline{I\text{-}E}$-restricted clones have so far been specific for the Lys 140 epitope whereas all of the I-A restricted clones have been specific for the Glu 109 epitope. This emphasizes the importance of T cell recognition of Ia in the selection of specificity for different epitopes on exogenous antigens.

myoglobins with Glu 109, whereas T cells immune to horse myoglobin (bearing Asp at 109) were stimulated only by myoglobins with Asp 109. Thus, instead of finding enormous heterogeneity in the T cell population, we identified residue 109 as an immunodominant site seen by the majority of B10.S T cells (Berkower et al., Proc. Natl. Acad. Sci. USA 79:4723, 1982).

Current Results:

T Cell Specificity and Myoglobin-Specific T Cell Clones: In order to investigate the role of Ir genes in controlling the epitope-specificity of the T cell response, we examined the H-2^d high responder strain, B10.D2, for which two Ir genes had been mapped, by the sme approach mentioned above for the B10.S stain (H-2^s) for which only one myoglobin Ir gene has been identified. B10.D2 lymph node T cells immune to sperm whale myoglobin demonstrated the same pattern of crossreactivity with a panel of about 12 myoglobins that had been seen in the B10.S strain, again pointing to an immunodominant antigeneic determinent (or epitope) centered around Glutamic acid 109. However, in this case, California sea lion myoglobin did not stimulate even though it carries Glu 109. A comparison of the sequence of sea lion myoglobin with that of closely related harbor seal, which did stimulate, revealed that a substitution of Gln for His at position 116 could destroy crossreactivity. Thus, the immunodominant epitope for B10.D2 T cells spans the region from Glu 109 to His 116, and most probably contains other residues as well, although it does not seem to go as far as residue 118, at which substitutions appear to have no effect.

The immunodominance of the Glu 109 epitope was mapped to the I-A^d (or K) region of H-2 using the B10.GD recombinant strain (I-A^d, I-E^b, I-C^b), which manifested the same fine specificity as the B10.D2 T cells. In contrast, the recombinant strain B10.A(5R) (I-A^b, I-E^k, I-C^d) showed a completely different pattern of fine specificity, crossreacting only with dwarf-sperm whale myoglobin, but none of the other myoglobins in the panel. This pattern was not sufficient to map the epitope unambiguously, but the most likely candidates for critical residues were 45, 66, and 151. Since the B10.D2 possesses both the I-A^d Ir gene of the B10.GD and the I-C^d Ir gene of the B10.A(5R), is the latter specificity pattern maps to I-C^d, one might expect the B10.D2 T cells to display a combination of both crossreactivity patterns. However, the subset of myoglobins which cross-stimulate the B10.A(5R) T cells are completely contained within the set which cross-stimulate B10.GD and B10.D2 T cells, so no additional corssreactivities could be expected for the combination of B10.GD (I-A^d) and B10.A(5R) (I-C^d ?) patterns compared to the B10.GD alone. Therefore, we cannot be sure whether B10.D2 T cells display both specificities. Alternatively, the B10.A(5R) pattern may depend on the hybrid I-$E^{b\,k}_{\beta\,\alpha}$ molecule of the B10.A(5R).

In contrast to the effect of H-2 linked genes on fine specificity, we could detect no effect of genes linked to the immunoglobulin heavy chain structural

repertoire of any strain of T cells capable of recognizing myoglobin in association with low responder Ia antigens or B cells (Kohno and Berzofsky, J. Exp. Med. 156:791, 1982)

In order to identify the structual features of sperm whale myoglobin that make it immunogenic in high responders and nonimmunogenic in low responders, we studied the Ir gene control of the response to other myoglobins. The antibody response to equine myoglobin, which shares the three-dimensional structure and 87% of the sequence of sperm whale myoglobin, was found to be under completely different Ir gene control from that to sperm whale myoglobin. Mice (such as B10.BR) that were low responders to sperm whale myoglobin were high responders to equine myoglobin. Moreover, the Ir genes mapped in different loci. The antibody response to equine myoglobin was controlled by complementing Ir genes genes in I-A and H-2D. We were surprised to find an Ir gene for an antibody response to a soluble protein antigen mapping in H-2D, rather than the I region, since none had mapped there before. Thus, either the mechanism of action of this Ir gene is different from those in the I region, or equine myoglobin is the first soluble protein antigen recognized in association with a class I (H-2K/D) rather than Ia antigen, or there is a class II (Ia) antigen mapping close to the H-2D locus (Berzofsky et al., J. Immunol. 128:737, 1982).

In order to understand the mechanism of action and specificity of these Ir genes, we have also been studying the myoglobin-specific receptors of the T cells and B cells involved in the response. A previous annual report described six monoclonal antibodies to myoglobin, all of high affinity (10^8 to 10^9 M^{-1}). We were able to identify the antigenic determinants of myoglobin recognized by 3 of these. At least two of the antibodies recognize topographic antigenic determinents consisting of amino acid residues from distant parts of the primary sequence, brought together by the folding of the molecule (Berzofsky et al., J. Biol. Chem., 257:3189, 1982). These determinants are distinct from those reported by others. In a previous report we also described the idiotypes of these monoclonal antibodies. Interestingly, some which have distinct antigenic specificity nevertheless share idiotypes, some in the combining site (Kohno et al, J. Immunol. 128:1742, 1982).

Finally, to study the sites of myoglobin recognized by T lymphocytes, we examined the crossreactivity of T cells immunized with one myoglobin and stimulated to proliferate with a series of myoglobins with known amino acid substitutions. B10.S mice are high responders to both sperm whale and horse myoglobin, and antibodies to one crossreact extensively with the other. Nevertheless, B10.S T cells immune to sperm whale myoglobin do not cross-react with horse myoglobin and vice versa. Examining a series of 15 myoglobins sharing some of the sequence differences between sperm whale and horse, we found a strict correlation with residue 109; that is, T cells immune to sperm whale myoglobin (bearing Glu at 109) were stimulated only by

cells, even though they could be helped by FGG-specific T cells (Kohno and Berzofsky, J. Exp. Med. 156:1486, 1982).

Since this type of T cell-B cell genetic restriction, as opposed to a T-cell macrophage restriction, had been seen previously in non-Ir gene controlled systems in cases in which $Lyb5^-$ but not $Lyb5^+$ B cells functioned (Singer et al., J. Exp. Med. 154:501, 1981; Asano et al., J. Exp. Med. 154:1100, 1981), we examined the Lyb phenotype of the B cells functioning in our in vitro secondary response by comparing the responses of $Lyb5^-$ populations from (CBA/N x DBA/2) F_1 male mice, bearing the x-linked CBA/N defect, with those of $Lyb5^+$ and $^-$ B cells from male progeny of the reciprocal cross carrying normal x chromosomes, but otherwise genetically identical. The response of the $Lyb5^-$ B cells was actually slightly higher than that of the mixed B cell population, an indication that the response in our culture system may involve primarily $Lyb5^-$ B cells. This result supports the hypothesis that Ir gene control of T cell-B cell interaction, as opposed to T cell-macrophage interaction, will be seen when the B cells participating are primarily $Lyb5^-$.

In order to explore the ability of low responder T cells to function if provided with F_1 or high responder B cells, but to do so without allogeneic effects and without intentionally altering the T cell repertoire by use of chimeras, we used T cells from mice neonatally tolerized to the high responder H-2 antigens. Newborn mice, within 24 hours of birth, were injected i.v. with 2×10^7 unirradiated spleen cells from adult F_1 mice. They were immunized with myoglobin at 8-12 weeks of age, and at the time of the experiment were confirmed to be tolerant by the absence of a mixed lymphocyte reaction (MLR) or cell-mediated cytotoxicity (CML) against the tolerated H-2 hyplotype. However, the spleen cells from these mice were shown by fluorescence activated cell sorting (kindly performed by S.O. Sharrow of the Immunology Branch, NCI) to contain 2-4% residual F_1 cells, whereas the thymuses of these mice contained no detectable F_1 cells.

Using this experimental model, we found that T cells from both high responder and low responder mice, when tolerant to F_1 (and therefore to both high and low responder H-2 types), had the same phenotype as each other and as the F_1 T cells. All these types of T cells could help F_1 and high responder B cells but not low responder B cells, even in the presence of adequate F_1 macrophages. Control experiments excluded artifacts such as allogeneic effects on help, or help by residual F_1 T cells in the tolerized animals. Thus, operationally, the Ir gene defect was manifested by the inability of low responder myoglobin-specific B cells (which could be demonstrated to be present using carrier-specific help) to receive effective help from myoglobin-specific (as opposed to carrier-specific) T cells of any origin, high responder, low responder, or F_1 hybrid. However, this result could be due either to an intrinsic H-2-linked B cell defect in presenting myoglobin to T cells for help, or to the absence (or rarity) from the T cell

of soluble antigen. This stimulation manifested the same Ir genetic restriction for macrophage source, and could be inhibited by inclusion of appropriate monoclonal or conventional anti-Ia antibodies, without complement, during the pretreatment with antigen (Berzofsky and Richman, J. Immunol. 126:1898, 1981).

The limitation of T cell proliferative responses is that they involve only T cells and macrophages, not B lymphocytes which produce antibody. In order to study the mechanisms of determinant-specific Ir gene control of the antibody response to myoglobin in vitro, we have now developed a modified Mishell-Dutton culture system in which secreted antibody specific for myoglobin can be measured in the culture supernatants by a solid phase radioimmunoassay. By this method, we can also measure the fine specificity of the supernatant antibodies for different fragments of myoglobin. The in vitro spleen cell antibody response to sperm whale myoglobin was found to be controlled by the same two Ir genes in I-A and I-C which controlled the in vivo response, at the level of individual determinants on different fragments of myoglobin. In addition, we extended the mapping to recombinant H-2 haplotypes not previously studied. Also, we found this in vitro antibody response to be macrophage and helper T-cell dependent, as shown by experiments depleting these cells, and reconstituting with purified T cell or macrophage populations.

In order to determine which cells and cell interactions are involved in mediating this determinant-specific Ir gene control of the antibody response to myoglobins, we selectively mixed T cells, B cells, and macrophages from different sources. In most cases, two of the cell types derived from (high responder x low responder) F_1 hybrid mice, and the origin of the third cell was varied. When the T cells were of F_1 origin, no allogeneic effects were to be expected. However, when the T cells were the cell being varied, T cells from neonatally tolerized mice had to be used to avoid allogeneic effects. First, myoglobin-immune helper T cells from (high responder x low responder) F_1 hybrid mice (plus F_1 macrophages) would help T cell-depleted populations of B cells plus macrophages from myoglobin immune high responder mice but not myoglobin immune low responder mice. To be sure that the low responder B cells were adequately primed in vivo, we used B cells from mice immunized with myoglobin coupled to an immunogenic carrier, fowl gamma globulin. We demonstrated that low responders immunized with myoglobin-fowl gamma globulin (FGG) responded as well as high responders, and that their B cells would make antimyoglobin when cultured with myoglobin-FGG if provided with FGG-specific syngeneic helper T cells. Thus, low responder mice contained competent, myoglobin-specific B cells. However, even these B cells cells were not helped by myoglobin-specific T cells of syngeneic or F_1 origin even in the presence of F_1 macrophages. The presence of suppressor cells in the B cell population was ruled out by showing that the low responder B cells did not suppress the response of unseparated F_1 spleen cells. We were forced to conclude that one site of Ir gene function was the failure of competent, myoglobin-specific B cells to be helped by myoglobin-specific T

with 1 x 10^6 irradiated spleen cells, more myoglobin, and 5% IL-2-containing culture supernatant from EL4 T lymphoma cells stimulated with phorbol myristic acetate. After 7 to 10 days, the wells showing growth were expanded into large wells (24-well plate, Costar No. 3524) with additional antigen, spleen cells and 1% IL 2. After one week of further expansion, the cells were rested one week. These T cell clones were expanded by rounds of stimulation and rest without IL 2, and tested for antigen specificity and H-2 restriction in the standard proliferation assay, except that 1 x 10^4 T cells were cultured with 5 x 10^5 irradiated syngeneic spleen cells in the presence of various myoglobins, or with 5 x 10^5 allogeneic or partially allogeneic spleen cells in the presence or absence of sperm whale myoglobin.

Major Findings:

Background: We have previously studied the in vivo serum antibody response and in vitro T-lymphocyte proliferative response to a natural protein antigen, sperm whale myoglobin (Mb), and shown that both of these responses are controlled by the same Ir genes (Berzofsky, J. A., J. Immunol. 120:360, 1978). Using inbred strains of mice which differ only in the H-2 complex (known as congenic strains), we demonstrated that two different genes, mapping in distinct subregions (I-A and I-C) of the I region of H-2, controlled the responses to different antigenic determinants (distinct chemical sites) on the same antigen molecule - the first example of such independent control of responses to different chemical moieties on the same protein molecule. Moreover, the gene which controlled the T-cell proliferative response to a given determinant of myoglobin also controlled the production of antibodies specific for that same region of the molecule - an indication of parallel control of T-lymphocyte recognition and activation of B lymphocytes with similar specificity (Berzofsky et al., Proc. Natl. Acad. Sci 76:4046, 1979).

One level at which this Ir-gene-controlled choice of antigenic determinants recognizable is mediated is the recognition by T-lymphocytes of antigen in association with cell surface structures on macrophages, which serve as antigen presenting cells. These surface structures, which differ in different inbred strains of mice, are encoded by genes also mapping in the I region of H-2 , and are therefore designated Ia (for I-associated) antigens. In our experiments, myoglobin-immune lymph node T cells, depleted of macrophages, from (high responder X low responder) F_1 hybrid mice were held constant as the source of responding (proliferating) T cells, and the cultures were reconstituted with liver macrophages from high responder parental mice with both Ir genes, low responder parental with neither gene, or recombinant mice with only one gene or the other. We found that the magnitude of response, and the selection of which fragments of myoglobin could stimulate these F_1 T cells in vitro was determined by the source of the macrophages, and corresponded exactly to the response pattern of the strain of mice from which the macrophages were obtained (Richman et al., J. Immunol. 124:619, 1980). Macrophage pretreated with antigen could also stimulate immune T cells in the absence

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB04018-07 MET

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Study of Human Immune Defense Mechanisms and Its Control

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Andrew V. Muchmore, M.D., Senior Investigator, Metabolism Branch, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH Metabolism Branch

SECTION

Cellular Immunology

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

A

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

These studies are designed to explore the role of cell surface lectin-carbohydrate interactions, in cellular recognition, co-operation and regulation. Special emphasis is placed on the role of complex carbohydrates and glycoproteins in the regulation of immune response during human pregnancy. A mannose 1-6 dimer of mannose and a more complex glycoprotein of approximate molecular weight 55 kilo daltons have been purified from human pregnancy urine. Both compounds are being extensively characterized for their immunoregulatory properties.

A second set of studies is examining a T independent antigen specific model of human antibody production in vitro. These studies are concentrating on 1) cellular requirements, 2) B cell subset diversity, and 3) fine specificity of V region products.

Other Professional Personnel:

Samuel Broder, M.D.	Deputy Clinical Director		NCI
R. Michael Blaese, M.D.	Senior Investigator	MET	NCI
Basil Golding	Clinical Associate	MET	NCI
Dean Mann, M.D.	Senior Invest.	IMM	NCI
Jean Decker, B.S.	Chemist	MET	NCI
Bo Nielsen	Visiting Scientist		

Project Description:

Objectives: Recent evidence from numerous laboratories has confirmed and elaborated on our early studies which suggested that cellular co-operation, recognition and regulation may in part be mediated by endogenous lectins and complex carbohydrate receptors. These early studies examined the potential role of carbohydrate recognition in assays of spontaneous monocyte mediated cytotoxity, antigen specific T cell proliferation, and DR antigen recognition. By blocking various in vitro immunologic reactions with simple sugars we could begin to dissect the role of carbohydrate recognition. These studies; however, were limited by the simple (but defined) nature of these blocking sugars. We have undertaken a major project aimed at purification of complex carbohydrates and glycoproteins from human pregnancy urine in an effort to characterize more complex sugars with immunoregulatory capacity. We predict that some of these purified complex saccharides will in fact represent specific receptors for endoginous lectins. We chose human pregnancy urine as a starting material because 1) previous work from our laboratory has shown the presence of immunoregulatory glycoproteins, 2) urine is a rich source of partially purified complex sugars and glycoproteins, and 3) such immunoregulatory molecules may play an important role in maintanance of the fetal allogroft.

A second project is utilizing a sensitive assay for the production of TNP specific human antibody using TNP - brucella abortus as an antigen. These studies are examining the cellular requirements, the fine specificity, the precursor frequency, and the B cell subsets involved in the production of specific antibody in vitro. This assay is also being used to screen the T independent response of B cells from a variety of patients with immune deficiency.

Methods Employed: In vitro cellular cytotoxicity systems have been developed for antibody dependent cellular cytotoxicity, mitogen induced cellular cytotoxicity, cell mediated lympholysis, and spontaneous monocyte mediated cytotoxicity using a sensitivity micro ^{51}Cr release assay for various target cells. These results are correlated with standard in vitro and in vivo assays of cell mediated and humoral mediated immunity. We have also employed standard methods of protein and carbohydrate purification including high pressure liquid chromotography, thin layer

chromatography, SDS acrilyamide gels, and ion exchange chromatography coupled with sensitive protein detection and radio iodination procedures.

Major Findings: Biochemical characterization. First morning void specimens from pregnant patients were pooled and run over insolubilized concanavalin A columns. Material which adhered to the column was eluted with 250 mM methyl mannose and lypholyzed. The alpha methyl mannose was separated from larger molecular weight material on a Sephadex G-10 column. This material was lyopholyzed and separated On a G-75 column in a volatile acetate buffer. Immunosuppressive activity was found in the first and fourth peaks. The smaller m.w. material was further separated on a 120 cm Fractogel 40S column in distilled water. Finally, the smaller suppressive material was purified using thin layer chromatography and spots were scrapped and eluted. This small m.w. material was sent to Dr. B. Nielsen who using a combination of mass spectroscopy, hydrolysis and methylation, has deduced the structure to be mannose 1-6 mannose. We are currently confirming this observation with a separate purification. The larger molecular weight material was further purified using DEAE ion exchange chromatography and final purification on Fractogel 55S. Immunosuppresive activity was found in the 55KD region. Analysis of this material on 12.5% SDS gels shows 2-3 lines with one line composed of contaminating albumim. Initial attempts to raise a hetero-antibody have failed and a major effort is in progress to raise an appropriae antibody.

Biologic activity. The manose dimer reversibly suppress antigen specific proliferation at concentrations of 1-5 microgram/cc. This material has no effect on the generation of spontaneous mediated cytotoxicity. The material is heat stable.

The larger molecular weight material inhibits both antigen specific proliferation and spontaneous monocyte mediated cytotoxicity at concentrations as low as 50ng/ml. This material is heat labile and only active if added at the initiation of culture. Delayed addition (>24 hours) fails to inhibit. This material is stable to a pH of 4.5 for 24 hours.

Major Findings: T cell independent antibody production in vitro: This assay has been adopted to a micro culture technique and has been successfully employed to measure B cell precursor freqency in cord blood and patients with the Wiskott-Aldrich syndrome.

Spontaneous cytotoxicity. We have continued our interest in spontaneous cytotoxicity and have characterized several cell lines as well as developed a unique B cell line which is spontaneously lytic and constitutively synthesis IL-1.

Significance to Biomedical Research:

Our studies on both monocyte mediated cellular cytotoxicity and factor B dependent cell mediated cytotoxicity have had several major implications. Our data suggest that a variety of "nonspecific" in vitro killing assays are in reality quite specific and represent target specific cell surface sugar recognition. Our phylogenetic studies imply this phenomonen is ubiquitous. We believe this type of cytotoxicity represents an important aspect of host immune defense which is independent of prior antigen exposure. Our studies with immunoregulatory compounds from human pregnancy urine offer the exciting opportunity to gain important insights into immunologic maintenance of the human placenta as well as offering the theoretical possibility that these materials may prove to be safe non toxic naturally occuring immunosuppressive agents.

Proposed Course:

Our laboratory will continue to characterize the nature of carbohydrate receptors and the effect of blocking these receptors on in vitro and in vivo immune reactivity. We will extend our studies to an in vivo model in efforts to control immunologic responses in an intact animal in order to gain insight into possible approaches and therapy of diseases which are the result of over active immune responses. We will continue to biochemically characterize the 55KD material from pregnancy urine and we plan to develop an ELISA or radioimmunoassay for measurement of various human tissues as well as serum and urine. Once the 55 KD material is purified to homogenity, a major emphasis will be placed on its biologic significance in pregnancy.

Publications:

Hall, R.E., Blaese, R.M., Davis, A.E., Decker, J.M., Jack, B.F., Colter, H.R. and Muchmore, A.V.: Co-operative interaction of factor B and other complement components with mononuclear cells in the antibody independent lysis of xerogeneic erythrocytes. J. Exp. Med., 156:834-843, 1982.

Muchmore, A.V. and Kleinerman, E.S.: Spontaneous monocyte mediated cytoxicity in man: evidence for T helper activity in MK cells and other natural effector cells. R. Herberman, (Ed.): Academic Press, 1982, pp. 815-819.

Hall, R.E., Muchmore, A.V., Decker, J.M., and Blaese, R.M.: Induction of cytotoxic effector activity in the HL60 promyelocytic cell line by incubation with PMA: A model system of human spontaneous monocyte mediated cytotoxicity. Cell. Immunol. 76: 58-68, 1983.

Blaese, R.M., Tosato, G., Greene, W.C., Fleisher and Muchmore, A.V.: The role of cell surface lectin-carbohydrate interactions in cellular recognition cooperation and regulation. The American Journal of Pediatric Hematology/Oncology 5: 199-206, 1983.

Littman, B.H., Hall, R.E., and Muchmore, A.V.: Lymphokine and PMA regulation of complement synthesis using U937. Cellular Immunology. In press.

Muchmore, A.V., Megson, M., Decker, J.M., Knudson, P., Mann, D.L., and Broder, S. Inhibitor a tivity of antibodies to human Ia-like determinants: comparison of intact and pepsin digested antibodies. J. Immunol. In press.

Broder, S., Megson, M., Muul, L., Muchmore, A.V., Blaese, R.M. and Mann, D.L.: Activation of regulatory cells by antibodies to humans Ia-like antigens. In Ballieux (Ed.): Human B Lymphocyte Function: Activation and Immunoregulation. Raven Press, 1982, pp. 289-296.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CB04017-07 MET

PERIOD COVERED
October 1, 1982 through September 31, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
David L. Nelson, M.D., Senior Investigator, Metabolism Branch, NCI, NIH

COOPERATING UNITS *(if any)*

LAB/BRANCH
Metabolism Branch, DCBD, NCI

SECTION
Immunophysiology Section

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	7	

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

A

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

Studies were undertaken to measure specific cytotoxic T-cell responses and humoral antibody responses by B-cells in vitro in normal individuals and patients with immunologic deficiency states. Patients were heterogeneous with regard to their ability to generate cytotoxic T-cells in vitro. Most patients with hypogammaglobulinemia produced cytotoxic T-cells normally, while patients with the Wiskott-Aldrich syndrome and ataxia-telangiectasia produced almost no cytotoxic T-cells. Cells from normal individuals produce specific antibody in vitro when stimulated with antigen. This was also true for 5 of 11 patients with hypogammaglobulinemia who produced no antibody in vivo. Thus a subset of hypogammaglobulinemic individuals have an immune deficiency which can be corrected in vitro. In contrast none of 7 ataxia-telangiectasia patients and none of 5 Wiskott-Aldrich syndrome patients produced antibody in vitro. Defects in the production of cytotoxic T-cells and specific antibodies may contribute to the increased incidence of neoplasia observed in immunodeficiency diseases.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:

Robert Yarchoan, M.D.	Investigator	MET	NCI
Claudia Quijano, M.D.	Guest Worker	MET	NCI
Brian R. Murphy, M.D.	Senior Investigator	LID	NIAID

Project Description:

Objectives: Exposure to infectious agents and other foreign antigens elicits biologic response phenomena in host tissues which may confer protection to the host organism upon subsequent exposure to the same antigen(s). These biologic phenomena are termed immune responses and have generally been divided into two major types 1) cell-mediated responses which require the immediate presence of immune cells for their effects to be manifest, and 2) humoral responses in which cells elaborate soluble molecules such as antibody which may exert their protective effects at some distance from the immune cells which produce them. Studies were undertaken to investigate the biology of cellular and humoral immune responsiveness in normal individuals and in patients with a variety of immunodeficiency diseases in which congenital and acquired defects in host reponsiveness are associated with an increased incidence of infection and malignant tumors. Particular emphasis was placed on studying the mechanisms underlying perturbations of immune function in these patients which might account for their increased incidence of neoplasia.

Methods Employed: Over the past year, special emphasis was placed on the study of antigen-specific cellular and humoral immune responses by human peripheral blood leukocytes in vitro. Two major classes of immune responses have been under investigation: 1) cell-mediated cytotoxicity responses by leukocytes whose development is thymus dependent (T-cells), and 2) humoral antibody responses produced by a second class of leukocytes, (B-cells) whose development is bone marrow dependent and requires T-cells for full maturation. Both T-cell and B-cell immune responses are highly dependent on monocytes which are a third type of mononuclear leukocyte. In vivo, these cellular and humoral responses normally occur concomitantly in a highly ordered fashion with multiple cellular interactions occurring among sub-populations of T-cells, subpopulations of T-cells with B-cells and macrophages, and B-cells with macrophages. These cellular interactions result in a finely regulated response which is promptly initiated and appropriately terminated following antigenic exposure.

A major component of host responsiveness to a variety of antigens may take the form of tissue destruction (cytolysis) whereby antigen bearing cells are lysed and eliminated. Cells capable of directly mediating such cytolytic events are termed cytotoxic effector cells. Although we have demonstrated that several types of leukocytes can function as cytotoxic killer cells, we have recently focused our attention on cytotoxic T-cells which possess specific cell surface receptors for antigens expressed on those cells which undergo lytic events (target cells). Cytotoxic immune T-cells are undetectable in the peripheral blood of non-immunized individuals. Human cytotoxic T-lympho-

cytes (CTL) with receptor specificities for surface antigens present on the cells of unrelated individuals may be generated during seven days of in vitro culture in which density gradient centrifugation prepared responder peripheral blood mononuclear leukocytes are immunized by coculture with mononuclear leukocytes from a non-related individual whose proliferative capacity has been inhibited by irradiation. In such cultures, responder CTL precursors proliferative and mature into cytotoxic effectors whose lytic activity is measured by a 6 hour radioisotopic release assay employing ^{51}Cr labelled target cells from the stimulating cell donor. These immune CTL recognize a series of target cell surface molecules termed transplantation or histocompatibility antigens, which differ among individuals of the same species (alloantigens) and these cytotoxic effectors are therefore termed alloimmune CTL. Alloimmune CTL probably play a major role in the host rejection of histoincompatible allografts such as that occurring in kidney or heart transplantation and also in graft versus host disease in which an immunoincompetent host receives a graft of immunocompetent T-cells and the donor graft attacks host tissues.

As such reactions are distinctly uncommon in nature, it has been difficult to explain these strong alloimmune responses. Recent studies have suggested that alloimmune responses are reactions to foreign MHC antigens which happen to "cross react" with self-MHC and foreign antigen (i.e., self-MHC restricted responses, vide infra). Thus the alloimmune repertoire was probably originally generated and continues to be maintained by stimulation with a series of exogenous antigens. As such, it may represent a summation of the immune repertoire for self MHC plus X.

Immune CTL have also been produced with receptor specificities for various chemical and viral antigens. Studies have demonstrated that individuals immunized either in vivo or in vitro with chemical haptens or viral antigens produce CTL which will lyse homologous antigen bearing autologous (self) target cells but will lyse antigen bearing target cells from other individuals only when the immune CTL and the target cell share genetically determined histocompatibility antigens. The relevant histocompatibility antigens which must be shared between target and immune CTL are those encoded by a genetic locus termed the major histocompatibility complex (MHC) which in humans codes for the human leukocyte antigens (HLA) A, B, C, DR, and D.

These cytotoxic T-cells with receptor specificities for foreign antigen plus autologous MHC antigens are termed self-MHC restricted CTL. Evidence from studies in experimental animals has suggested that self-MHC restricted CTL play a major in vivo role in the recovery from virus infections, immunologic surveillance against neoplasia, and deleterious autoimmune phenomena. In humans we have produced CTL with receptor specificities for chemical haptens (trinitrophenol-TNP) and autologous MHC antigens in vitro by seven days primary immunization of responder peripheral blood mononuclear leukocytes with hapten modified autologous leukocytes, followed by five additional days of secondary in vitro stimulation with fresh or cryopreserved autologous haptenated luekocyes. Lytic T-cell activity is detected by a 6 hour radioiso-

tope release assay employing ^{51}Cr labelled TNP-modified target cells. Human CTL with specificities for viral antigens and self MHC gene products are produced in vitro by culturing responder peripheral blood mononuclear leukocytes with infectious allantoic fluid containing influenza A/Hong Kong virus or irradiated influenza virus infected autologous leukocytes for seven days. Such cultures proliferate and generate CTL whose lytic activity is tested on autologous or allogeneic virus infected targets in a short term ^{51}Cr release assay.

The second major area of emphasis centered on the assessment of humoral immune function by human peripheral leukocytes in vitro. In these studies we concentrated on measuring specific humoral antibody production by B-lymphocytes. The presence of specific antibody in biological fluids can be detected by a variety of methods including agglutination with particulate antigens, hemolysis employing antigen coated erythrocytes and radioimmunoassays. Each of these methods offers differing levels of sensitivity in detecting specific antibody. Recently, a very sensitive technique for measuring specific antibodies in the nanogram to picogram per milliter range has been described which involves the detection of antigen bound immunoglobulin through an enzymeconjugated second antibody directed against the bound first antibody. This technique has been termed the Enzyme Linked ImmunoSorbent Assay (ELISA). In a usual assay, nanogram to picogram per milliter range has been described which involves the detection of antigen bound immunoglobulin thru an enzyme conjugated second antibody directed against the bound first antibody. This technique has been antigen (i.e. virus) is first allowed to bind nonspecifically in the wells of a mutliwell plastic plate and non-bound antigen is washed away. A source of putative antibody (human serum or in vitro human leukocyte culture supernatant) against the bound antigen is then added, incubated, and non-bound antibody is washed away. Next, an enzyme (alkaline phosphatase) conjugated heterologeous (rabbit) antibody directed against human antibody is added, incubated, and unbound conjugated antibody is washed away. Finally, enzyme substrate (paranitrophenolphosphate-PNPP) is added, incubated, and the conversion of colorless PNPP to yellow paranitrophenol (PNP) product is measured in a spectrophotometer. The amount of substrate PNPP converted to product PNP is related to the amount of enzyme conjugated antibody bound which in turn is proportional to the amount of human anti-virus antibody bound to the immobilized antigen. Such an ELISA employing purified whole influenza virus as the antigen was chosen for the measurement of human specific antibody production in vitro. The ELISA which has been developed is rapid, sensitive and antigen specific. To assess specific anti-virus antibody production in vitro by human B lymphocytes peripheral mononuclear leukocytes are first washed extensively with tissue culture media to remove residual traces of serum antibody adherent to the cells. Then, mononuclear leukocytes or purified subpopulation of these leukocytes (T-cells, B-cells) are cultured in vitro with infectious alloantoic fluid containing the antigenically distinct influenza viruses A/Hong Kong (A/Hong Kong/8/68-x-31 [H3N2]) or B/Hong Kong (B/Hong Kong/8/73/); or with formalin-inactivated, zonally purified A/Aichi (A2/Aichi/68 MN 25241 [H3N2]) and B/Hong Kong (B/HK/15/72 Rx 3560-1). After twelve days of in

vitro culture, cumulative secretion of anti-influenza A or anti-influenza B virus antibody into culture supernatants is detected by ELISA.

In order to better place these in vitro studies of antibody production in perspective with regard to in vivo events, a limited number of studies were also performed to analyze the serum and secretory humoral immune responsiveness of volunteers undergoing intranasal immunization with live cold-adapted influenze A/Alaska/77(H3N2) or A/Hong Kong/77 (H1N1).

In other studies, total immunoglobulin of various isotypes (IgG, IgA, IgM, IgE) secreted into culture supernatants by human mononuclear cells and subsets of such cells activated in vitro by a series of polyclonal activators was measured by a "sandwich" ELISA. In such an assay the ladder of reagents from the solid phase up are: 1) anti immunoglobulin, 2) immunoglobulin, 3) alkaline phosphatase-conjugated-anti-immunoglobulin and, 4) substrate PNPP. The amount of PNPP converted to product PNP by anti-immunoglobulin bound alkaline phosphatase is proportional to the quantity of immunoglobulin in the middle of the "sandwich".

Major Findings: Cytotoxic T-Cell Responses by Human Peripheral Blood Leukocytes In Vitro: Studies were continued to define the cellular requirements for the in vitro generation of alloimmune and self-MHC restricted CTL, and the potential of cells from patients with immunodeficiency diseases to produce self-MHC restricted CTL. Previously, we had demonstrated that highly purified human T-cells were necessary and sufficient as responders in the production of alloimmune and self-MHC restricted CTL when stimulated with irradiated allogeneic leukocytes and irradiated virus infected autologous leukocytes, respectively. Additional studies were undertaken to define the requirement for monocytes in both cytotoxic systems. Peripheral blood mononuclear leukocytes containing T-cells, B-cells and monocytes were rigorously depleted of monocytes by sequential removal of cells capable of ingesting particulate iron and then cells possessing receptors for the chrystalyzable fragment (Fc) of immunoglobulin G. Such monocyte-depleted lymphocytes did not generate self MHC restricted CTL when influenza virus itself was added to these cultures but could generate virus specific CTL when mixed with irradiated adherent monocytes which themselves did not contain CTL precursors. Thus the in vitro generation of virus-specific self MHC restricted CTL was shown to be dependent on both T-cells and monocytes. In additional studies, the presence of adherent monocytes bearing antigenic specificities recognized by the heteroantiserum anti-p, 23,30 which recognizes human antigens similar to murine Ia (immuneassociated) antigens were shown to be required for the generation of influenza specific CTL. When responder cells for the generation of alloimmune CTL were equally rigorously monocyte-depleted, the generation of CTL was diminished but not abolished. However, when both responder and the irradiated stimulator cells were monocyte-depleted no CTL were produced. The addition of irradiated adherent monocytes from either the responder or the stimulator individual restored the response even though neither population of monocytes contained CTL precursors. Thus the generation of

human alloimmune CTL in mixed leukocyte cultures was dependent on monocyte function in the responder cells only when the irradiated stimulator population was monocyte-depelted. Since mixed leukocyte cultures and the generation of alloimmune CTL are routinely used for the assessment of immunocompetence in many patient studies, this observation is critical to the interpretation of these results. Monocyte defects within the responder cell population may only be observed when a stimulator cell population of peripheral blood leukocytes is devoid of monocytes. On the other hand, when virus immune CTL are produced by the direct addition of virus to peripheral blood mononuclear leukocytes, the absence of CTL production can result from T-cell and/or monocyte defects in the responder cell population.

We first focused our studies on examining the capacity of peripheral blood leukocytes from immunodeficiency disease patients to produce antigen-specific self-MHC restricted CTL. Given the observation that these CTL are restricted to recognize antigen and self-MHC gene products, one might ask how these receptor specificities are acquired. Studies of the cellular maturation processes and cell-cell interactions required for the production of virus-specific self-MHC restricted CTL in experimental animals have suggested the following scheme: 1) bone marrow stem cells differentiating into T-cells within the thymus develop receptor specificities for those MHC gene products expressed on the epithelium of the thymus prior to antigenic exposure, 2) these receptor specificities which then constitute the repertoire of MHC gene products considered as self are stable in the peripheral post thymic T-cell population, 3) T-cells expressing receptor specificities for foreign antigens plus self-MHC gene products are then selected from this peripheral T cell pool when antigen is encountered in association with irradiation resistant lymphoreticular cells (? monocytes), and 4) the maturation of such antigen-specific self restricted cells and may be positively and negatively regulated by other T-cell subsets termed helper-and suppressor-T cells; respectively.

Since results in experimental animals suggested that self-MHC restricted CTL play a major role in the recovery from viral infections and the lysis of autologous tumor cells, studies were undertaken to assess virus-specific self-HLA restricted reponses in patients with immunodeficiency diseases who have frequent infections and an increased incidence of neoplasia. One such immunodeficiency disease is termed common variable hypogammaglobulinemia. These patients have low levels of antibodies (gammaglobulin) in their serum and fail to make specific antibodies following in vivo antigenic challenge. In contrast to this observed functional B-cell defect, the patients exhibit a variable pattern of T-cell immunocompetence. Of eleven common variable hypogammaglobulinemic individuals studied thus far, the peripheral blood mononculear leukocytes of nine patients have produced influenza virus-immune CTL capable of lysing autologous virus-infected target cells. Since the assay system employs virus immune CTL tested on infected autologous target cells, negative results could be caused by either defective CTL generation and/or target cells which did not express the relevent antigenic structures (HLA antigens, virus antigens) recognized by immune effectors. In the two

patients in which virus-immune CTL for autologous targets were not detected, this lack of responsiveness was shown to be a defect in effector CTL generation since virus-infected target cells from both individuals were lysed by HLA compatible immune CTL from normal donors. Moreover, this deficiency within the effector cell population was not simply attributable to the lack of T-cells since both patients possessed normal proportions of T-cells within their cultured peripheral blood mononuclear leukocytes. Thus the majority of patients with common variable hypogammaglobulinemia possessed leukocytes capable of generating virus-immune, HLA restricted CTL; an experimental finding in accord with clinical observations that these patients are predominantly troubled by recurrent bacterial rather than viral infections.

Another human immunodeficiency disease studied for virus-specific CTL production in vitro was ataxia-telangiectasia. This disease is an autosomal recessive disorder characterized by progressive spinocerebellar degeneration leading to a clumsy gait (ataxia), oculocutaneous dilated blood vessels (telangiectasia), recurrent infections, and a high incidence of neoplasia. Histopathologic examination of the thymus gland from ataxia-telangiectasia patients reveals a thymus which is fetal in appearance and almost totally devoid of epithelial elements. Since the aforementioned studies of the development of virus-immune CTL in experimental animals suggested a critical role for the thymic epithelium in the maturation of self-MHC receptor specificties on CTL precursors, we were particularly interested in whether cells from ataxia telangiectasia patients could produce antigen-specific self-HLA restricted CTL. Of the 11 ataxia telangiectasia patients studied thus far, the mononuclear leukocytes of ten have failed to produce significant lysis on autologous virus infected target cells. In these ten patients, this lack of CTL function was shown to be a defect in effector CTL production since virus-infected patients' cells were lysed by normal HLA-related immune CTL. This defect was not readily attributable to lack of T-cells within the responder leukocytes since the proportion of T-cells among the patients' cultured leukocytes was not significantly different than normal. Additional studies were therefore undertaken to investigate the mechanism of this unresponsiveness in ataxia-telangiectasia patients. The serum of nearly all ataxia-telangiectasia patients contains elevated levels of alpha-fetoprotein, a fetal protein not normally found in adult serum. Since immunosuppressive effects have been ascribed to alpha-fetoprotein, we considered the possibility that the non-responsiveness of these patients might be due to serum inhibitory effects. Therefore, normal peripheral blood mononuclear leukocytes were cultured with virus in the presence of 5% normal pooled plasma (usual dulture conditions), or 5% pooled plasma from ataxia-telangiectasia patients with elevated alpha-fetoprotein levels.

We found that normal cells generated the same virus-immune CTL activity in plasma from ataxia-telangiectasia patients as in normal plasma. Thus ataxia-telangiectasia plasma did not appear to extend marked immunosuppressive effects on normal cells and the non-responsiveness of patient's leukocytes was not easily attributable to plasma inhibitory effects. An additional mechanism which might account for the non-responsiveness of ataxia-telan-

giectasia leukocytes is the presence of excessive cell-mediated immunosuppression. In order to evaluate the potential of excessive suppressor cell activity, coculture experiments were undertaken in which cells from one ataxia-telangiectasia patient which did not produce virus immune CTL were mixed in an equal proportion with cells from an MHC identical normal sibling which were capable of producing virus-immune CTL. Such MHC-matched patient-sibling combinations are required for these experiments to avoid the generation of alloimmune CTL. If excessive cellular suppression was responsible for the non-responsiveness in the patient then this suppression might be expected to reduce the CTL activity generated by the sibling's cells. This experiment demonstrated that the MHC matched sibling's cells generated the same CTL: activity whether or not they were cocultured with the patient's cells. Thus in this one available patient-sibling combination, excessive cellular suppression did not appear to be the mechanism whereby leukocytes from the patient failed to generate self-MHC restricted CTL. The mechanism of this lack of responsiveness to viruses in association with self-HLA antigens in patients with ataxia-telangiectasia is still under investigation. Defective monocyte and/or T-helper cell functions remain to be teseted. However, this unresponiveness to viruses and self-MHC antigens may play a role in these patients undue susceptibility to recurrent infections and neoplasia.

The third immunodeficiency disease studied for virus-immune self-MHC restricted CTL production in vitro is the Wiskott-Aldrich syndrome. The Wiskott-Aldrich syndrome is a sex-linked recessive disorder characterized by low peripheral blood platelet counts (thrombocytopenia) recurrent infections, a skin conditions termed eczema, and an increased frequency of malignant neoplsms. Common viral infections such as chickenpox may be life-threatening in these patients and in one case neoplasia was associated with persistent urinary excretion of a papova virus and the same virus was isolated from the patient's tumor. In seven Wiskott-Aldrich syndrome patients studied thus far, the peripheral blood lekocytes of six failed to lyse influenza virus-infected, autologous target cells. The one patient who responded produced only minimal CTL activity which was greater than 16 fold less than simultaneously assayed normal controls. In all six patients not manifesting CTL activity, this defect was attributable to deficient production of immune CTL effectors since the virally infected patients' target cells were lysed by virus immune CTL from normal, HLA-related individuals. To investigate whether this defect in the production of immune CTL in the Wiskott-Aldrich syndrome is limited to viruses, experiments have been initiated to investigate the capacity of patient's cells to mediate TNP-specific cytotoxicity. One patient studied thus far also lacked peripheral blood leukocytes mediating TNP-specific cytotoxicity. This non-responsiveness was attributable to defective CTL effector production since the patient's TNP modified target cells were lysed by the TNP-immune, HLA-related normal CTL. Thus patients with the Wiskott-Aldrich syndrome are deficient in their ability to produce immune cytotxic T-lymphocytes in vitro.

Recently, immunohematologic normalization has been reported in patients with the Wiskott-Aldrich syndrome following bone marrow ablative therapy and transplantation of bone marrow from MHC identical siblings. Two patients whose cells lacked the capacity to produce virus-immune CTL prior to such therapy generated normal influenza immune CTL activity six months and 18 months following transplantation therapy which was performed by Drs. O'Reilly and Good at Sloan-Kettering Institute. An additional Wiskott-Aldrich patient who received immunosuppressive therapy (without bone marrow ablation) and bone marrow transplantation from a MHC indentical sibling, also possesses a normal potential to generate influenza immune CTL in vitro. While the precise mechanism(s) of non-responsiveness to viruses and self-MHC gene products in the Wiskott-Aldrich syndrome remains unresolved, this defective production of virus-immune CTL is correctable by bone marrow transplantation following either immunosuppression or bone-marrow ablative therapy.

These studies of virus-immune self-MHC restricted CTL production demonstrate a heterogeneity in responsiveness among immunodeficiency disease patients. Most hypogammaglobulinemic patients but not patients with ataxia-telangiectasia on the Wiskott-Aldrich syndrome are capable of generating immune CTL in vitro. In large part these studies of antigen specific self-MHC restricted proliferation and cytotoxic responses have been limited to TNP and influenza A/HK. Therefore studies have been initiated to examine alloimmune proliferative and cytotoxic responses (which may represent a summation of the antigen specific self restricted repertoire) in those pa tients lacking responses to TNP and/or influenza A/HK. Studies have employed as stimulators either irradiated unseparated PBMC, macrophage-depleted PBMC, and T-cell depleted PBMC, the latter two populations presumably to uncover macrophage and T-cell defects, respectively, in the responder populations being tested. The results thus far are similar regardless of the stimulator population. Each of five WAS patients exhibited a weak proliferative response and generated no detectable cytotoxic activity. Of three A-T patients studied all three mounted weak proliferative responses and only one of the three patients produced CTL activity. These alloimmune studies confirm the existence of profound defects in the ability of A-T and WAS patients to generate immune CTL effectors and suggest that this defect is not limited to a few antigenic specificities. These defects in the production of immune CTL may contribute to the pathogenesis of recurrent infections and the high incidence of neoplasia in some of these patients.

Humoral Immune Responses by Human Peripheral Blood Leukocytes In Vitro: Studies were initiated to develop methods for the assessment of specific humoral antibody production by human peripheral blood mononuclear leukocytes in vitro. Antibody molecules belong to a class of serum proteins termed the gammaglobulins. Since these gammaglobulins can confer immunity they are often termed immunoglobulins (Ig) and several distinct sub-classes of immunoglobulins (IgM, IgG, IgA, IgD and IgE) are known to exist. Immuno-globulin secreting cells are derived from a class of leukocytes called B-cells which possess cell surface immunoglobulin as antigen receptors. Studies in experimental animals have shown that transition of B-cells into

immunoglobulin secreting cells is antigen-dependent, monocyte-dependent, and for most antigens requires the positive influence of T-helper cells and is negatively regulated by T-suppressor cells.

Much of our understanding of the maturation and immunoregulation of the human humoral immune response has derived from in vitro studies employing polyclonal activators wuch as pokeweed mitogen (PWM) and the Epstein-Barr virus which activate immunoglobulin secreting cells through receptors which are not antigen-specific. We have developed a method for studying antibody production by human PBMC in vitro which is antigen-induced and does not require the presence of polyclonal activators. For these studies, we have again employed influenza viruses as antigens. Cultures of peripheral blood mononuclear cells from >95% of normal adult individuals produce specific anti-influenza virus antibody in vitro in the presence of type A influenza viruses. Antibody secretion requires de novo protein synthesis, begins about day 5 of culture and reaches maximal rates between days 5 and 7 of culture. Antibody synthesis can be induced by both live influenza type A and B viruses as infectious allantoic fluid or formalin-inactivated, zonally purified type A and B viruses. Antibody generation was both antigen-dependent and virus-specific at the induction phase since: 1) cultures in media alone or stimulated with normal allantoic fluid produced no antibody and 2) cultures stimulated with type A viruses produced anti-influenza A antibody but not anti-influenza B antibody and vice versa. The production of antibody was shown to require the cooperative ineraction of T-cells, B-cells and monocytes in culture. This requirement for T helper cells capable of promoting the maturation of B-cells into antibody secreting cells was further investigated using a series of hybridoma derived monoclonal antibodies directed at predominantly distinct subsets of human T-cells. Using this methodology the human T-helper cell for anti-influenza antibody responses was shown to bear the specificity recognized by the monoclonal antibody OKT4 but not the specificity recognized by the monoclonal OKT8 which recognizes virus immune CTL effectors. Thus a subset of human T-cells comprising 40-60% of the total T-cell population was identified as the helper cell subset for specific antibody responses by B-cells in vitro.

Studies were also undertaken to investigate the fine specificity of the human in vitro antibody response to influenza virus. Cultures of PBMC were stimulated with the following purified formalin-inactivated whole influenza viruses: A/Aichi/68(H3N2), A/Bankok/79/(H3N2), A/USSR/77/(H1N1) and B/Hong Kong. Antibody production was assayed by ELISA on plates coated with each of the viruses as well as plates coated with purified viral hemagglutinin (H3 and H1) molecules. Antibody production was influenza type specific in that cultures stimulated with B/Hong Kong made little or no antibody against any of the type A viruses and vice versa. There was also specificity among influenza virus subtypes - that is, cultures stimulated with A/USSR/(H1N1) made only 29% as much antibody directed against A/Aichi(H3N2) as was directed against the stimulating H1N1 virus and cultures stimulated with A/Aichi(H3N2) made only 19% as much antibody against A/USSR(H1N1) as against the homologous virus. Using the ELISA for antibody to purified virus hemagglutinin (one of

the two major viral surface glycoproteins and the one responsible for virus attachment to cells), antibody to hemagglutinin was demonstrated in cultures stimulated with whole viruses of H3N2 and H1N1 subtypes. Moreover, cultures stimulated with H1N1 virus produced only 10% as much antibody to purified H3 as they did to purified H1 and cultures stimulated with H3N2 virus produced only 2% as much antibody to purified H1 as they did to purified H3. And lastly, when early (A/Aichi/68[H3N2]) and late (A/Bankok/78/[H3N2]) strains of H3N2 virus were studied it was observed that 3 of 4 individuals made more antibody to A/Aichi than to A/Bangkok when stimulated with either virus - perhaps reflecting the phenomena of "original antigenic sin". Two major conclusions can be drawn from these in vitro studies of the B-cell repertoire to influenza virus: 1) the in vitro response faithfully recaptiulates the in vivo response and 2) unlike our previous studies demonstrating that influenza-immune cytotoxic T-cells were cross-reactive between subtypes (i.e. cells stimulated with H3N2 virus lyse H3N2, HON1 and H1N1) the B-cell repertoire for influenza is largely non-cross reactive among subtypes.

Since studies employing certain polyclonal activators such as pokeweed mitogen had demonstrated that allogeneic T and B cells could cooperate for the synthesis of immunoglobulin and a large body of evidence in experimental animals suggested that this might not be the case for specific antibody synthesis, studies were undertaken to examine the effects of allogeneic T-cell and B-cell interactions on the human in vitro specific antibody response to influenza virus. In eight allogeneic combinations studied, mixtures of 5×10^6 B-cells with 2×10^6 allogeneic T-cells produced only 25% as much specific antibody as the same B-cells with autologous T-cells ($p<.005$). Experiments to examine the mechanism of this effect demonstrated that irradiation of the T-cells reduced antibody response with autologous T-cells and resulted in increased antibody responses with allogeneic T-cells. These studies with high T-cell density (2×10^6) suggested suppression as the mechanism for reduced antibody production with allogeneic T-cells. When cultures with low T-cell density were studied; however, a different pattern was observed. Cultures with $.5 \times 10^6$ B-cells and $.125 \times 10^6$ allogeneic T-cells made more antibody than cultures with autologous T-cells. Thus mixtures of B-cells and allogeneic T-cells can result in both positive and negative allogeneic effects, and depending on the culture condition, either can predominate.

Studies were also initiated to study the ontogeny of in vitro immunologic responsiveness to influenza viruses. These experiments were begun by examining the antibody resopnses of cord blood mononuclear cells (CBMC). CBMC from six neonates made no measurable in vitro antibody response to influenza virus. In addition, unlike adults, CBMC failed to proliferate to influenza virus demonstrating that the virus is not a non-specific mitogen. Moreover, CBMC made little or no antibody when stimulated with EBV. These results are consistent with lack of previous exposure to influenza and/or immaturity of the newborn immune system. However, cocultures of irradiated cord blood T-cells with allogeneic adult B-cells produced specific antibody to influenza virus. Thus, in spite of lack of previous exposure to influenza

virus, cord blood T-cells are able to mediate positive allogeneic effects for specific in vitro antibody production.

Investigation of the immune response to influenza virus in vivo was limited to a study of 17 children undergoing a primary infection with a live, cold adapted influenza A candidate vaccine virus. Serum and nasal wash anti-hemagglutinin antibody responses of IgM, IgG, and IgA isotypes were measured. All 17 of the vaccinated children had serum IgG responses, 16 had an IgM response and 13 had an IgA antibody response. With regard to nasal-wash antibody, 14 had an IgA response, 13 had an IgM response and 9 had an IgG response. Most of the nasal wash IgA and IgM antibody was secreted while only some of the IgG nasal wash antibody was secreted.

In previous studies we had also shown that during respiratory infection with influenza virus, cells which spontaneously secrete IgG and IgA anti influenza antibody are present in peripheral blood at day 6 following infection and disappear by day 27. Since secretory immune responses (particularly of the IgA class) are important in preventing influenza virus infections, we bagan a series of studies to examine the immunoregulation of antigen-specific IgA antibody responses by human peiripheral blood mononuclear cells. When stimulated in vitro with influenza A/Aichi/68 (H3N2) virus PBMC from 8 donors produced 39.1 (1.3) ng/ml (G.M. X/÷ SEM) IgG and 5.0 (1.5) ng/ml of IgA anti-virus antibody. Using a limiting dilution technique and Poisson analysis to calculate precursor frequencies of antibody producing cells we found that the mean precursor frequencies of B-cells producing IgM-, IgG-, and IgA-antibody were 1:183,000, 1:148,000 and 1:1, 534,000 respectively. Thus the lower amount of IgA vs. IgG antibody produced in vitro could be explained by a lower B-cell precursor frequency. To determine whether the same B-cell precursors make gM, IgG and/or IgA antibodies, multiple replicate wells were established to contain < 1 anti-influenza precursor per well. In these cultures, the production of IgM, IgG, and IgA were independentindicating that precursors make IgM, IgG, or IgA and that antigen-driven human peripheral blood B-cels make only one isotype of antibody and do not undergo "isotype switching". These studies will further our knowledge of the immunoregulation of IgA antibody responses.

Studies have been initiated to utilize this method to study maturational and immunoregulatory abnormalities in patients with immunodeficiency disease. Three patients studied with X-linked hypogammglobulinemia and isolated growth hormone deficiency failed to produce antibody in vitro in spite of having the capacity to mount a specific cytotoxic T-cell response to the same virus. In addition all three patients' cells could provide help to normal allogeneic B-cells and did not inhibit antibody production by cells from normal individuals. Thus these patients have an immune defect involving antibody secreting cells or their precursors. Of eleven hypogammaglobulinemic patients studied with a different disease termed common variable hypogammaglobulinemia (CVH), five made measurable antibody responese in vitro. Since CVH patients make specific antibody responses poorly in vivo, the finding of intact in vitro antibody responsiveness suggests that some patients have a host envir-

onmental abnormality as the cause of their hypogammaglobulinemia which can be overcome in vitro.

Specific antibody response were also sought in patients with ataxia-telangiectasia and the Wiskott-Aldrich syndrome who lacked influenza specific CTL responses. Six of seven patients with the Wiskott-Aldrich syndrome failed to produce specific antibody as did four of five patients with ataxia-telangiectasia. Further studies were undertaken to define the cellular basis of this non-responsiveness in three ataxia-telangiectasia patients. One of the patients was shown to have intact monocyte function since his irradiated adherent cells reconstituted antibody resopnses in macrophage depleted cultures of his MHC matched sibling's cells. All three patients had immunocompetent B-cells since their cells produced antibody when: 1) stimulated with the polyclonal B-cell activator Epstein-Barr virus, and 2) provided T-cell help in the form of allogeneic irradiated T-cells. These results suggested a defect in T-helper cells as partial cause for the immunodeficiency in ataxia-telangiectasia. However, when T-cells from two patients were added to purified allogeneic B-cells both were capable of "helping" antibody synthesis. These results suggest the existence of two subsets of T-cells, one which can help allogeneic B-cells and the second which helps autologous B-cells and that ataxia-telangiectasia patients are lacking the latter subset. These patient's T-cells behave as the previously mentioned cord blood mononuclear cells, again providing evidence for maturational arrests in ataxia-telangiectasia.

Patients with a selective deficiency of IgA were shown to make IgG and IgM but not IgA antibodies in influenze virus in vitro, again demonstrating the biological relevance of this assay.

Thus, we developed a method to study specific antibody responses by human peripheral blood mononuclear cells in the absence of polyclonal activators. This assay in combination with assays of self-MHC restricted CTL function toward the same antigens will prove to be a powerful tool for investigating maturational and immunoregulatory events in humans.

Studies were also initiated to investigate the immunobiology of B-cell activation by polyclonal activators that were T-cell and monocyte independent (B95-8 strain of EBV) or T-cell and monocyte dependent (PWM). For these studies, total IgM and IgG immunoglobulin were measured in cell culture supernatants by ELISA. To investigate the EBV viral requirements for human B-cell activation, multiple microwells containing non-limiting numbers of B-cells (10^5/well) were cultured for 12 days with serially greater dilutions of the B95-8 strain of EBV. A plot of the number of wells producing either IgM or IgG versus the virus dilutions was consistent with a "single hit" model by Poisson analysis indicating that one infectious virus particle was sufficient to induce a B-cell to produce immunoglobulin. Additional studies using cultures containing limiting dilutions of B-cells, saturating concentrations of EBV, and "feeder" layers of irradiated T-cells showed that the mean frequency of EBV activatable B cells for IgM secretion was 1 in 146 (range 1:58 to

1:219) and for IgG secretion, 1 in 284 (range 1:152 to 1:420). When PWM was used instead as the polyclonal activator, the mean frequency of activatable B-cells for IgM was 1 in 1596 (range 1:222 to 1:3212) and for IgG was 1 in 1470 (range 1:172 to 1:3755). Thus the frequency of B-cells activatable by EBV is greater than that of B cells activatable by PWM. Moreover, when multiple replicate cultures containing limited numbers of B-cells and saturating amounts of EBV were tested for both IgM and IgG, the B-cell precursors secreted IgM or IgG, but not both isotypes. Similar results were obtained with PWM. Thus, human peripheral blood B-cell do not undergo isotype switching with either T-cell dependent (PWM) or T-cell independent (EBV) polylconal activators.

Proposed Course: Studies will be continued to assess T-cell mediated specific cytotoxic responses in normal individuals and in patients with immunodeficiency. Studies in experimental animals and man have suggested that the T-cell subsets involved in the production of immune CTL (i.e. CTL precursors, T-helper cells, T-suppressor cells) may be separable on the basis of cell surface antigens. Recently methods have been developed whereby antibodies recognizing only sub-populations of human peripheral blood T-cells can be produced (hybridoma antibodies). Such antibodies will be tested for their ability to recognize T-cell subsets in normal individuals which are required for the generation of immune CTL and also to identify immune CTL effectors. If antibodies useful in dissecting CTL responses in normal individuals are found, these reagents will be used to further probe the mechanism(s) or defective specific CTL responses in immunodeficiency diseases (i.e. lack of CTL precursors, T-helper cells or monocytes, excessive T-suppressor cells). Attempts will be made to assess hapten-specific CTL responses generated in vivo by immunization with the cutaneous application of dinitrochlorobenzene. Additional studies of hapten-specific CTL generation in vitro will be undertaken in patients with the Wiskott-Aldrich syndrome and ataxia-telangiectasia to further asess the scope of defective CTL production in these individuals.

With regard to humoral antibody responses in vitro, studies will be expanded to further define the proportion of normal individuals capable of responding with antibody production to the influenza viruses and other antigens and the optimal in vitro culture conditions for generating such responses. The aforementioned studies employing hybridoma antibodies recognizing T-cells and additional antibodies possibly recognizing monocyes and B-cells will be expanded in attempts to further define the cellular interactions necessary to generate a response. Using purified virus antigens (i.e. virus hemagglutinin, virus neuraminidase), the specificity of the in vitro secreted antibody will be further defined. Attempts will be made to establish T-cell lines which may be cloned to proved purified subsets of T-helper and T-suppressor cells for further analysis. Studies of specific humoral immune responsiveness in vitro will be continued in patients with immunodeficiency diseases, particularly those patient groups already defined as having defective specific CTL responses (ataxia-telangiectasia, Wiskott-Aldrich syndrome) and those hypogammaglobulinemic patients with normal CTL

responses. Specific CTL reponses and specific antibody responses will then be studied in parallel to further elucidate the mechanism(s) involved in these patients susceptibility to recurrent infection and cancer.

Publications:

Yarchoan, R., Biddison, W.E., Schneider, H.S. and Nelson, D.L.: Human T cell subset requirements for the production of specific anti-influnza virus antibody in vitro. J. Clin. Immunol. 2: 118-125, 1982.

Lucas, C.J., W.E. Biddison, D.L. Nelson, and S. Shaw: Killing of measles virus-infected cells by human cytotoxic T-cells. Inf. Immun. 38: 226-232, 1982.

Greene, W. C., T.A. Fleisher, D.L. Nelson, and T.A. Waldmann: Production of human suppressor T-cell hybridomas. J. Immunol. 129: 1985-1992, 1982.

Murphy, B.R., D.L. Nelson, P.F. Wright, E.L. Tierney, M.A. Phelan, and R.M. Chanock: Secretory and systemic immunological response in children infected with live attenuated influenza A virus vaccines. Infection and Immunity 36: 1102-1108, 1982.

Yarchoan, R., G. Tosato, R.M. Blaese, R.M. Simon, and D.L. Nelson: Limiting dilution analysis of Epstein-Barr virus-induced immunoglobulin production by human B-cells. J. Exp. Med. 157: 1-14, 1983.

Nelson, D.L., W.E. Biddison, and S. Shaw: Defective in vitro production of influenza virus-specific cytotoxic T-lymphocytes in ataxia-telangiectasia. J. Immunol. 130: 2629-2635, 1983.

Arnaud-Battandier, F., and Nelson, D.L.: Immunologic characteristics of in testinal lymphoid cells of the guinea pig. Gastorenterology. 82: 248-253, 1982.

Yarchoan, R. H.S. Schneider, B.B. Wray and D.L. Nelson: Specific anti-influenza virus antibody production in vitro by lymphocytes from a subset of patients with hypogammaglobulinemia. J. Clin. Invest. In press.

Yarchoan, R. and D.L. Nelson: A study of the functional capabilities of human neonatal lymphocytes for in vitro specific antibody production. J. Immunol. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB04016-11 MET

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Mechanism of Anabolic Action of Insulin-like Growth Factors.

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Peter Nissley, M.D., Senior Investigator, Metabolism Branch, NCI

COOPERATING UNITS *(if any)*

Laboratory of Biochemical Pharmacology, NIADDK
Laboratory of Pathophysiology, NCI

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2	2

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.)*

A workable purification scheme has been devised for the isolation of a large molecular weight form of rat insulin-like growth factor-II (IGF-II) also known as multiplication stimulating activity (MSA). Human fetal fibroblasts have been shown to produce at least two size classes of IGF's as well as an IGF binding protein. An IGF-II receptor purified from chondrosarcoma chondrocytes has been shown to be fully active and greater than 90% pure.

Other Professional Personnel:

Lynne Gaynes, M.D.	Medical Staff Fellow	MET	NCI
Matthew M. Rechler, M.D.	Senior Investigator	LBP	NIADDK
Wayne Anderson, Ph.D.	Senior Investigator	LP	NCI
Lalitha Nagarajan, Ph.D.	Visiting Scientist	LP	NCI

Project Description:

Objectives: The understanding of normal growth may be a prerequisite to understanding malignant growth. A member of the insulin-like growth factor family (IGF) called somatomedin-C or IGF-I has been shown to mediate the anabolic action of growth hormone in vivo. Therefore, elucidation of the mechanism of action of insulin-like growth factors in vitro should reflect the normal growth process in vivo. The object of the project is to purify one of the insulin like growth factors, to study the regulation of production of IGF, to characterize cell surface receptors for IGF, and study the biochemical events accompanying growth stimulation by IGF in cell culture systems. Using human skin fibroblasts, we also hope to be able to identify patients with end-organ resistance to insulin-like growth factors.

Methods Employed: MSA or multiplication stimulating activity (rat IGF-II) is being purified from serum-free medium conditioned by a rat liver cell line (BRL-3A) using ion exchange chromatography, gel filtration, and high pressure liquid chromatography (HPLC). Human fetal fibroblasts derived from skin and lung are being used to study the production of insulin-like growth factors. The IGF's produced by the human fetal fibroblasts are being characterized by bioassay (tritiated thymidine incorporation into DNA in chick embryo fibroblasts), radioreceptor assay and radioimmunoassay. An IGF-II receptor is being purified from Swarm rat chondrosarcoma cells by subcellular fractionation, detergent solubilization from a membrane fraction, affinity chromatography on an IGF-Sepharose column, and Sepharose 6B get filtration. Receptor purification is followed by mesurement of binding of radiolabeled IGF and separation of bound tracer from free tracer with albumin coated charcoal. Skin fibroblasts from patients with possible end-organ resistance to IGF are being cultured from punch biopsies. IGF receptors on these patients' fibroblasts are being characterized by competitive binding studies and biologic response to IGF is being measured (tritiated thymidine incorporation into DNA, amino acid and glucose transport).

Major Findings:

Purification of a large form of rat IGF-II. As purified from human plasma the insulin-like growth factors are small polypeptides (Mr=7500). Since the purification schemes are multiple step procedures involving exposure to acid it is possible that the circulating forms of the IGF's may be larger than Mr=7500. In the medium conditioned by the BRL3A rat

liver cell line, we described larger forms of MSA (rat IGF-II) of Mr=8700 and 16,300 in addition to the Mr=7400 form. We are purifying the Mr=16,300 form from BRL3A conditioned medium to determine the specific biologic activity of this species and document a precursor:product relationship between the Mr=16,300 and Mr=8700 and 7400 forms. Our preliminary experiments indicate that the Mr=16,300 species can be purified to homogeneity by a combination of ion exchange chromatography, gel filtration on Sephadex G75, and high pressure liquid chromatography.

Characterization of insulin-like growth factors produced by human skin fibroblasts. MSA or rat IGF-II may be fetal growth factor in the rat since levels of MSA are high in fetal rat blood and decline postnatally to reach low levels by day 20 of extrauterine life. We recently reported that rat embryo fibroblasts synthesize MSA, have cell surface receptors for MSA and respond to MSA with increased DNA synthesis, thus consituting an autocrine or paracrine system. In addition, we showed that placental lactogen stimulates the production of MSA by the rat embryo fibroblasts. Placental lactogen has extensive amino acid sequence homology with growth hormone and has been proposed as the fetal counterpart to growth hormone in regulating the production of a fetal somatomedin or insulin-like growth factor.

In order to see whether or not these findings in the rat might have parallels in man, we have begun a series of studies in human fetal fibroblasts. We have obtained human fetal fibroblast strains (skin and lung) from cell repositories and have collected serum-free medium conditioned by these cells in monolayer culture. We have also screened amniocytes obtained for prenatal diagnosis, however, in general, these cultures have not grown as rapidly as the fibroblast strains nor do these cell strains tolerate serum-free conditions as well. The conditioned medium from the human fetal fibroblasts was gel filtered under acid conditions to dissociate and separate insulin-like growth factors from a binding protein. Column fractions were tested in a bioassay (tritiated thymidine incorporation into DNA in chick embryo fibroblasts), a radioreceptor assay which is relatively specific for IGF-II and a radioimmunoassay for IGF-I. Our preliminary experiments indicate that human fetal fibroblasts are producing insulin-like growth factors of a least two size classes as well as relatively large amounts of an IGF binding protein.

Purification of an IGF-II receptor from chondrosarcoma cells. There are two types of IGF receptors. One type preferentially binds IGF-I compared to IGF-II and interacts weakly with insulin. Affinity crosslinking studies have shown that the binding subunit of this type I IGF receptor has a Mr=130,000 similar to the binding subunit of the insulin receptor. A second type of IGF receptor preferentially binds IGF-II compared to IGF-I and does not interact with insulin. Affinity crosslinking studies have shown that the binding subunit of this receptor has a Mr=260,000.

The Swarm rat chondrosarcoma has been shown to be under hormonal control in vivo. We have shown that chondrosarcoma cells in monolayer culture respond to insulin and insulin-like growth factors with increased proteoglycan synthesis and have insulin receptors as well as type I and type II IGF receptors. The number of type II receptors on the chondrosarcoma chondrocyte is about ten fold higher than the number on most cells in culture, consequently this cell is a convenient source for the purification of the type II receptor. We solubilized the type II receptor from a membrane fraction and purified the receptor on an MSA-Sepharose affinity column and by gel filtration on Sepharose 6B. When the purified receptor was analyzed by sodium dodecyl sulfate polyacrylamide get electrophoresis (SDS-PAGE) and the gel stained for protein by a silver staining technique, a major component of Mr=260,000 was seen as well as a minor component of Mr<68,000. When radiolabeled IGF was affinity crosslinked to the purified receptor preparation and the ^{125}I-IGF receptor complex analyzed by SDS-PAGE and authoradiography, the radioactive band coincided with the Mr=260,000 species identified by silver staining. Competitive binding experiments indicated that the relative order of potency of insulin and IGF's was IGF-II>IGF-I and insulin did not compete. This pattern is identical to that observed for the type II receptor on intact cells. In addition, the binding affinity of the purified receptor for rat IGF-II was the same as the value determined for the receptor on intact cells and binding capacity measurements suggest that the purified reeceptor is fully active with a binding stochiometry of 1:1.

Significance to Biomedical Research:

The purification of one of the larger IGF species will permit determination of whether or not this species is more active than smaller forms already purified.

The finding that human fetal fibroblasts produce IGF's should allow characterization of these IGF's and possible lead to the discovery of a new fetal IGF. This system should also permit study of the hormonal regulation of IGF production by these fatal cells.

The ability to purify one of the IGF receptors should lead to the development of monoclonal antibodies, characterization of receptor strucure and chemistry and study of receptor biosynthesis and turnover.

Proposed Course:

We plan to complete the purification of the large molecular weight form of rat IGF-II (MSA) and then determine its specific biologic activity and its role as a precursor to the smaller forms of rat IGF-II.

The IGF's produced by the human fetal fibroblasts will be characterized to see whether they represent already described IGF's or a new fetal IGF.

We will explore the regulation of IGF production by the fetal fibroblasts; in particular, whether human placental lactogen stimulates the production of IGF.

We plan to develop monoclonal antibodies to the IGF-II receptor and use these antibodies to demonstrate the importance of the receptor in growth responses to IGF in cells in culture and to study receptor biosynthesis and turnover.

We will continue to try to identify patients with possible end-organ resistance to insulin-like growth factors and study skin fibroblasts from these patients in culture.

Publications:

White, R.M., Nissley, S.P., Short, P.A., Rechler, M.M., and Fennoy, I.: Developmental pattern of a serum binding protein for multiplication stimulating activity in the rat. J. Clin. Invest. 69: 1239-1252, 1982.

Adams, S.O., Nissley, S.P., Kasuga, M., Foley, T.P., and Rechler, M.M.: Receptors for insulin-like growth factors and growth effects of multiplication-stimulating activity (rat insulin-like growth factor II) in rat embryo fibroblasts. Endocrinology. 112: 971-978, 1983.

Adams, S.O., Nissley, S.P., Greenstein, L.A., Yang, Y. W-H., and Rechler, M.M.: Synthesis of multiplication-stimulating activity (rat insulin-like growth factor II) by rat embryo fibroblasts. Endocrinology. 112: 979-987, 1983.

Adams, S.O., Nissley, S.P., Handwerger, A. and Rechler, M.M.: Developmental patterns of insulin-like growth factor I and II synthesis and regulation in rat fibroblasts. Nature. 302: 150-153, 1983.

Stevens, R.L., Austen, K.F., and Nissley, S.P.: Insulin-induced increase in insulin binding to cultured chondrosarcoma chondrocytes. J. Biol. Chem. 258: 2940-2944, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB04015-13 MET

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Studies of Host Defense Mechanisms in Immunodeficiency and Malignancy

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

R. Michael Blaese, M.D., Head, Cellular Immunology Section, MET:C:DCBD

COOPERATING UNITS *(if any)*

Arthritis and Rheumatism Branch, NIAMDD
Walter Reed Army Medical Center
Laboratory of Clinical Investigation, NIAID

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	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 our studies of the immunobiology of the Epstein-Barr virus in order to clarify the defense mechanisms which regulate the capacity of this potentially oncogenic virus to induce uncontrolled B lymphocyte proliferation, differentiation for immunoglobulin secretion, and cellular transformation. In the past we have identified four distinct suppressor T cell immunoregulatory mechanisms which can affect EBV induced B cell differentiation and have characterized the cellular control processes which occur during acute infection (i.e. infectious mononucleosis) and in convalescent EBV immune normal subjects. We have extended these studies with the development of sensitive limiting dilution techniques for determining the frequency of EBV infected B lymphocytes present during acute infection (500-5000/10^6 B cells) and after recovery (1-10/10^6 B cells). Patients with rheumatoid arthritis were found to have a striking defect in their suppressor T cell response directed specifically at EBV and as a consequence have ten times the normal burden of EBV infected B cells in the circulation. Interestingly, although essentially all B lymphocytes have receptors for EBV, we found that only about 1% of these cells become activated to produce immunoglobulin after infection and only 0.3% of B cells will ultimately become transformed. We have studied 27 patients with the chronic infectious mononucleosis syndrome and have been able to group these patients with three subgroups based on their cellular response to EBV. In one of these patient groups, a unique antisuppressor T cell activity was found. Further, we have found that normal monocytes may also have anti or contrasuppressor activity for certain T cell functions in man. These observations help us to define yet another major immunoregulatory cell circuit in the human immune system in which defects may predispose or contribute to disease.

PHS 6040 (Rev. 1/83)

GPO 895-100

Other Professional Personnel

Andrew V. Muchmore, M.D.	Senior Investigator	MET	NCI
Giovanna Tosato, M.D.	Expert	MET	NCI
Frank M. Orson, M.D.	Medical Staff Fellow	MET	NCI
Alfred D. Steinberg, M.D.	Senior Investigator	A&R	NIAMDD
Thomas A. Waldmann, M.D.	Branch Chief	MET	NCI
Thomas A. Fleisher, M.D.	Asst. Chief Allergy/Immunology, Walter Reed Army Medical Center		
Robert Yarchoan, M.D.	Medical Staff Fellow	MET	NCI
David L. Nelson, M.D.	Senior Investigator	MET	NCI
Steven E. Straus, M.D.	Senior Investigator	LCI	NIAID

Project Description

Objectives: The objectives of this study were to determine mechanisms important to the development and function of both humoral and cellular immune responses in man and animals; to determine the nature of the deficiency in immune function characterizing such disease states as hypogammaglobulinemia, ataxia telangiectasia, the Wiskott-Aldrich syndrome, intestinal lymphangiectasia, and advanced malignancy; and to develop approaches to prophylaxis and/or therapy of the infections and neoplastic disease frequently associated with defective immune processes. This plan is directly related to Objective 2, Approach 1 of the National Cancer Plan.

Methods Employed: Antibody responses of experimental animals and man were determined after immunization with a variety of antigens and the antibodies were detected by standard techniques or methods developed in our laboratory. Cellular immune responses were measured by delayed hyper-benzene and by skin allograft rejection. Proliferative responses in vitro were tested using nonspecific mitogens, specific antigens, and allogeneic cells in mixed lymphocyte culture. Immunoglobulin secretion by activated lymphocytes was measured by a reverse hemolytic plaque assay and this test was applied to cultured cells stimulated with a variety of mitogens, viruses, and chemical agents. Studies of the characteristics of various cell surface receptors on immunocompetent lymphocytes and monocytes-macrophages utilized autoradiography, immunofluorescence microscopy, and cellular rosette formation.

Major Findings:

Studies of the immunobiology of the Epstein-Barr virus (EBV) have been an active subject of research on the Branch for the past five years. EBV is a virus of the herpes group which infects B lymphocytes specifically and as a consequence these infected B cells are induced to proliferate, differentiate for immunoglobulin production, and eventually transform into permanently growing B cell lines in culture. The virus is suspected in the etiology of Burkitt's lymphoma and nasal pharyngeal carcinoma and has been demonstrated in polyclonal B cell malignancies occurring in patients receiving immuno-

suppressive drug therapy, children with inherited immunodeficiency disorders, and in adults with the AIDS syndrome. Since this virus persists in the genome of some B cells for the life of the host following primary infection, immune mechanisms which prevent B cell tranformation by this virus are critically important to survival.

To determine the frequency of endogenously EBV-infected B cell, capable of transforming into continuously growing cell lines and to further characterize the mechanisms which control these cells, we have developed sensitive techniques for determining the rate of spontaneous B cell transformation in the human peripheral blood lymphocyte population. Using limiting dilution cultures of purified B cells grown on a feeder layer of autologous irradiated T cells, the frequency of endogenously EBV infected B cells in sero-positive normal subjects capable of spontaneous transformation was found to range from 1 to 10 cells per million B cells. In EBV sero-negative adults and children, as expected, no spontaneously transforming B cells were found. In patients with acute EBV infection (i.e., infectious mononucleosis) 500 to 5000 cells per million B cells spontaneously transformed. When unirradiated autologous T cells were added to these cultures, essentially no B cells transformed from the normal EBV sero-positive donors, demonstrating the effectiveness of these immune T cells in controlling virus induced B cell transformation. By contrast autologous T cells from patients with the acute infectious mononucleosis were totally without effect in preventing endogenous EBV infected B cells from transforming, despite the fact that these T cells have profound suppressor activity for both immunoglobulin production and transformation when exogenous EBV was added to the cultures. These observations indicate that suppressor T cells activated in acute infectious mononucleosis, while capable of preventing previously uninfected B cells from being activated by EBV, are unable to control cell growth by B cells which acquired EBV infection before this suppressor mechanism became activated. Clearly then, additional defense mechanisms must be present to control these B cells which were infected by EBV early in the disease.

In addition to its association with certain B cell lymphomas and acute infectious mononucleosis, EBV also has an as yet undefined association with the common form of the adult rheumatoid arthritis. For example, patients with rheumatoid arthritis have high titers of antibody to EBV associated antigens. When cultures of peripheral blood lymphocytes from normal EBV sero-positive donors are infected with EBV, initially the virus activates B cells to produce immunoglobulin which peaks at 8 to 10 days of culture and then this production rapidly falls as a consequence of the EBV immune suppressor T cells present in these patients. By day 14 of culture, the presence of EBV immune suppressor T cells inhibits immunoglobulin production in these cultures by 90% compared with EBV infected B cells cultured alone. By striking constrast, when lymphocytes from EBV sero-positive rheumatoid arthritis are stimulated with EBV in vitro, immunoglobulin production progressively increases throughout the 14 day culture period indicating a defect in EBV specific suppressor T cell activity in these patients. When the

number of endogenously EBV infected B cells in these rheumatoid arthritis patients was determined by precursor frequency analysis they were found to have an average of 10 times as many spontaneously transforming B cells as the normal subjects. Thus, the in vitro demonstration of defective suppressor T cell activity in these patients correlates well with the presence of an increased burden of EBV infected B cells in vivo and again suggests that many of the immune abnormalities in patients with rheumatoid arthritis are related to defective T cell control of this virus and the consequent effects of virus induced B cell activation.

In addition to utilizing precursor frequency analysis to determine the number of endogenously EBV infected B cells, this technical approach has been very useful for answering several other questions relating to B cell activation. One question for which this technique has proved to be especially valuable has been in the determination of the frequency of B cells capable of reponding to various polyclonal B cell activators. Using limiting dilutions of B cells in the presence of non-limiting concentrations of irradiated helper T cells, the frequency of B cells responding to stimulation with pokeweed mitogen was determined. In normal adult peripheral blood between 1 in 200 and 1 in 3000 B cells respond to pokeweed mitogen with production of IgM and between 1 in 200 and 1 in 4000 respond with IgG production. Analysis of these results further showed that the precursors of IgM and IgG segregated independently and that there was no evidence of isotype switching from the production of one class to the production of another class of immunoglobulin. When EBV was used as the stimulant a higher proportion of IgM and IgG precursors were found (between 1 in 60 and 1 in 200, and 1 in 150 and 1 in 400 respectively for IgM and IgG). Again, no evidence of isotype switching was found. Since essentially all B cells have receptors for EBV, it is intriguing that only 1% or less of these cells can be activated to produce immunoglobulin by this virus. To further address the relationship between B cell activation and EBV infection, limiting dilution analysis was used to measure the frequency of precursors for EBV induced immunoglobulin production compared with EBV induced B cell transformation. In these experiments the vast majority of cells which were induced to transform also produced immunoglobulin. However, twice as many precursors for immunoglobulin production were found indicating that tranformation is not an essential prerequisite for EBV induced immunoglobulin production. In only about 2% of the transformed B cells no immunoglobulin was observed. The possibility that these non-producing transformed B cell lines may have non-productively rearranged all of their immunoglobulin genes is currently being investigated.

We have previously described four distinct types of suppressor T cell activity capable of controlling EBV induced immunoglobulin production. In non-immune EBV sero-negative donors, no regulatory T cell activity demonstrable. In acute infectious mononucleosis intense polyclonal, polyisotypic suppressor activity is found which inhibits not only EBV, but also other polyclonal B cell activators like pokeweed mitogen. In EBV seropositive immune donors after recovery from infectious mononucleosis, the intense nonspecific polyclonal suppression is no longer demonstrable and in its place an EBV specific, late acting suppressor T cell is found.

Chronic infectious mononucleosis is a syndrome which is becoming increasingly recognized. Patients with chronic mononucleosis have recurrent or continuous symptoms suggestive of acute infectious mononucleosis which may persist for months or years following the initial acute disease. They frequently show serological evidence of continuing EBV infection and often have persistent weakness, lasitude, lymphadenopathy, and fatigue. We have studied 27 patients with this syndrome to determine there pattern of cellular immune reactivity to EBV and have found three distinctive patterns of reactivity. About 1/5 of these patients have cellular responses to EBV consistent with the findings in normal EBV immune adult donors. In another 1/5, the responses are identical to patients with acute infectious mononucleosis characterized by profound non-specific suppressor T cell activity. The remaining 60% of patients have a unique pattern of cellular reactivity. These patients' B cells respond normally to EBV and they have apparently normal, late acting T suppressor cell activity. Further, these patients mononuclear cells produce immnoglobulin normally in response to pokweed mitogen. However, each of these patients also has strong suppressor T cell activity capable of inhibiting the response of allogeneic normal lymphocytes to pokweed mitogen in cocultures. Thus, these patients profoundly suppress allogeneic cells while not suppressing their own response and preliminary experiments indicate that the allogeneic suppression is not seen in cocultures with HLA identical donors. These results suggest that an MHC restricted contrasuppressor mechanism may be active in these patients and further studies may help us to define yet another immunoregulatory cell circuit in man.

Anti or contrasuppressor activity has been found in another aspect of the in vitro response to EBV. The late acting suppressor T cell observed in normal EBV immune donors is specific for EBV induced B cell responses and is inhibited by x-ray and cyclosporin A. In experiments involving removal or addition of autologous monocytes to EBV activated lymphocyte cultures, a striking effect on late acting T suppressor activity was observed. Removal of macrophages from these cultures resulted in a marked enhancement of suppressor T cell activity and conversely addition of monocytes reversed this suppressor cell activity. Excessive numbers of monocytes have been shown previously to inhibit pokeweed mitogen induced immunoglobulin production, but this is the first demonstration that monocytes may also inhibit suppressor T cell activity and thus augment immunoglobulin production by certain B cell activators.

Another area of major activity in the cellular immunology section over the past few years has been an attempt to define the nature of the cellular structures involved in self-self and self-non-self recognition and in intracellular communication in immune and non-immune host defense processes. Dr. Muchmore's laboratory has identified a system of recognition of foreign targets by non-immune mononuclear phagocytes based on the interaction of effector cell surface lectin-like receptors with carbohydrate

determinants on the target cell. This recognition system appears to be phylogenetically ancient in that it can be found in primative invertebrates such as the starfish as well as in mammals and man. We have exended these studies to explore the possibility that such lectin-carbohydrate interactions might also be involved in immunoregulatory signals between lymphoid cells. As described earlier, T cells from patients with infectious mononucleosis are profoundly inhibitory to the process of B cell activation in vitro by mitogens or by EBV. In order to test for a possible lectin-sugar interaction in this immunoregulatory process, suppressed lymphocyte cultures containing normal B cells, infectious mononucleosis T cells, and pokeweed mitogen were supplemented with high concentrations of a variety of monosaccharides, disacchardies and oligosaccharides and subsequent immunoglobulin production was measured. Over 30 tested sugars showed no activity in this system. However, two related monosaccharides were found which had a striking effect. D-mannose and alpha-methyl-D-mannose almost totally reversed the suppression caused by the infectious mononucleosis T cells permitting immunoglobulin production to occur in these cultures. At 25 millimolar these sugars reversed the suppression by an average of 85% and substantial activity could be detected with sugar concentrations as low as 2 millimolar. In preliminary studies a variety of synthetic mannose analogs have been tested and several compounds show activity at concentration as low as 50 micromolar. We have also studied two other situations in which similar suppressor phenomena are observed, the human newborn and in some patients with common variable hypogammaglobulinemia. The activity of suppressor T cells present in human cord blood is also removed by mannose and alphamethyl-mannoside. In suppressed indicator cultures containing cells from agammaglobulinemic patients, additional evidence for sugar-lectin mediated immunoregulation was found. Suppressor T cell activity from two patients was reversed by mannose or yeast mannan and suppressor monocyte activity in another patients was reversed by n-acetyl-d-glucosamine. These data appear to support the hypothesis that sugar lectin recognition system that evolved in primative invertebrate mononuclear phagocytes as a means of distinguishing foreigness has been preserved and expanded in higher vetebrates to form the basis of certain immunoregulatory cellular interactions as well.

Additional studies of patients with common variable hypogammaglobulinemia have led to an entirely new series of observations relating to the influence of certain steroid hormones on B cell differentiation in vitro. In studies of the suppressor T cells present in certain patients with hypogammaglobulinemia, it has been observed that these suppressor cells are sensitive to both irradiation and to hydrocortisone. Thus steroids or irradiation are able to block the suppressor activity of such cells. During the course of these experiments we observed that hydrocortisone alone cultured with normal lymphocytes seemed to induce immunoglobulin production in culture. Human peripheral blood lymphocytes cultured with hydrocortisone or many of its analoges including dexamethasone, prednisolone, and methyprednisolone were found to produce in culture as many immunoglobulin

secreting cells as did cultures stimulated with pokeweed mitogen. Kinetic studies showed that this response to steroid hormone in vitro began to appear at a about the 4th day and peaked somewhat later than pokeweed mitogen at the 8th to the 10th day of culture. Other steroid hormones such as dyethylstilbesterol and testosterone did not induce the activation of B cells in culture. Aldosterone, a steroid hormone with weak glucocorticoid activity, stimulated B cells to produce immunoglobulin only when used at very high concentrations in vitro. In order to determine the cellular basis for such stimulation, peripheral blood lymphocyte populations were separated into T cells, B cells and monocytes. The glucocorticoids were found to be unable to stimulate immunoglobulin production by purified B lymphocytes alone. Both monocytes and T lymphocytes were required in the cultures for glucocorticoids to have their stimulatory activity on B lymphocytes. Since glucocorticoids are able to reverse the suppressor T cell activity seen in certain pathologic conditions and also appear to be able to reverse normal suppressor T cell activity, a potetential mechanism for the action of steroid hormones in inducing B cell activation would be the inactivation of a suppressor cell maintaining such B lymphocytes in an inactive state. T cells irradiated with 2000 R to inactivate the radiosensitive suppressor T cell were mixed with B lymphocytes in culture. Such cell mixtures produced no more immunoglobulin than that produced by B lymphocytes alone. However, when corticosteroids were added to such mixtures a marked increase in immunoglobulin production was observed. Thus although we have not been able to directly demonstrate that corticosteroids are not simply inactivating a suppressor T lymphocytes, other procedures which inactivate supressor T cells do not result in the activation of immunoglobulin production by B cells without the addition of the glucocorticoid hormones.

To further examine the cellular sites of action of the steroid effect, soluble factors produced by cultured T cells were tested for their ability to substitute for intact T cells in this response. Supernatants from T cells cultured without stimulants for 24-48 hours were added to B cell cultures. Such supernatants were incapable of substituting for intact T cells when the cultures were stimulated with pokeweed mitogen, but they were able to replace T cells when corticosteroids were used as the stimulant. Thus, it appears that the principal site of action of steroid hormones in this system is upon the B lymphocytes. However, the B cell response to steroids is also clearly restricted to only a subpopulation of B cells. For example, human neonatal B cells do not respond with immunoglobulin production following corticosteroid stimulation. In addition, IgE production is only minimally enhanced in normal subjects with steroid stimulation while large amounts of IgE are produced by by lymphocytes from allergic subjects when stimulated with steroids. This lack of stimulation of cord blood lymphocytes, the preferential stimulation of IgE production in allergic subjects, and the lack of detectable proliferation in steroid stimulated cultures actively producing immunoglobulin, have led us to propose that steroids are inducing a subset of memory B cells to produce immunglobulin in vitro.

Significance to Biomedical Research:

The present studies extend our understanding of the diverse, yet interrelated mechanisms contributing to the development and normal functioning of immune system in animals and man. They indicate that the normal expression of immune function is dependent on multiple processes and that defects in immunity may be the result of factors influencing a variety of these processes. For example, disfunction of the immune system can occur through such diverse factors as defects in differentiation on the one hand or through the mediation of exogenous viral pathogens on the other. Thus human disease may result from a deficiency of immune elements as in certain forms of agammaglobulinemia or from an excess of certain immune functions as in other types of agammaglobulinemia associated with excessive suppressive T cell activity. Suppressor T cell function may be part of a normal defense mechanism in such diseases as infectious mononucleosis and a deficiency of normal suppressor T cell activity may lead to the expression of certain autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus. Our new insights into the role of sugar-lectin interactions as immunoregulatory signals offers potential for precise definition of this communication process and for future therapeutic manipulation of these signals.

Proposed Courses:

Continue the studies directed toward the understanding of the pathways involved in the development and function of normal immune responses and the application of insights gained through such studies for the development of new approaches for the prevention and therapy of human disease.

Publications:

Blaese, R.M., and Tosato, G.: Four Suppressor T Cell Systems in Man Acting by Direct Suppression at the B Cell Level. In Fauci, A., and Balleux R.E. (Eds.): Human B Cell Function. New York, Raven Press, 1982, pp. 259-267.

Blaese, R.M., Pike, S., and Tosato, G.: Suppressor T cell function in man: Production by the direct action of immunoregulatory T cells on the B cells in four distinct systems. Clin. Immunol. and Immunopath. 23: 162-171, 1982.

Stein, M., Steeg, P., Oppneheim, J.J., Stiehm, E.R., Mann, D., and Blaese, R.M.: Modulation of Human Cord Blood Monocyte Dr Antigen Expression In Vitro by Lymphpokines and Interferon. In Landy, M. and Oppenheim, J.J. (Eds.): Interleukins, Lymphokines and Cytokines. New York, Academic Press. In press.

Stiehm, E.R., Mann, D., Stein, M.B., Steeg, P.S., Oppenheim, J.J., and Blaese, R.M.: Deficient Dr Antigen Expression on Human Neonatal Monocytes: Reversal with Lymphokines. In Wedgewood, R., and Rosen, F. (Eds.): Proceedings of the 4th International Workshop on Primary Immunodeficiency Diseases. New York, National Foundation. In press.

Blaese, R.M., Muchmore, A.V., and Tosato, G.: Lymphoid Cell Derived Lectin-Like Receptor Molecules as Immunoregulatory Signals in Immunodeficiency. In Wedgewood, R., and Rosen, F. (Eds.): Proceedings of the 4th International Workshop on Primary Immunodeficiency Diseases. New York, National Foundation. In press.

Blaese, R.M., Tosato, G., Greene, W.C., Fleisher, T.A., and Muchmore, A.V.: The role of cell surface lectin-carbohydrate interaction in cellular recognition, cooperation, and regulation. Amer. J. Pediat. Hematology and Oncology. 5: 199-206, 1983.

Broder, S., Megson, M., Muul, L., Muchmore, A.V., Blaese, R.M., and Mann, D.L.: Activation of Regulatory Cells by Antibodies to Human Ia-Like Antigens. In Fauci, A.S., and Ballieux, R.E. (Eds.): Human B-Lymphocyte Function: Activation and Immunoregulation. New York, Raven Press, 1982, pp. 289-298.

Hall, R.E., Muchmore, A.V., Decker, J.M., and Blaese, R.M.: A model system of human spontaneous monocyte cytotoxicity: The HL-60 promyelocytic cell line becomes cytotoxic for erythrocyte target cells after incubation with phorbol myristate acetate. Cell. Immunol. In press.

Hall, R.E., Blaese, R.M., Davis, A.E., Decker, J.M., Tack, B.F., Colten, H., and Muchmore, A.V.: Cooperative interaction of factor B and other complement eomponents with mononuclear cells in the antibody independent lysis of xenogeneic erythrocytes. J. Exp. Med. 156: 834-843, 1982

Ladisch, S., Poplack, D.G., and Blaese, R.M.: Inhibition of human lymphoproliferation by intravenous lipid emulsion. Clin. Immunol. & Immnopathol. 25: 196-202, 1982.

Yarchoan, R., Tosato, G., Blaese, R.M., Simon, R.M., and Nelson, D.L.: Limiting dilution analysis of Epstein-Barr virus-induced immunoglobulin production by human B cells. J. Exp. Med. 157: 1-14, 1983.

Hall, R.E., Muchmore, A.V., and Blaese, R.M.: Monocyte cytotoxicity: Evidence for multiple mechanism of in vitro erythrocyte target killing. Trypan blue can both inhibit and enhance target lysis. J. Ret. Endothiel. Soc. 32: 233-246, 1982.

Tosato, G., Pike, S.E., and Blaese, R.M.: Reversal of infectious mononucleosis associated suppressor T cell activity of D-mannose. J. Exp. Med. In press.

Blaese, R.M.: Immunodeficiency Diseases. In Altman, L. (Ed.): Clinical Allergy and Immunology. Boston, G.H. Hall. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB04004-22 MET

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Regulatory Functions of Amino Acids

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

James M. Phang, M.D., Senior Investigator, Metabolism Branch, NCI

COOPERATING UNITS *(if any)*

Grace C. Yeh, M.D. - Clinical Pharmacology Branch, DCT, NCI
David Valle, M.D. - John Hopkins University School of Medicine
Robert J. Smith, M.D. - Joslin Research Laboratories

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	2	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 enzymes catalyzing the inteconversion of proline and pyrroline-5-carboxylate provide a mechanism for the intercompartmental, intercellular, and interorgan transfer of redox potential. Mediated by the transfer of redox potential, PC regulates PRPP and nucleotide production thereby linking amino acid and nucleotide metabolism. The catalytic stimulation of nucleotide synthesis by the proline cycle suggests that PC may be a second messenger intracellularly or a "primitive hormone" intercellularly.

PHS 6040 (Rev. 1/83)

GPO 895-100

Other Professional Personnel:

Alexander Fleming, M.D. Medical Staff Fellow MET NCI

Project Description:

Objectives: Recent reports have emphasized the primary role of metabolic regulation in malignant transformations of mammalian cells. The transport, biosynthesis and degradation of amino acids may play an important role in this metabolic regulation. More importantly amino acids are not only necessary building blocks for proteins but also are regulators in intermediary metabolism. This project emphasizes the biosynthesis and degradation of proline because the metabolism of proline has unusual, even unique features. The metabolic intermediates of proline provide an important link of carbons betwen the TCA and urea cycles. In addition, these intermediates, functioning as redox couples, can regulate a number of redox-independent metabolic pathways e.g., the production of nucleic acids. These studies may elucidate changes in growth rates, energy allocation and malignant transformations in tumors. The project is relevant to objective 3, Approaches 1 and 5 of the National Cancer Program.

Methods Employed: A variety of approaches are used to pursue the aforementioned objectives in animal tissues, freshly isolated human cells and cells maintained in long-term tissue culture. Cultured cell lines are especially useful since they can be cloned to insure genetically homogenous populations. Mutant cells are isolated by standard techniques or are obtained from patients with defined inborn errors of metabolism. Many biochemical techniques are utilized in these studies. Uptake of amino acids are assessed by using labeled amino acids and rates of protein and collagen synthesis are obtained by incubating cells with L-proline-^{14}C and separation of incorporated labeled amino acids by ion-exchange column chromatography. Methods have been develped to measure ornithine production from proline and glucose formation from lactate, proline and alanine. Nucleotides and their precursors, labeled and unlabeled, are quantitated using high-pressure liquid chromatography. The enzymes of biosynthesis and degradation of proline are assayed using specific radioisotopic methods. Biochemical studies on specific enzymes include the isolation of mitochondria, solubilization of enzymes from mitochondrial particles and purification. Physicochemical characteristics of these enzymes are studied by gel filtration and affinity chromatographic techniques.

Major Findings:

Clinical and Basic Research Goals: Our laboratory has shown that proline and its metabolite, pyrroline-5-carboxylate, regulates major metabolic pathways. Pyrroline-5-carboxylate, the obligate intermediate in the direct interconversions of proline, ornithine, and glutamate can initiate a redox-dependent metabolic cascade. This cascade includes 1) the activation of the pentosephosphate pathway, 2) increased formation of phos-

phoribosyl pyrophosphate (PRPP) and 3) increased production of nucleotides. Furthermore, we have shown that the interconversions of proline and pyrroline carboxylate constitute a metabolic cycle in which oxidizing potential in the form of pyrroline-5-carboxylate, can be generated and transferred between cellular compartments as well as between different cells with asymmetric enzyme capacities. Thus, the proline-P5C cycle by catalytically generating oxidizing potential can open metabolic gates necessary for cellular activation. Based on our findings, we hypothesized that proline and pyrroline-5-carboxylate may act as a primitive but general hormone system which allow for intercompartmental and intercellular exchange of metabolic information. We propose that derangement of this regulatory mechanism is involved in the pathogenesis of certain inborn errors of metabolism. For example, type 2 hyperprolinemia and gyrate atrophy of the choroid and retina. This mechanism also may be important in the pathogenesis of malignant tumors in man.

Pyrroline-5-Carboxylate as the second messenger in mitogenesis. Our previously published work demonstrated a metabolic cascade activated by pyrroline-5-carboxylate which includes the activation of the pentophosphate pathway, increased production of PRPP and increased incorporation of purines into nucleotides. Since other workers have previously shown that these are early events in the mitogenic activation of quiescent cells, we considered that pyrroline-5-carboxylate may be a involved in the activation process. Cultured human fibroblasts were made quiescent by serum deprivation. With the addition of 10% fetal bovine serum to quiescent cells, PRPP rapidly rose. Significant increases over control levels were seen as early as 30 minutes. The addition of L-pyrroline-5-carboxylate also increased PRPP levels. The levels of PRPP rose proportionately with P5C concentrations and with saturating concentrations of P5C the PRPP levels attained a magnitude about 40% of that produced by 10% fetal bovine serum. Interestingly, P5C and fetal bovine serum were synergistic in increasing the levels of PRPP. We also examined whether the effects of P5C on human fibroblasts were dependent on the cell cycle. Accordingly we defined the S phase for DNA synthesis by activating quiescent cells with 10% fetal bovine serum. In human fibroblasts, DNA synthesis begins at about 14 hours and peaks at about 18 hours. Strikingly, when P5C was superimposed on cells undergoing transitions throught the cell cycle, the magnitude of the augmentation by pyrroline-5-carboxylate was greatest in late G1 and early S. Pyrroline-5-Carboxylate also stimulated nucleotide formation from preformed purines, a step which is PRPP-dependent. We examined the incorporation of C14 hypoxanthine into C14 nucleotides using high pressure liquid chromotography to separate the respective nucleotides in the acid soluble pool. We found that P5C not only increased PRPP levels but also increased the cellular incorporation of hypoxanthine into nucleotides. Quiescent cells treated with P5C showed marked increase in the incorporation of hypoxanthine into ADP and ATP. Incorporation of hypoxanthine into total nucleotides was increased almost two fold. Thus, in quiescent cells where PRPP synthesis has been described as an early event in mitogenesis, P5C activates cells to increase their cellular complement

of PRPP and thereby increase the incorporation of preformed purines into nucleotides. These studies suggest that P5C may play a role as a second messenger in the activation of cultured cells. Furthermore, the synergistic effects of serum and P5C may depend on the compartmentation of P5C or the activation of specific P5C metabolizing enzymes to allow for maximal stimulation of PRPP formation.

Measurements of P5C in Human Plasma and Biologic Fluids. We have shown that P5C is an effector molecule. In addition we have described a proline-P5C metabolic cycle, which catalytically transfers oxidizing potential between cellular compartments as well as between cells. As a regulatory system these findings suggest that P5C and proline function as a "hormone system" for one cell to regulate the metabolism of another cell at an adjacent or distant site. This hypothesis demands that P5C be present as an extracellular moiety in mammalian species. Extension of this hypothesis into a physiologic framework has been difficult because of the unavailabilty of a P5C assay sensitive enough to detect it in biologic fluids. Therefore, we developed a radioisotopic enzyme-coupled assay for P5C which allows measurements of P5C in the picamole range. Using this assay we have measured P5C levels in human venous plasma, saliva, urine, as well as other biologic fluids. Normal plasma levels are 250 $\pm$ 20 pmol/ml. The levels in males are slightly but significantly higher than that found in females. We also measured P5C in the venous plasma of two patients with Type 2 hyperprolinemia, an inborn error of metabolism with deficiency of P5C dehydrogenase. In these patients, the levels of circulating P5C were 15 to 20 fold elevated over normals. Thus, for the first time, a relatively simple, highly specific and highly sensitive assay for P5C is available for studies of the regulatory role of proline and P5C metabolism in patients.

Significance to Biomedical Research:

The linkage of the proline metabolic pathway to glucose (energy) and nucleotide metabolism has far-reaching implications. Since the HMP pathway is the source of pentose for nucleic acids, the proline-PC interconversions provide a mechanism for adjusting the synthesis of macromolecules. Derangements in the linkage may be involved in the altered metabolism of malignant cells. Furthermore, the demonstrated proline-PC cycle provides not only a mechanism for ATP generation from the HMP pathway, but also serves as a model for small molecules acting as metabolic signals between cells. These studies provide a new approach in understanding pathophysiologic mechanisms in several diseases including: 1) metabolic derangements in sepsis, 2) increased collagen production in hepatic fibrosis, 3) retinal degeneration in gyrate atrophy and 4) oncogensis. The recent finding of P5C in biologic fluids places the intercellular proline-P5C cycle in a physiologic context. We propose that the system functions as a "hormone" by which metabolic information is transferred between cells.

Proposed Course:

Recent studies on the mechanisms of oncogenesis (Src gene, etc.) have re-emphasized the importance of studies in the area of metabolic regalation. We think that the regulatory effects of the PC-proline axis is an important new facet of metabolic regulation. During the coming year we will emphasize the following specific areas:

1) We will focus on the physiologic and/or pathophysiologic implications of the catalytic proline cycle. It is likely that the proline cycle is important mainly under "stress conditions," e.g. with increases in temperature, trauma, etc. We will use the differential metabolism of [5-^{3}H] proline and [U-^{14}C] proline to monitor cycling. The "stress conditions" may be produced in both cultured cells and in animals.

2) The PC-initiated stimulation of nucleotide formation. We will study the relationship between cell activation (mitogenesis) and the PC effect. The possibility that cell activation requires PC-initiated events will be examined physiologically and biochemically. Alterations in PC reductase e.g. kinetic properties, differential use of pyridine nucleotides (NADPH vs. NADH) and association of the enzyme with cell membranes will be examined. Using cultured cells with defined fluxes of P5C synthesis we will determine whether the P5C-mediated events are a necessary component of mitogenic activation by specific growth factors. Our ability to measure P5C will add important insight into the production, utilization, and release of P5C during activation of cultured cells.

3) Pathophysiologic mechanisms in gyrate atrophy. The deficiency in P5C production in this disorder with propressive retinal degeneration and lenticular opacities serves as a model not only for ocular diseases but also for CNS disorders. Transmission of metabolic information between various ocular tissues may be an important function for P5C metabolism. We will study P5C levels in ocular fluids as well as its metabolism in specific ocular tissues.

4) The development of a sensitive P5C assay by our laboratory now allows studies of the physiologic and pathophysiologic role of P5C in human diseases. We will first concentrate on patients with known enzyme defects e.g. Type II hyperprolinemia, gyrate atrophy, etc. These studies will then be extended to patients in which clinical manifestations suggest a pathophysiologic derangement in P5C metabolism.

5) Protein chemistry of PC reductase. We previously showed that PC reductase is present at high levels in erythrocytes and the enzyme in these cells has kinetic characteristics and regulatory features distinct from the enzyme found in other tissues. Since these findings suggest the existence of isozymes of PC reductase, we hope to identify isozymes by physiochemical methods. Purification of the enzyme from erythrocytes may provide suitable substrate for studies directed at identifying possible post-translational modification (phosphorylation, etc.). Production of antibodies to human or animal P5C reductase will be a requisite initial step for our attempts to clone the P5C reductase gene.

Publications:

Hagedorn, C.H. and Phang, J.M. Transfer of reducing equivalents into mitochondria by the interconversions of proline and ^{1}-pyrroline-5-carboxylate. Arch. Biochem. Biophys. In press.

Yeh, G.C. and Phang, J.M. Pyrroline-5-carboxylate stimulates the conversion of purine antimetabolites to their nucleotide forms by a redox dependent mechanism. J. Biol. Chem. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB04003-27 MET

PERIOD COVERED

October 1, 1982 through September 30, 1983

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

Studies of Porphyrin Metabolism in the Tumor-Bearing Host and Porphyria

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Donald P. Tschudy, M.D., Senior Investigator, Metabolism Branch, NCI, NIH

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	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 main objectives are to study the mechanisms controlling heme biosynthesis and biochemical aspects of porphyrin metabolism in experimental porphyria, tumors and tumor bearing hosts.

PHS 6040 (Rev. 1/83)

GPO 895-100

Project Description:

Objectives: To study mechanisms controlling heme biosynthesis and biochemical aspects of porphyrin metabolism in experimental porphyria, tumors and tumor-bearing hosts. To study the immunosuppressive and growth inhibitory mechanisms of succinylacetone.

Methods Employed: Quantitative enzyme determinations, chemical determination of porphyrins, porphyrin precursors and tissue heme; cell culture methods; measurement of lymphocyte proliferation in response to mitogens and antigens; measurement of antibody levels.

Major Findings: The profound immunosuppressive activity of succinylacetone (SA) has been studied further. Repeated studies of formation of antibodies to sheep red blood cells by rats show that SA administration can inhibit antibody formation by 99.9% or greater. In mice, SA did not markedly inhibit antibody formation. For this reason pharmacological studies of total body turnover of SA were performed in rats and mice. Concentrations of SA were 3-4 times higher in rats than in mice. This suggests that the failure of SA to exhibit profound immunosuppressive activity in mice results from pharmacological factors in the metabolism and excretion of the compound. This may also explain why SA exhibits weaker antitumor activity in mice than in rats. A single large dose (500 mg/kg) maintained body water concentrations in rats of 0.3-0.4μ M beyond 48 hours. This is in the concentration range which has been shown to inhibit lymphocyte transformation by antigens and the mixed lymphocyte reaction using human cells.

Because tumor cells (L1210) grown in the presence of SA gradually increase their capacity for porphyrin uptake, the question arose as to whether this resulted from a gradually induced metabolic effect of SA or a slow uptake of SA by tumor cells. Kinetic studies of SA uptake by L1210 cells demonstrated rapid uptake during a 10 minute period. This suggests that SA produces a gradual increase of tumor cell capacity for porphyrin uptake through a gradually induced metabolic effect on tumor cells.

When SA is added to human peripheral lymphocyte cultures 24 or 48 hours after antigen, lymphocyte transformation is inhibited, showing that SA exhibits immunosuppressive activity after the immune response has been initiated. SA at a concentration of 1mM inhibited thymidine incorporation by more than 80% and at 3 μM inhibited up to 100%.

Studies done in collaboration with Dr. David Nelson's laboratory show that SA prevents the development of cytotoxic ("killer") cells during the mixed lymphocyte reaction using human cells. This effect occurs at lower concentrations than those which inhibit thymidine incorporation during mitogen and antigen stimulated human lymphocyte transformation. In some experiments SA at a concentration of 30 μM completely inhibited the develop-

ment of cytotoxic cells as measured by cell mediated lysis. Considerable inhibition was seen with SA at a concentration of 3 μM.

Growth curves of viable human lymphocytes during pokeweed mitogen stimulation with and without SA present indicate that SA does not have an acute cytotoxic action on these cells. In the presence of 0.3, 1 and 3 μ M SA, viable cell number was greater after 24 hours of pokeweed stimulation than controls, although on days 3 and 6 the number of SA treated cells was below controls.

When rats are treated with SA and given sheep erythrocytes, antibody production to the erythrocytes is blocked. When the same animals are given erythrocytes at a later time, but without SA, antibody production occurs, but is less than the response of controls in the original primary response. The possibility of partial clonal deletion by repeated antigen challenge with SA treatment is raised by these data. When SA and antigen are both given to rats a second time, antibody production is markedly inhibited by SA, but not as effectively as occurs on the first administration of antigen.

Dr. Michael Blaese, collaborating on the studies of SA, has been in communication with investigators who have discovered an unexplained immunodeficiency in minks with hereditary tyrosinemia. We think this is an example of the immunosuppressive effect of SA which is known to be produced in some types of human heredity tyrosinemia.

Three pyrroles have been synthesized and tested for their ability to inhibit growth of L1210 cells in culture. These are: 1) 2-methyl-4-carboxyethyl pyrrole, 2) 2-succinyl-3-carboxyethyl-5-methyl pyrrole, and 3) 2-methyl-3-acetyl-5-(1,2,3,4 tetrahydroxybutyl) pyrrole. They were compared with SA for growth inhibitory activity. Pyrroles 1) and 2) were less effective than SA at equal concentrations, but pyrrole 3) was much more effective than SA and the other pyrroles.

Publications:

Ebert, P. S., Frykholm, B. C., Hess, R. A. and Tschudy, D. P.: Characteristics of hematin uptake in malignant, embryonic and normal cells. Cancer Biochem and Biophys. 6:157-65, 1983.

Tschudy, D. P.: Disorders of Porphyrin Metabolism. In Stein, et al. (Eds.): Internal Medicine. Little, Brown, and Co., 1983, first edition, pp. 1910-1915.

Tschudy, D. P., Ebert, P. S., Hess, R. A., Frykholm, B. C. and Atsmon, A.: Growth inhibitory activity of succinylacetone: Studies with Walker 256 carcinosarcoma, Novikoff hepatoma and L1210 leukemia. Oncology 40:148-54, 1983.

Tschudy, D. P.: The Porphyrias. In Williams, W.J., Beutler, E., Erslev, A. J., and Lichtman, M. A. (Eds.) Hematology. McGraw-Hill Book Co., 1983. In press.

Ebert, P. S., Smith, P.D., Bonner, R. F., Hess, R. A., Costa, J. L. and Tschudy, D. P.: The effect of defined wavelength and succinylacetone on the photoinactivation of leukemia L1210 cells in vitro by hematoporphyrin. Photobiochemistry and Photobiophysics. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB04002-14 MET

PERIOD COVERED

October 1, 1982 through September 30, 1983

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 Dysfunction

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Thomas A Waldmann, M.D., Chief Metabolism Branch, NCI, NIH

COOPERATING UNITS *(if any)*

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	3	3

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

A

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

Studies were directed toward defining the role of disorders of lymphocyte maturation and of immunoregulatory cell interactions in the pathogenesis of immune dysfunctions. Recombinant DNA technology has been applied to the study of the arrangement and rearrangement of immunoglobulin genes in lymphocytic leukemias. A hierarchy of gene rearrangements was suggested with a rearrangement of immunoglobulin heavy chain genes preceeding light chain genes and kappa gene rearrangments or deletions preceeding lambda. All hairy cell leukemic cells studied and lymphoid blast crisis cells from patients with chronic myelogeneous leukemia were shown to be B cell presursors on the basis of their immunoglobulin gene rearrangements. Furthermore, non-T non-B cell leukemic cells that did not react with anti-T-cell monoclonal antibodies represented B cell precursor leukemias. Using a monoclonal antibody, anti-Tac, the human receptor for T cell growth factor (TCGF, interleukin-2) has been purified to homogeneity. The receptor is a 55,000 dalton glycoprotein composed of a 33,000 dalton peptide backbone that is post translationally modified by introduction of N and O linked carbohydrate as well as sialic acid yeilding mature receptors. The anti-Tac monoclonal inhibits (1) in vitro T cell proliferation induced by antigens, the development of cytotoxic T cells and T cell dependent B cell immunoglobulin production. Leukemias of helper T cells (Sezary leukemia cells) are Tac antigen negative. In contrast the adult T cell leukemia which is associated with the type C retrovirus (human T cell leukemia/lymphoma virus, HTLV) universally displays large numbers of TCGF receptors on the cell surface. The consistant display of TCGF receptors which may be aberrant in size on adult T cell leukemia cells may play a role in the uncontrolled growth of these cells.

Other Professional Personnel:

Samuel Broder, M.D.	Deputy Clinical Director	MET	NCI
Andrew Arnold, M.D.	Medical Staff Fellow	MET	NCI
Stanley J. Korsmeyer, M.D.	Senior Investigator	MET	NCI
Ajay Bakhshi, M.D.	Medical Staff Fellow	MET	NCI
Warner Greene, M.D., Ph.D.	Expert	MET	NCI
Warren Leonard, M.D.	Senior Staff Fellow	MET	NCI
Joel Depper, M.D.	IPA	MET	NCI

Project Description:

Objectives: The objectives of the study were: to determine the sequential steps involved in the differentiation of uncommitted stem cells into B cells and plasma cells that are the cells that synthesize and secrete a particular immunoglobulin molecule. Major efforts were directed toward defining the disorders of immunoglobulin gene rearrangements associated with leukemias of B cell precursors that are unable to mature into B cells and to utilize an analysis of immunoglobulin gene rearrangements to categorize leukemias that were previously of controversial origin and to make an early diagnosis and a definition of the lineage of aberrant lymphocytes. Other objectives were to purify and characterize the membrane receptor for T cell growth factor to study the expression of this receptor in varying lymphoid malignancies, to determine the effect of a monoclonal antibody (anti-Tac) to the T cell growth factor receptor on the human immune response in vitro and to determine the efficacy of the anti-Tac monoclonal in the treatment patients with the of adult T cell leukemia. Finally, major efforts were directed toward determining the role of the regulatory network of suppressor T cells, helper T cells and macrophages in the control of the maturation of B cells into plasma cells and to define the disorders of these suppressor and helper interactions that underlie primary immunodeficieny, allergic, autoimmune and malignant disease in humans.

Methods Employed: Recombinant DNA techniques with ^{32}P labelled probes to the human constant kappa, lambda light chain and heavy chain V,D,J and C chain genes were used to study the immunoglobulin gene rearrangements that occur during B cell maturation and to analyse disorders in the arrangements of such genes in lymphocytic leukemias and immunodeficiency states. Hybridoma procedures have been used to produce monoclonal antibodies to the T cell growth factor receptor and to study biological modifiers of the immune response (lymphokines) produced by T cells. In vitro culture techniques of mitogen stimulated peripheral blood lymphocytes have been developed for the study of the terminal differentiation of B lymphocytes into immunoglobulin secreting cells and for the analysis of helper and suppressor T cell and monocyte activity. These techniques have been applied to the analysis of helper and suppressor function of T cell leukemias and to the analysis of immunoregulatory antibody to the T cell growth factor receptor.

Major Findings: Major efforts under this project over the past few years have been directed toward defining the organization of the immunoglobulin genes and the rearrangement of these genes that occur as a stem cell matures into a B cell and into the regulatory T cell circuit involved in the control of B cell maturation and of immunoglobulin synthesis. These studies have placed special emphasis on the analysis of leukemias of B cells and their precursors and of T cells that retain regulatory function in order gain insights the nature of the cells of the lymphoid system and the disorders of these cells that lead to leukemia. Additional studies were performed in patients with immunodeficiency diseases associated with a high incidence of malignancy in order to define factors in the normal and abnormal state controlling the production of antibodies and the synthesis of immunoglobulin molecules.

The human immunoglobulin light chain genes in their embryonic or germline state are orgainzed in a discontinuous system of multiple germline variable (V_L) regions alternative joining (J_L) segments and a single or even multiple constant (C_L) regions. Heavy chains are similarly organized but have an additional diversity (D_H) segment incorporated between the V_H and J_H regions. During the process of differentiation of a stem cell into a mature B cell there is a rearrangement of the cellular genome to combine a single (V_H) and a single (D_H) and a single (J_H) to form an active heavy chain gene. Subsequently a single light chain variable region (V_K or V_L) combines with an appropriate J_K or J_L region to activate a light chain gene. ^{32}P-labelled clones of human constant, joining, diversity and variable region genes were used as probes to study the gene arrangements in B cell, T cell and non-T, non-B forms of lymphocytic leukemias or cell lines. All B cell leukemias have rearranged the immunoglobulin genes of at least one set of heavy chain genes and one set of light chain genes. In addition, in many cases aberrant rearrangements of other light and heavy chain genes may be present. In contrast, all human T cell lines and leukemias retain germline patterns of both kappa and lambda genes and usually (18 of 20 cases) display germline heavy chain genes. Acute myelogenous leukemias as well as histocytic, monocytic and promyelocytic leukemias display germline heavy and light chain genes. Thus human hematopoietic cells pursuing non-B cell pathways of development usually retained their immunoglobulin genes in the germline configuration whereas cells in the B cell series always have immunoglobulin gene reorganizations. A series of leukemias of controversial origin were analyzed with the immunoglobulin gene probes to define their origin. All eight cases of hairy cell leukemia examined had heavy chain gene rearrangements when examined with J_H probe. All of these cells also had a kappa or lambda chain gene rearrangement. The appropriate light chain RNA was demonstrable in cells displaying surface kappa or lambda chains. Thus hairy cell leukemia is a malignancy of mature B cells committed to immunoglobulin production at the immunoglobulin gene level. Immunoglobulin genes were also examined in the cells of patients with chronic myelogenous leukemia and chronic myelogenous in blast crisis. In the chronic granulocytic phase or in myloid blast crisis the immunoglobulin genes remained in the germline configuration. In contrast in virtually all cases of lymphoid blast crisis

there was a rearrangement of the heavy chain genes and in three of eight cases of the light chain genes as well. Thus in most cases lymphoid blast crisis in patients with chronic myelogenous leukemia reflects a B cell precursor form of leukemia. In certain cases serial examination of a single patient revealed lymphoid blast crises with different clonal patterns of immunoglobulin gene rearrangement showing that the stem cell which represents which reflects the site of the malignant transformation in chronic myelogenous leukemia has the capacity to mature along different pathways including-different lineages of B cell development. Since all of the immunoglobulin gene reorganization and regulatory events appear to be completed by the time B cells are synthesized we have examined acute non-T, non-B lymphocytic leukemia, a leukemia that consists of cells at early stages of differentiation. Twelve of the 37 patients with acute lymphocytic leukemia displayed T cell associated antigens and had germline light chain genes and in 11 of 12 cases had germline heavy chain genes as well. In contrast, all 25 cases of "non-T, non-B" ALL, which lacked definitive T cell markers and surface immunoglobulin had rearranged heavy chain genes and in 11 cases had progressed to light chain gene rearrangements. The observations of patients with heavy chain gene rearrangements without light chain gene rearrangements but the failure to observe any patients with light chain gene rearrangements who did not also have heavy chain gene rearrangements supports the view that heavy chain gene rearrangements preceed those of light chains. Furthermore, the obsevation that seven leukemias had aberrant rearrangements of the kappa light chain genes or deletion of the kappa light chain genes with the lambda genes remaining in the germline configuration whereas there were no cases where there were aberrant lambda rearrangements in a kappa producing cell supports our view that kappa gene rearrangements and deletions preceed those of lambda light genes. That is the light chain rearrangements preceed in an order kappa before lambda. We have correlated the immunoglobulin gene rearrangements with the development of surface markers characteristic of B cells. The cases with the most difficult phenotype for classification which express HLA-Dr but lack the common acute lymphocytic leukemia antigen, cALLA, appear to represent the earliest recognizable B cell precursors with rearranged heavy chain genes but have their light chain genes in the germline configuration. Despite the fact that all 25 cases of common acute lymphocytic leukemia had heavy chain gene rearrangments and that 11 of these 25 had light chain gene rearrangements as well only 5 produced mu chains, one produced gamma chains and one produced lambda light chains. None of these cell populations manifest surface immunoglobulins nor secreted immunoglobulins. In some of the 19 cases that did not produce cytoplasmic immunoglobulin heavy chains there had been rearrangements of both sets of heavy chain genes. Those cells that have had a V, D, J aberrant joining on both of the 14th chromosomes would have deleted the residual D regions as well as the signal flanking hepta- and nonanucleotides required for immunoglobulin gene reorganization. Thus, these cells may not have the residual genetic D-region material nor the important flanking signals for gene reorganization that are required to reproduce an immunoglobulin molecule and may thus, be frozen in the B cell precursor stage of maturation as a result of these aberrant rearrangements. This would explain the failure of maturation of B cell precursors in

certain patients with acute B cell precursor lymphocytic leukemia.

Clonal populations of B cells, B cell precursors and plasma cells have specific individual immunoglobulin gene rearrangements. We have shown that the detection of such rearrangements by the identification of a new band on Southern hybridization blots with immunoglobulin gene probes provides a sensitive marker for both clonality and B cell lineage within lymphoid tissue lacking expresssion of diffinitive surface phenotypes. We have utilized these genetic markers to (1) establish a diagnosis of lymphoma in a malignancy of uncertain type, (2) show that some lymphomas previously classified as of T cell type in fact contain monoclonal B cells, (3) detect clonal B cell populations within lymphomatous tissues of undertain immmuno-type and with an atypical lymphoreticular hyperplasia having no other clonal surface markers. These sensitive and unique indicators of clonality located directly at the DNA level are capable of providing insights into the cellular origin and aid in early detection of tumors of the B-cell series.

Following the rearrangements discussed above, effective genes capable of inducing the synthesis of a particular heavy and a particular light chain are produced. As these events progress at a DNA level a stem cell matures into a pre-B cell, a cell with mu chain demonstrable in its cytoplasm but no surface immunoglobulins. Following the initiation of light chain synthesis, complete IgM molecules are produced, and incorporated into the cell surface as the pre B cell matures into a immature B cell. The immature B cell then develops into a mature B cell with a series of surface membrane rceptors. The union of appropriately presented antigen, lectin or the Epstein-Barr virus with their surface receptors triggers subsequent events which include B cell terminal differentiation into antibody producing plasma cells. This B cell maturation is carefully regulated in both a positive and negative fashion by interacting mononuclear cells. Many antigens and polyclonal activators require the presence of cooperating helper T cells as well as B cells to induce the maximal immunoglobulin response. More recently it had been recognized that a network of suppressor T cells acts a negative regulator of B cell maturation thus, inhibiting immunoglobulin production. Over the past years we have been studying the elements of the complex regulatory T cell circuit.

A major accomplishment that has been made under this project has been the definition of T cell growth factor receptors on the cells of the immuno-regulatory T cell circuit. T cell growth factor (TCGF for interleukin-II) is a 14,000 dalton glycoprotein hormone critical to the evolution of normal human immune responses that has been characterized and whose gene has been cloned. This factor is essential for the expansion and continued proliferation of cytotoxic, suppressor and some helper T cells. The normal pathway of T cell activation involves the interaction of antigen or lectin with macrophages which are induced to synthesize and secrete the monokine interleukin-I. In the presence of antigen or lectin and interleukin-I, inducer/helper T lymphocytes are activated to synthesize and secrete TCGF. Resting T cells do not manifest receptors for T cell growth factor, however, as T

cells are activated, they are induced to express T cell growth factor receptors. In the presence of T cell growth factor, the cells with the newly induced receptors are then capable of proliferation and continued growth.

We have developed a monoclonal antibody using hybridoma technology that we term anti-Tac that appears to identify the T cell growth factor receptor. We have utilized this antibody to elucidate the role of the T cell growth factor receptor in the development of various lymphocyte functions. The antibody was selected on the basis of its ability to bind to activated T cells, but not to bind to resting T cells or various B cell and pre-B cell lines. This pattern of cellular reactivity was essentially identical to that of TCGF receptors. We therefore hypothesized that anti-Tac recognizes the human receptor for T cell growth factor.

Our other data that we have developed in support of this hypothesis are as follows: 1) anti-Tac blocks 80% of the TCGF induced DNA synthesis of TCGF dependent continuous T cell lines but does not inhibit DNA synthesis of TCGF independent T cell lines, 2) anti-Tac but not control monoclonal antibodies block over 95% of the binding of ^{3}H-TCGF to Hut-102B2 cells and PHA lymphoblast that manifest T cell growth factor receptors; 3) TCGF in high concentration blocks the binding of ^{3}H-anti-Tac but not ^{125}I-anti OKT11 to PHA lymphocyte lymphoblasts; 4) after TCGF is covalently cross linked to Hut 102B2 cells both anti-Tac and anti-TCGF can immunoprecipitate a band that is 12-14,000 daltons larger than the receptor immunoprecipitated by anti-Tac in the absence of crosslinking. We believe this additional band represents TCGF crosslinked to its receptor;

Utilizing anti-Tac monoclonal antibody, we have defined those reactions that require an interaction of T cell growth factor with its inducible receptor on activated T cells. We find that anti-Tac 1) blocks T cell proliferation induced by soluble antigens, autologous antigens and allogenic HLA antigens; 2) partially inhibits T cell proliferation induced by mitogenic lectins; 3) abrogates the generation of cytotoxic T lymphocytes in allogenic cell cultures and; 4) inhibits the immunoglobulin production by B cells activated by T cell dependent polyclonal activators such as pokeweed mitogen and wheat germ agglutinin. In contrast, anti-Tac does not inhibit the immunoglobulin synthesis by the T helper cell independent activator, the Epstein-Barr Virus. The first three actions discussed above can be interpreted as the prevention of the binding of TCGF by activated T cells. The inhibition of immunoglobulin synthesis by B cells stimulated by T helper cell dependent activators may either reflect an inhibition of the secretion of a B cell growth and differentiation factors by the helper T cells or a direct action on the B cells themselves. The issue as to whether B cells can be controlled directly by T cell growth factor is a controversial one. We have evidence that under certain circumstances B cells may manifest an antigen recognized by the anti-Tac monoclonal antibody. All Hairy Cell Leukemic cells that are clearly B cells as discussed above on the basis of their immunoglobulin gene reorganization are Tac (TCGF receptor) positive.

Certain B cell lines from patients with Burkitt's lymphoma or with HTLV induced T cell leukemia or even B cell lines from normal individuals induced with Epstein-Barr Virus can be induced to manifest the Tac antigen by exposure to phorbol esters. On the basis of preliminary studies, it appears that the binding of the anti-Tac monoclonal to Tac positive B cells is inhibited by purified TCGF supporting the view that in these B cells the tac antigen represents a receptor for TCGF. In aggregate, these data provide additional support for the hypothesis that anti-Tac recognizes the human T-cell growth factor receptor and illustrate ways in which this antibody can be used to modulate the human immune response.

As noted above, the regulatory T cell network of normal individuals represents a complex array of different cells with different and at times opposing functions. It has, therefore, been quite difficult to analyze the function, phenotype and mode of action of inividual T cells with a unique specific function. In order to get a better understanding of this regulatory network, we have been studying clonal T cell leukemias with retained immunoregulatory function. Over the past year we have been examining leukemias of mature T cells. The first type, the Sezary syndrome, is characterized by erythroderma, generalized lymphadenopathy, and circulating pleomorphic malignant T lymphocytes with a propensity for epidermal infiltration. The second leukemia, the adult T cell leukemia (ATL), is also a malignancy of mature T cells that has a tendency to infiltrate the skin. However, certain clinical features aid in distinguishing these syndromes. First, ATL has a more aggressive course and is often complicated by hypercalcemia and pulmonary infiltrates. Second, cases of ATL are clustered geographically, occurring in the South west of Japan, the Carrabian basin and in certain areas of the Southeastern United States. Recently, a unique human type C retrovirus, human T cell leukemia lymphoma virus (HTLV) has been isolated from the neoplastic T cells of patients with ATL from multiple areas of the world. In the present study we compared the immunoregulatory function and cell surface phenotype of leukemic cells from 10 patients with the clinical diagnosis of adult T cell leukemia and 2 patients with the Sezary syndrome who had circulating antibodies to HTLV with the function and cell surface phenotype of leukemic cells from 10 patients with the Sezary syndrome lacking serum antibodies to HTLV. Leukemic cells of both groups were of the T3, T4+, T8- phenotype. Despite the similar phenotype HTLV negative Sezary leukemic cells frequently functioned as helper T cells whereas, most HTLV+ ATL cells functioned as suppressors of immunoglobulin biosynthesis. The ATL cells functioned as suppressors even when the target cells were depleted of T8 cells an observation that reduces the likelihood that the leukemic T cells induces cells among the normal T cells to become the effectors of suppression. In addition, we have noted that a long term T cell growth factor dependent line from the leukemic cells of one of the patients with HTLV associated leukemia secretes a 70 to 90,000 molecular weight suppressor protein termed SISS-B that inhibits pokeweed mitogen induced immunoglobulin synthesis by B cells that are cultured with irradiated T cells. It appears from these studies as well as from more extensive analyses from other laboratories that the cells defined by the T4 and T8 antibodies are

complex populations that are not solely helper/inducer and suppressor/cytoxic populations respectively. They support the view that cell populations that react with T4 and T8 monoclonal antibodies differ not in terms of their function per se, but in terms of the class of the antigen with which the T cell interacts; i.e., T8+ cells appear to be involved in those cellular interactions and functions that are controlled by class I HLA (i.e. HLA A or B molecules) whereas, T4+ cells are involved in interactions that concern class II molecules (i.e., HLA-DR or SB). In this scheme, T4+ cells would not only be helper cells in their interactions with other lymphoid cells but might also act as cytotoxic or suppressor cells when HLA-Dr molecules are involved.

Since the T3, T4 and T8 monoclonal antibodies were not of value in differentiating HTLV negative Sezary leukemic cells from ATL cells we examined these leukemic cells populations using other monoclonal antibodies to a series of activation antigens on T cells including the T9 antigen that defines the transferrin receptor, the Ia antigen present on resting B cells, monocytes and on activated T cells and the T10 antigen present on primative hematopoietic cells, thymocytes and activated T cells. Both T9 and Ia antigens were present on the leukemic cells of the majority of patients with either HTLV positive ATL or the Sezary leukemias without serum antibodies but were not present on normal resting T cells. Thus these monoclonal antibodies helper to identify both of these groups of leukemic cells as activated rapidly proliferating cells but they are not of value in differentiating these leukemias from each other. The monoclonal T10 was of greater value in differentiating these leukemias. The 10 HTLV antibody negative Sezary leukemic cell populations had reduced proportions of T10 whereas 9 of 10 HTLV positive adult T cell leukemias or Sezary leukemic populations examined had a higher percentage of T10 positive T cells than did normal individuals.

The monoclonal, anti-Tac, which was developed in our laboratory and which appears to react to the membrane receptor for T cell growth factor was of value in differentiating cells from the patients with ATL and the two Sezary patients with antibodies to HTLV from cells of the remaining Sezary patients who were HTLV negative. The Sezary T cell leukemic populations from patients that did not have antibodies to HTLV were Tac antigen negative whereas all of the leukemic T cell populations from patients with antibodies to HTLV were Tac antigen positive. Thus the HTLV associated mature T cell leukemia cells were Tac antigen positive and thus manifest the receptor for T cell growth factor. The fact that all ATL cell poplations as well as HTLV induced T cell lines manifest the Tac antigen that is associated with the inducible receptor for T cell growth factor may have implications for our understanding of one of the pathogeneic factors associated with the leukemic transformation and the rapid proliferation of these malignant T cells. The infection of mature T cells with HTLV leads to the production of T cell growth factor by some of these cell poplations in culture and to the expression of TCGF receptors in all cases. This production of T cell growth factor in some cases and the induction of the

receptor required for its action may play a role in the pathogenesis of the uncontrolled growth of these neoplastic cells. For those cells producing TCGF there may be a self stimulatory circuit in which the same cell produces and responds to this growth factor for T cells. More recently it has been shown that certain HTLV transformed cord lines do not release detectable T cell growth factor. It is thus possibile that in certain cases HTLV infection leads to a bipassing of the TCGF-TCGF receptor system causing altered growth by mechanisms that have not been defined. Alternatively HTLV may directly affect the receptor changing its form so that it behaves as if T cell growth factor were bound to it and thus directly stimulates the growth of the malignant T cell. In accord with this latter observation we have isolated the receptors from a variety of human T cell leukemia/ lymphoma virus infected T cell lines. While all are glycoproteins with intra-chain dilsulfide bonds we have found significant size hetrogeneity. The most abberant receptor (found on Hut 102 B2 cells) is 5,000 daltons smaller and has a slightly more basic PI than the normal receptor on PHA activated lymphoblasts. Pulse chase and tunacamycin experiments demonstrate that both the Hut 102-102B2 and PHA lymphoblasts receptors have ostensively the identical precursor peptide of 33,000 daltons and that differences in post translational processing resulting in differences in the size of the receptors are present. It is conceivable that the constant presence of receptors on these cells and/or the abberancy of these receptors may contribute to the uncontrolled growth of these malignant cells.

Using the in vitro biosynthesis procedure with polyclonal activation we have developed, we have continued to identify patients with disordered immunoglobulin synthesis due to a variety of mechanisms including disorders of intrinsic B cell activity, disorders of helper T cell function, and disorders of the network of interacting T cells involved in immune suppression. Individuals with common variable hypogammaglobulinemia with excessive suppressor cell activity have been shown to have a marked increased in the number of T8 Ia-bearing T cells. Certain of the cell lines from such patients as well as lines from patients with HTLV associated T suppressor leukemia have synthesized a suppressor lymphokine that we term SISS-B. This suppressor lymphokine is also produced by Concanavalin A stimulated peripheral blood mononuclear cells, continuous cloned human T cells grown in Interleukin-II as well as human T-T cell hybridomas that we have prepared. The humoral suppressor factor produced 40-80% inhibition of polyclonal antibody synthesis. This factor is: (1) a protein with a molecular weight of 70-90,0000 daltons, (2) noncytotoxic, (3) present as early as eight hours after exposure to Concanavalin and, (4) reversed in its action by the monosaccharide L-rhammnose.

Significance to Biomedical Research: The studies of leukemias using recombinant DNA technology are providing insights into the earliest events of B cell maturation, are aiding in classification of malignancies that were previously of controversial origin. They are providing methods for the early diagnosis and the definition of the lineage of lymphoid malignancies and are providing insights into the causes that underlie the maturation failure of

lymphoid malignancies. Taken into conjunction with studies of transposed onc-genes they are providing insights into the nature of the malignant transformation itself. The studies relating to the definition of the T-cell growth factor receptor are important in our understanding of the mechanism involved in the control of T cell proliferation and in the development of T cells into cytotoxic, suppressor and helper T cell subpopulations. Furthermore, they are providing important new information concerning the pathogenesis of the uncontrolled growth of the leukemic T cells of patients with the HTLV associated T cell leukemia. Finally, the devlopment of techniques for the study of the effect of helper and suppressor T cells on the maturation of B cells and their application to the study of patients with immunogloblin deficiency disorders have been of great value in defining the critical stages of B cell maturation and of the network of immunoregulatory cells that controls this maturation process. A series of new pathogenic mechanisms have been defined to explain the defects in patients with immunodeficiency disorders that are associated with a high incidence of malignancy. In general these studies are providing a scientific basis for the development of rational strategies for the therapy of immunodeficiency and of malignant diseases.

Proposed Course: Studies directed toward understanding the pathways and regulatory mechanisms controlling the sequential development of stem cells into B cells and then into immunoglobulin synthesizing plasma cells. Special emphasis will be placed on the immunoglobulin genes and their rearrangement that control immunoglobulin synthesis. Here studies of patients with heavy chain disease to define these disorders of a genetic level will be extended. Analysis of autosomal recessive immunodeficiency diseases using restriction length polymorphisms will be persued to defined those genetic immunoglobulin deficiency diseases with an abnormal gene on chromosome 14, the site of the heavy chain immunoglobulin genes. Further studies will be performed in patients with immunodeficiency disease with probes for genes that are uniquely expressed in T cells or B cells but not on the opposing cell. Such clones have been obtained using subtraction library approaches. In addition we will continue our work on the network of immunoregulatory T cells that control human immune responses. Here efforts will be made to try to clone the gene that encodes the T cell growth factor receptor. In additional studies, the efficacy of the anti-Tac monoclonal in the treatment of patients with Tac positive T cell leukemias will be evaluated. In addition, studies of the nature and mode of action of the lymphokine, SISS-B on the immune response will be extended.

Honors and Awards:

The Roger Orson Memorial Lecture, The University of Minnesota, 1982
First Julius H. Sedlmayr Lecture, Roosevelt Hospital, Columbia Unviersity, 1983
Sigma Xi Lecture, University of Connecticut, 1983
The William Dameshek Visiting Professorship and The Third William Dameshek Memorial Lecture, Tufts University, 1983
Distinguished Service Medal, DHHS, 1983

Publications:

Waldmann, T.A.: Protein-losing gastroenteropathy. In Wagner, H.N. (Ed.): Principles of Nuclear Medicine, 2nd Edition. W.B. Saunders. In press.

McIntire, K.R. and Waldmann, T.A.: Measurement of alpha-fetoprotein. In Rose, N.R. and Freedman, H. (Eds.): Manual of Clinical Immunology, 2nd Edition. American Society for Microbiology, Washington, D.C., 1980, pp. 936-943.

Waldmann, T.A. and Herberman, R.: Tumor markers in the diagnosis and in monitoring the therapy of malignancy. In Holland, J. and Frie, E.J. (Eds.): Cancer Medicine, 2nd Edition, 1982, pp. 1068-1088.

Broder, S., Muul, L., Megson, M., and Waldmann, T.A.: Abnormalities of immunoregulatory cells in immunodeficiency disease and cancer. In Sell, K. (Ed.): The Lymphocytes. Alan R. Liss, Inc., 1981, pp. 93-130.

Waldmann, T.A., Korsmeyer, S.J., Hieter, P.A., Ravetch, J.V., Broder, S., and Leder, P.: Regulation of the human immune response: From immunoglobulin genes to regulatory T cell networks. Fed. Proc. 42: 2498-2503, 1983.

Korsmeyer, S.J., and Waldmann, T.A.: The immunoglobulin gene organization and assembly. In Fudenberg, H.H., and Stobo, T. (Eds.): Basic and Clinical Immunology, 4th Edition. Los Altos, California, Lange Medical Publications, 1982, pp. 43-51.

Kirsch, J.R., Ravetch, J.V., Hieter, P.A., Korsmeyer, S.J., Waldmann, T.A., and Leder, P.: Genetic rearrangements in the humoral immune system. In Serrou, B. (Ed.): Elsevier, North Holland, Biochemical Press, 1982, pp. 113-124.

Korsmeyer, S.J., Hieter, P., Sharrow, S.O., goldman, C.K., Leder, P. and Waldmann, T.A.: Normal human B-cells display ordered light-chain gene rearrangements and deletions. J. Exp. Med. 156: 975-985, 1982.

Leonard, W.J., Depper, J.M., Uchiyama, T., Smith, K.A., Waldmann, T.A., Greene, W.C.: A monoclonal antibody, anti-Tac blocks the membrane binding and action of human T cell growth factor. Nature 300: 267-269, 1982.

Greene, W.C., Fleisher, T.A., Depper, J.M., Leonard, W.J., Stanton, G.J. and Waldmann, T.A.: Characterization of a soluble suppressor of human B cell function produced by a continuous human suppressor T cell lines. II. Evidence for suppression through a direct action of CTC-SISS B on human B cells. J. Immunol. 129: 1120, 1982.

Waldmann, T.A., and Korsmeyer, S.: Hierarchy of immunoglobulin gene rearrangements and deletions in human lymphoid leukemia. International Symposium on Leukemia Research, 1983, pp. 77-84.

Waldmann, T.A., Broder, S., Goldman, C.K., Frost, K., Korsmeyer, S.J., and Medici, M.A.: Disorders of B cells and helper T cells in the pathogenesis of the immunoglobulin deficiency of patients with ataxia-telangiectasia. J. Clin. Invest., 1983, 71:282-295.

Mihich, E., MacDonald, J., Oettgen, H., Waldmann, T.A., Jasmin, C., Serrou, B., Grimm, E., Blomgren, H.: Cancer Immunotherapy: Clinical Studies. In Current Chemotherapy and Immunotherapy. Proceedings of the 12th International Congress of Chemotherapy. Amer. Soc. for Microbiol. Press, Washington, D.C., 1982, pp. 1150-1154.

Korsmeyer, S.J., Arnold, A., Bakhshi, A., Ravetch, J.V., Siebenlist, U., Hieter, P.A., Sharrow, S.O., LeBien, T.W., Kersey, J.H., Poplack, D.G., Leder, P., and Waldmann, T.A.: The rearrangement of immunoglobulin genes and cell surface antigen expression of acute lymphocytic leukemia of T-cell and B-cell precursor origin. J. Clin. Invest. 71: 301-313, 1983.

Waldmann, T.A.: Common variable immunodeficiency in current therapy in allergy and immunology. In Liectestein, L. and Fauci, A.S. (Eds.): B.C. Deckers Inc., Philadelphia Publishers, 1983, pp. 293-296.

Waldmann, T.A.: Science Citation Classic: "Waldmann, T.A., Broder, S., Blaese, R.M., Durm, M., Blackman, M., and Strober, W.: The role of suppressor T cells in the pathogenesis of common variable hypogamma-globulinemia. The Lancet II:609-613, 1974." Current Contents 18:17, 1983.

Waldmann, T.A.: Intestinal lymphongiectasia. In Birth Defects Compendium, 3rd Edition. In press.

Depper, J.M., Leonard, W.J., Smith, K.A., Waldmann, T.A., and Greene, W.C.: Monoclonal anti-Tac blocks the action and membrane binding of human interleukin-2. In Oppenheim, J.J. and Cohen, S. (Eds.): Interleukins, Lymphokines and Cytokines. In press.

Depper, J.M., Leonard, W.J., Waldmann, T.A., and Greene, W.C.: Blockade of the Interleukin-2 receptor by anti-Tac antibody: Inhibition of human lymphocyte activation. J. Immunol. In press.

Waldmann, T.A., Korsmeyer, S.J., Hieter, P.A., Ravetch, J.V., Arnold, A., and Leder, P.: Immunoglobulin gene rearrangements in human lymphocytic leukemia. Cold Spring Harbor Symposium. In press.

Fiorilli, M., Misiti, J. Pandolfe, F., Serenzato, G., and Waldmann, T.A.: In vitro interaction with antigen suppresses the differentiation of chronic lymphocytic leukemia cells secreting monoclonal anti-sheep red cell antibody. J. Immunol. In press.

Waldmann, T.A., Misiti, J., Nelson, D., and Kramer, K.H.: Ataxia telangiectasia: a multi-system hereditary disease with immunodeficiency impaired organ maturation defect, x-ray hypersensitivity and a high incidence of neoplasia. A Combined Staff conference of the NIH Annals of Internal Medicine. In press.

Waldmann, T.A.: The regulation of the human humoral immune response and functional assays for its assessment. In Wedgwood, R.J., and Rosen, F. (Eds.): Workshop on Immunodeficiency Diseases. The March of Dimes Birth Defects Press, Sunderland Mass. In press.

Wedgwood, R.J., Rosen, F., Auiti, F. Cooper, M., Good, M., Hansen, L., Hitzig, W.H., Matsumoto, S., Seligmann, M., Soothil, S.F., and Waldmann, T.A.: Report of WHO Panel on Primary Immunodeficiency Syndromes. Clinical and Experimental Immunology. In press.

Mariani, G., Sullivan, P.W., McGuire, E.A., McIntire, K.R., Adamson, R.H., and Waldmann, T.A.: The significance of in vivo and in vitro metabolic studies of tumor produced proteins. In Mariani, G. (Ed.): Pathophysiology or Plasma Protein Metabolism. In press.

Greene, W.C., Cossman, J., Leonard, W.J., Bakhshi, A., Waldmann, T.A., and Korsmeyer, S.J.: Hairy Cell Leukemia: a malignant proliferation of B cells which express Tac antigen. In Golde, D.W. and Marks, P.A. (Eds.): Normal and Neoplastic Hematopoiesis. UCLA Symposia on Molecular and Cellular Biology, Volume 9. New York, New York, Alan R. Liss, Inc. In press.

Bakhshi, A., Arnold, A., Sharrow, S.O., LeBien, T.W., Kersey, J.H., Poplack, D.G., Leder, P., Waldmann, T.A., and Korsmeyer, S.J.: Correlation of cell surface antigen expressions and immunoglobulin gene rearrangements in acute lymphocytic leukemia. UCLA Symposia on Molecular and Cellular Biology, New Series. In press.

Greene, W.C., Leonard, W.J., Depper, J.M., and T.A. Waldmann: Studies of the human TCGF receptor with monoclonal anti-Tac. J. Clin. Immunol. Immunopath. In press.

Taub, R.A., Hollis, G.F., Hieter, P.A., Korsmeyer, S.J., Waldmann, T.A., and Leder, P.: The variable amplification of immunoglobulin lambda light chain genes in human populations. Nature. In press.

Korsmeyer, S.J., Greene, W.C., Cossman, J., Hsu, S., Neckers, L.M., Marshall, S.L., Jensen, J.P., Bakhshi, A., Leonard, W.J., Jaffe, E.S., and Waldmann, T.A.: Rearrangement and expression of immunoglobulin genes and expression of Tac antigen in Hairy Cell Leukemia. Proc. Natl. Acad. Sci. USA. In press.

Greene, W.C., Depper, J.M., Leonard, W.J., Fleisher, T.A., and Waldmann, T.A.: Construction of human suppressor T cell hybridomas. Lymphokine Research. In press.

Waldmann, T.A.: Protein losing gastroenteropathies. In Bockus: Gastroenterology, 4th Edition. In press.

Gallo, R., Wong-Staal, F., Greene, W.C., and Waldmann, T.A.: Letter to Editor. Blood. In press.

Lum, LG., Benveniste, E., Steinberg, A.D., Waldmann, T.A., and Blaese, R. M.: The proportion of T_A, T_G and T_M cells in various immunodeficiency and autoimmune disorders. Cell. Immunol. In press.

Rosen, F.S., Wedgwood, R.J., Auiti, F., Cooper, M.D., Good, R.A., Hanson, L.A., Hitzig, W.H., Matsumato, S., Seligmann, M., Soothill, J.F., Waldmann, T.A.: In Wedgwood, R.J., Rosen, F.S., and Paul, N.W. (Eds.): Primary Immunodeficiency Diseases. Birth Defects Original Series. In press.

Depper, J.M., Leonard, W.J., Waldmann, T.A., and Greene, W.C.: A monoclonal antibody which recognizes the human receptor for T-cell growth factor: functional effects and have as a probe to investigate receptor regulation Lymphokine Research. In press.

Leonard, W.J., Depper, J.M., Waldmann, T.A. and Greene, W.C.: A monoclonal antibody to the human receptor for T cell growth factor. In Cuatrecases, P. and Greaves, M. (Eds.): Receptors and Recognition: Antibodies to Receptors. In press.

Korsmeyer, S.J., Bakhshi, A., Arnold, A., Siminovitch, K.A., and Waldmann, T.A.: Genetic rearrangements of human immunoglobulin genes. In Greene, M.I. and Nisonoff, A. (Eds.): Idiotypic Networks in Immunobiology. Plenum Publishing Company, New York. In press.

SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY REPORT
DCBD, NCI
October 1, 1982 through September 30, 1983

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 seven 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:

During the past year we have continued our studies of the immunopathology of skin diseases in two major areas. The first is in cell mediated immunity and the role of epidermal Langerhans cells in the induction and expression of cell mediated immune responses. In vivo studies indicate that Langerhans cells play an integral role in the induction of contact hypersensitivity. In vitro Langerhans cells have been shown to have strong stimulatory activity in TNP specific and allostimulatory proliferation and cytotoxic T lymphocyte systems. So far we have been unable to demonstrate their ability to perform accessory cell functions i.e., in the restoration of a CTL response in an Ia^+ adherent cell depleted culture system. We have also shown that ultraviolet light irradiation modulates the antigen presenting function of Langerhans cells in such a way so that UV irradiated epidermal cells stimulate allogeneic T cells poorly if used immediately after irradiation and stimulate vigorously if taken 3 days after irradiation. We have also demonstrated that in certain types of immunologic reactions in the skin, namely in graft versus host disease (in mice) keratinocytes can be induced to synthesize Ia antigens. The Ia antigens on keratinocytes do not induce allogeneic T cell proliferation. The function(s) of these Ia positive keratinocytes is currently under study.

The second major area of study is the identification and characterization of antigens and antibodies involved in the pathophysiology of some of the so-called auto-immune blistering skin diseases. We are currently concentrating on the basement membrane zone constiuents involved in normal physiology and in the disease called pemphigoid. Using immunoprecipitation techniques and fluorography we had reported that pemphigoid antigen was a disulfide-linked glycoprotein of molecular weight of approximately 220K. With newer technology we have demonstrated that the pemphigoid antigen can be directly extracted from skin and is very similar to that which was immunoprecipitated from the cultured cells. We are also currently characterizing skin specific antigens which are identified by monoclonal antibodies produced in our laboratories. One of these is a unique stratified squamous epithelia specific lamina densa antigen which is identified by KF-1 antibody. This antigen is not detected in certain forms of epidermolysis bullosa.

Detection and Analysis of Circulating Immune Complexes:

Increasing evidence indicates that circulating antigen-antibody complexes play a role in the pathogenesis of a variety of dermatologic, rheumatologic, neoplastic and infectious disease states. We have identified and partially characterized the immune complexes which exist in several diseases i.e. Sjogren's syndrome, mixed cryoglobulinemia, mixed connective tissue disease, acute and chronic schistosomiasis, hepatitis and various types of cutneous and systemic vasculitis, utilizing two highly sensitive radioimmunoassays for detecting immune complexes i.e. ^{125}I-C1q binding assay and the Raji cell radioimmunoassay. Using a sensitive radioimmunoassay for the detection of IgA containing immune complexes we have demonstrated that patients with dermatitis herpetiformis, gluten-sensitive enteropathy and IgA nephropathy, among others, have circulating IgA containing immune complexes. In dermatitis herpetiformis these soluble immune complexes contain both IgA_1 and IgA_2. We have also examined the influence that certain genes of the major histocompatibility complex exert on immune function in vivo and in vitro in humans. In this regard we have demonstrated an Fc receptor reticuloendothelial system clearance defect in 50% of patients with dermatitis herpetiformis and have found that a high percentage of HLA-B8/DRw3 positive normal individuals also have delayed splenic clearance of IgG coated autologous erythrocytes indicating abnormal FcIgG receptor function of splenic macrophages. In attempts to determine the extent of this FcIgG receptor defect we studied lymphocyte receptors and found that normal HLA-B8/DRw3 positive individuals and HLA-B8/DRw3 positive dermatitis herpetiformis patients also have decreased numbers peripheral blood lymphocytes bearing receptors for the Fc portion of IgG. In addition, normal individuals who possess the HLA-B8/DRw3 antigens have increased T-helper cell/T-suppressor cell ratios. This is due to a statistically significant decrease in the number of T-suppressor cells. These HLA-B8/DRw3 patients and controls were also shown to have increased numbers of spontaneous immunoglobulin secreting cells in their peripheral blood as measured by a plaque forming assay.

Another of our major interests centers around the role of chemical mediators in the induction ofn inflammation. We have shown that human C5a is a potent mediator of cutaneous inflammation. It causes the rapid onset of a wheal and flare reaction when injected into normal skin. The wheal and flare reaction associated with C5a skin tests can be decreased by pretreatment of the individual with antihistamines but not by pretreatment with systemic corticosteroids. Injections of human C5a into normal skin cause the accumulation of polymorphonuclear neutrophils around the blood vessels in the superficial and mid dermis.

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. Oral 13-cis-retinoic acid was effective in the treatment of skin cancer, and a variety of disorders of keratinization, and cystic acne. An oral synthetic aromatic derivative of retinoic acid (RO-10-9359) was similarly tested and found to be more effective and less toxic than 13-cis-retinoic acid in the treatment of the disorders of keratinization. A high initial, low maintenance dosage of 13-cis-retinoic acid was found to be a comparably effective schedule but less

productive of toxicity than previously used continuous high-dosage schedules in the treatment of cystic acne. With regard to safety, we are continuing to closely monitor long term and short term side effects of the retinoids. Patients treated with 13-cis-retinoic acid showed small but significant elevations in plasma lipids and changes in lipoproteins. RO-10-9359 produced similar changes which were dose dependent and responsive to dietary management. One chronic toxicity, "Retinoid Hyperostosis" has been observed with long-term, high-dose inotretinoin. This shows anterior spinal ligament calcification and osteophyte formation of vertebrae.

In conjunction with these clinical studies we are continuing to evaluate the morphologic and biochemical effects of Vitamin A and its analogs on skin. Freeze fracture studies have indicated that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms. In addition, a specific cytosol retinol binding protein has been identified in mouse skin and human skin from patients with Darier's disease, psoriasis and basal cell carcinomas. A retinoic acid binding protein has also been identified in normal skin. Using split skin preparations and keratomed skin we have found that the binding proteins in human skin are predominantly in the epidermis.

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

UV-radiation is believed to be the major cause of the most common type of human cancer, cancer of the skin. Patients with xeroderma pigmentosum (XP) are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor development in XP patients would, therefore, elucidate the role of DNA repair in preventing, in normal humans, those cancers which may be due to certain chemical and physical carcinogens. As some XP patients develop neurological abnormalities which are due to the early death of neurons we have studied other neurodegenerative disorders in order to elucidate their pathogenesis as well as to develop pre-symptomatic diagnostic tests. We have used a trypan blue exclusion test to determine the number of lymphoblasts surviving after irradation with x-rays. We have now demonstrated hypersensitivity to X rays in lymphocyte lines from patients with the following degenerative diseases: Duchenne muscular dystrophy, Usher syndrome (recessively inherited retinitis pigmentosa and sensorineural deafness), Alzheimer and Parkinson diseases, and in Down syndrome. It is possible that the degeneration of excitable tissue in these hypersensitive disorders may be caused by the accumulation of unrepaired DNA damage as the result of faulty DNA repair mechanisms.

With Dr. Dominic A. Scudiero we have detected hypersensitivity to the lethal effects of N-methy-N-nitrosoguanidine (MNNG), a mutagenic and carcinogenic DNA-damaging chemical, in fibroblasts from 6 patients with Huntington disease, 4 with familial dysautonomia, 5 with muscular dystrophy and with Usher syndrome. We have increased the number of normal lines studied from 13 to 21, and we have studied 3 additional Usher syndrome and 4 additional muscular

dystrophy lines. Our results show that Usher syndrome and muscular dystrophy are diseases in which the patients' fibroblasts are significantly hypersensitive to the lethal effects of MNNG. Fibroblasts from patients with demyelination (e.g., multiple sclerosis, Cockayne syndrome, Charcot-Marie-Tooth disease) were not hypersensitive to MNNG. All our results show that hypersensitivity to MNNG is present in cells from patients with primary degeneration of post-mitotic excitable tissue (e.g., nerves, photoreceptors, muscle).

Biochemical Characterization of Mammalian Melanosomes:

We are continuing our studies of the abnormal proteins produced in malignant melanoma. These studies are aimed at elucidating the mechanism of formation of these atypical proteins, as well as their importance to the immunology of melanoma and possible immunotherapy potential. The melanosome in normal pigment cells is composed of multiple proteins, many of which are loosely bound and easily extracted. These probably constitute the proteins of the limiting membrane of the organelle. Other proteins are of lower molecular weight and are tightly bound to the granule, and probably constitute the structural, fibrillar protein; they appear to be the proteins which complexes with the melanin polymer. Melanosomal proteins from melanoma tissues vary in structure from those of normal tissues. A comparison of analogous proteins from normal and melanoma melanin granules resolved a slight, but significant, difference of isoelectric points and a difference of 10,000 MW between the two proteins; both have amino acid contents which are identical with respect to 13 amino acids, but differ significantly with regard to four amino acids. The carboxy and amino terminals of these proteins are identical, while peptide mapping has revealed that amino acid sequences are deleted in 3 or more regions of the tumor specific protein. Other proteins in these tissues seem to differ in a similar manner. It has been found that tumor-specific proteins similar to these can be found in the serologic fluids of melanoma patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro. It has been recently shown that one of these shed proteins has structural homology to the albumins, and may represent a normally occurring gene which is abnormally expressed in neoplastic tissues. There are implications that these tumor-specific proteins may have a critical immunologic importance to the survival of the tumor in the host.

We are also studying the role of tyrosinase in melanogenesis in normal tissues and the importance of the altered enzyme found in melanoma. Since this enzyme is essential for melanin biosynthesis, it is a unique system for the study of enzymatic control mechanisms in normal and malignant tissues. We have examined the status of enzymatic control of melanogenesis and have found that tyrosinase is the enzyme responsible for melanin synthesis in many different pigmentary systems. We have shown that L-DOPA is the natural activator of the enzyme in vivo; and that tyrosinase is extremely specific in its requirement for L-DOPA as a cofactor. It has been further shown that tyrosinase is under allosteric control and that phospholipids may play a part in the expression of the enzyme's activity. Our laboratory has provided evidence which supports the theory that the enzyme is additionally controlled by enzyme-associated factors which can further modify the production of pigment; these may prove to be critical to the control of pigment formation in mammals.

Tumor Virus Expression In Vitro and In Vivo:

Papilloma viruses are a common cause of epidermal tumors in man. Some lesions induced by these viruses may undergo malignant conversion. Little has been known about the functional organization of the genomes of these viruses or how lesions progress from a benign to a malignant state. The capacity of Bovine papillomavirus (BPV) DNA to transform mouse cells has been studied. The sequences responsible for transformation by BPV are not identical to those required for its maintenance as a multiple copy extra-chromosomal element. A 2.3 kb segment of the viral DNA which cannot by itself induce transformation can be activated by a retroviral LTR to be transforming. Cells transformed by such a construction contain integrated copies of the viral DNA. The demonstration that cells transformed by Bovine papillomavirus can be cured by their viral DNA by long term treatment with interferon has been applied clinically to a trial of human leukocyte interferon in patients with epidermodysplasia verruciformis, a disease of chronic widespread wart virus infection. In short term studies, intralesional or systemic treatment with human leukocyte interferon resulted in a marked diminution in the size of warts and a decrease of virus-positive cells in lesional skin.

The studies of viral and cellular oncogenes have also made considerable progress. We have cloned four different human p21 ras genes which are homologous to the p21 transforming genes of Harvey and Kirsten murine sarcoma viruses. Chromosomal mapping of these genes has demonstrated that they are dispersed to four different chromosomes. An activated form of one of these genes has been found to be a tumor oncogene. The highly oncogenic property of this activated gene has been localized by genetic studies and by DNA sequence analysis to a point mutation in the p21 coding region of the gene. Since elevated levels of the normal p21 protein encoded by this gene can also be oncogenic, these results indicate that p21 ras genes can be oncogenic by either of two mechanisms: increased levels of the normal gene product or normal levels of a structurally altered gene product.

Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments:

The polypeptide chains which comprise the subunits of the keratin filaments of normal bovine and murine epidermis have been isolated and individually characterized by standard protein chemical techniques. The unfractionated mixture of polypeptides or combinations of two of the purified polypeptides spontaneously polymerize in vitro in dilute salt solution into filaments that are uniformally 80 A wide and up to 40 μm long. The polypeptide composition of these filaments, and their structure, based on electron microscopy and X-ray diffraction, are the same as the in situ keratin filaments. The stoichiometry of the recombination experiments reveals that the polypeptides are present in the filaments in the precise molar ratios of 1:2. The X-ray diffraction and stoichiometric data implies that the filaments contain regions of three-chain coiled-coil α-helix; that is, the filament may consist of a three-chain building block unit. This structural concept has been supported by partial proteolytic digestion of filaments and the subsequent isolation and characterization of α-helix-enriched fragments. The structural unit is about 20A wide by about 500 A long and consists of three subunits aligned side-by-side with two coiled-coil α-helical segments each about 180 A long, interspersed by regions of non α-helix.

cDNA cloned probes that encode human and mouse epidermal keratins have been isolated and are being used to determine the amino acid sequences of the proteins, and to study the structure and expression of keratin genes. In collaboration with Dr. D.R. Roop the cDNA species complementary to mouse and human epidermal keratin subunits have been produced and cloned in the E coli plasmid victor pBr322. Cloned probes which respectively encode mouse keratins of 67, 60, 59, 55 and 50 kdaltons and a human keratin of 67 kdaltons in size have been identified. These cDNA species are being subjected to DNA sequence analyses by use of the Maxam-Gilbert procedures. Assignment of amino acid sequences to the DNA sequences is then possible by application of the genetic code. Preliminary results suggest that the keratin subunits do indeed contain two long stretches of α-helical regions that are separated by a region of non- α-helical sequences, and contain regions that are rich in glycine and serine residues at the amino and carboxyl-terminal ends of the proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB03657-09 D

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

S.I. Katz, Branch Chief, Dermatology Branch, NCI

COOPERATING UNITS *(if any)*

Dermatology Department, USUHS, Bethesda

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.0	5	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.)*

My clinical and laboratory endeavors involve three major areas of immunodermatology. The first deals with studies of patients with various forms of vesiculobullous diseases. We have not only provided detailed clinicoimmunopathological correlations of several heretofore poorly defined diseases, i.e. dermatitis herpetiformis, acquired epidermolysis bullosa and herpes gestationis but we have characterized the antigens to which the antibodies in some of these diseases bind, i.e., pemphigus and pemphigoid antigens. These studies are closely linked to my second major area of interest which is to provide an understanding of and to chemically characterize ultrastructurally-defined components of the epidermal basement membrane and to determine the function of each of these. We have demonstrated that epidermal cells synthesize both the skin specific pemphigoid antigen and the ubiquitous laminin, both of which are localized to the lamina lucida of the basement membrane zone. We have also recently described, by using monoclonal antibodies, another stratified squamous epithelial specific basement membrane protein which is defined by the KF-1 antibody. This basement membrane zone antigen is a noncollagenous component of the lamina densa which was previously thought to contain only type IV procollagen. In recent studies we have found that the antigen defined by KF-1 is specifically absent or markedly diminished in the dystrophic forms of epidermolysis bullosa which is a severely mutilating disease characterized by marked skin fragility and blisters. My third and major area of interest is the role of the epidermis as an immunological tissue. We have, using mouse, human and guinea pig skin, demonstrated that within normal epidermis Langerhans cells are the only cells which 1) synthesize and express Ia antigens, 2) can present both soluble antigens and haptens to sensitized T cells, 3) are capable of allogeneic T cell stimulation in a mixed epidermal-lymphocyte proliferation system, 4) can induce hapten and allogeneic cytotoxic T lymphocytes in vitro, and 5) are of a mesenchymal origin. We have also demonstrated that keratinocytes produce an Interleukin 1-like cytokine which may serve as a second signal in generating T cell responses.

Other Professional Personnel:

M. Iijima	Visiting Fellow	Derm NCI
S. Breathnach	Visiting Scientist	Derm NCI
J.D. Fine	Medical Staff Fellow	Derm NCI
K.D. Cooper	Senior Staff Fellow	Derm NCI

Project Description

Objectives:

1) To investigate the mechanisms involved in the expression of certain immunologic skin diseases, namely pemphigus, bullous pemphigoid, dermatitis herpetiformis and herpes gestationis.

2) To determine the cell surface characteristics of the lymphocytes and epidermal cells involved in these diseases.

3) To determine the ultrastructure and ultrastructural localization of antibodies in blistering skin diseases.

4. To better understand the distribution of collagen types in human skin and to characterize the chemical constituents of the basement membrane zone.

5) To determine the functional capabilities of Ia-bearing epidermal cells (Langerhans cells) in mice, guinea pigs, and humans as these cells play an integral role in antigen presentation and in allogeneic T cell activation.

6) To identify substances produced by epidermal cells which modulate immunological responses.

7) To identify and characterize normal cell surface, basement membrane and cytoplasmic structures in human skin and to determine their possible role in skin cancer, wound healing, and in blistering diseases.

Material:

Skin biopsies, either punch or Castroviejo keratome slices are used. Also the small intestine of patients with dermatitis herpetiformis are studied in order to determine their antigen binding characteristics. Blister fluid studies for inflammatory mediators are also under investigation. The roofs of suction blisters are also assessed for the distribution of Langerhans cells and production of immunomodulating factors. Guinea pigs, mice and rabbits are used for the identification of lymphocyte and epidermal cell surface antigens as well as for the production of antibodies. Mouse skin is used to prepare epidermal cell suspensions which are used for sensitization.

Methods Employed:

Direct and Indirect Immunofluorescence, Cell Mediated Cytotoxicity, Immunochemical methods for identifying immunoglobulins and immune complexes. Radioimmunoassays. Mixed leukocyte cultures and in vitro antigen priming studies. Delayed type hypersensitivity reactions including contact hypersensitivity are generated by skin painting and by the injection of haptenated cells into syngeneic mice. Radioimmunoprecipitation techniques are also employed as are standard techniques for the production of monoclonal antibodies.

Major Findings:

1) By cell culture and immunoprecipitation techniques we have demonstrated that the bullous pemphigoid antigen, a unique glycoprotein of stratified squamous epithelia, is produced by epidermal cells and has a molecular weight of 220Kd. Most recently we have demonstrated that this pemphigoid antigen can be directly extracted from skin and can be identified by PAGE and transfer to nitrocellulase paper.

2) In vivo studies indicate that Langerhans cells play an integral role in the induction of contact hypersensitivity. Epidermal cells devoid of Langerhans cells cannot perform this function. In vitro Langerhans cells have been shown to have strong stimulatory activity in TNP specific and allostimulatory proliferation and cytotoxic T lymphocyte systems. So far we have been uanble to demonstrate their ability to perform accessory cell functions i.e., in the restoration of a CTL response in an Ia^+ adherent cell depleted culture system.

3) Ultraviolet light irradiation modulates the antigen presenting function of Langerhans cells in such a way so that UV irradiated epidermal cells stimulate allogeneic T cells poorly if used immediately after irradiation and stimulate vigorously if taken 3 days after irradiation.

4) Keratinocytes devoid of Langerhans cells produce a factor which has Interleukin (IL) I activity; that is , it enhances PHA induced thymocyte proliferation. It has many of the same physicochemical characteristics of IL 1. We have termed this factor ETAF. ETAF serves to reconstitute UV induced abrogation of allogeneic T cell stimulation. It also induces fever (like endogenous pyrogen) and is chemotoctic for neutrophils.

5) We have produced monoclonal antibodies against normal epidermal cell constituents and are currently characterizing the antigens and studying their role in various pathologic states, such as wound healing and skin tumor formation. One such antibody KF-1 identifies a structure in the basement membrane zone which is defective in patients with epidermolysis bullosa.

6) We have demonstrated that in certain types of immunologic reactions in the skin, namely in graft versus host disease (in mice) keratinocytes can be induced to synthesize Ia antigens. The Ia antigens on keratinocytes do not induce allogeneic T cell proliferation. The function(s) of these Ia positive keratinocytes is currently under study.

Significance to Cancer Research:

A basic understanding of immunologic injury in various autoimmune disease states is important in interpreting and furthering current concepts in self-recognition. Pemphigus is associated with thymoma and myasthenia gravis and its study may provide a clue as to the association between pemphigus and other malignancies. Our in depth studies of herpes gestationis, an immunologic blistering disease of pregnancy, may provide important clues to maternal-fetal interactions. The role of Langerhans cells in immune reactions in in the skin and their possible function or dysfunction after ultraviolet light exposure should provide some insight into their role in skin tumor formation. The study of the basement membrane and its disruption by cancer cells is essential to the study of tumor invasion.

Proposed Course of Project:

Outlined above.

Publications:

Pehamberger, H., Levy, R.B., Heukart, P., Katz, S.I.: Induction of cell-mediated cytotoxic self responses by epidermal cells modified with a haptenic sulfhydryl reagent. Scandinavian J. of Immunology. 16: 323-341, 1982.

Sauder, D.N., Carter, C.S., Katz, S.I., Oppenheim. J.J.: Epidermal cell derived thymocyte activating factor (ETAF). J. Invest. Derm. 79: 34-39, 1982.

Stanley, J.R., Hawley-Nelson, P., Yaar, M., Martin, G.R., Katz, S.I.: Laminin and bullous pemphigoid antigen are distinct basement membrane proteins synthesized by epidermal cells. J. Invest. Derm. 78: 456-459, 1982.

Stanley, J.R., Beckwith, J.B., Fuller, R.P., Katz, S.I.: A specific antigenic defect of the basement membrane is found in basal cell carcinoma but not in other epidermal tumors. Cancer. 50: 1486-1490, 1982.

Hall, R.P., Lawley, T.J., Smith, H.R., Katz, S.I.: Bullous Eruption of Systemic Lupus Erythematosus: Dramatic response to Dapsone therapy. Ann. Int. Med. 97: 165-170, 1982.

Katz, S.I.: Commentary on Sulfoxone Treatment in dermatitis Herpetiformis. Arch Derm. 118: 809-912, 1982.

Katz, S.I.: Erythema elevatum diutinum in Dermatology in General Medicine. Fitzpatrick TB et al (Eds.) McGraw Hill Co. New York, 1983, pp. 20-27.

Katz, S.I: Immunogenetics of Dermatitis Herpetiformis in Proceedings of the XVI International Congress of Dermatology, University of Tokyo Press, Japan (in press).

Katz, S.I. and Lawley, T.J.: Advances in Dermatology: Dermatitis Herpetiformis in Proceedings of the XVI International Congress of Dermatology, University of Tokyo Press, Tokyo, Japan (in press).

Stanley, J.R., Yaar, M., Hawley-Nelson, P., Katz, S.I.: Pemphigus Antibodies Identify a Cell Surface Glycoprotein Synthesized by Human and Mouse Keratinocytes. J. Clin. Invest. 70: 281-288, 1982.

Pehamberger, H., Tamaki, K., Henkart, P., Katz, S.I.: Langerhans Cell Containing Epidermal Cells are not Cytotoxic in Antibody Dependent - and Mitogen Induced - Cellular Cytotoxicity. Immunobiology. 162: 229-236, 1982.

Sauder, D.N. and Katz, S.I.: Immune Modulation by Epidermal Cell Products: Possible Role of ETAF in Inflammatory and Neoplastic Skin Diseases. J. Amer. Acad. Derm. 7: 6b51-654, 1982.

Sauder, D.N. and Katz, S.I.: Strain Variation in the Induction of Tolerance by Epicutaneous Application of Trinitrochlorobenzene. J. Invest. Derm. 80: 383-386, 1983.

Hall, R., Lawley, T.J., and Katz, S.I.: Editorial: Bullous eruption of Systemic Lupus Erythematosus. J. of Amer. Acad. Derm. 7: 797-799, 1982.

Sauder, D.N., Noonan, F., DeFabo, E. and Katz, S.I.: Ultraviolet radiation inhibits alloantigen presentation by epidermal cells: partial reversal by the soluble epidermal cell product, epidermal cell derived thymocyte activating factor (ETAF). J. Invest. Dermatol. (in press).

Breachnach, S.M., Fox, P.A., Neises, G.R., Stanley, J.R. and Katz, S.I.: A unique epithelial basement membrane antigen defined by a monoclonal antibody (KF-1). J. Invest. Derm. 80: 392-395, 1983.

Iijima, M. and Katz, S.I.: Specific immunological tolerance to dinitrothiocyanobenzene: Modulation by suppressor T cells. J. Invest. Derm. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB03666-05 D

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Chemical Mediators of Inflammation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Thomas J. Lawley, M.D., Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(if any)*

LCI, NIAID, Metabolism Branch, NCI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.2	.8

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

B

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

Antigen-antibody complexes play important roles in a variety of human systemic and cutaneous diseases. This laboratory studies how immune complexes are formed, how they cause tissue damage, and how they are cleared from the circulation. We have identified and partially characterized the immune complexes which exist in several diseases i.e. Sjogren's syndrome, mixed cryoglobulinemia, mixed connective tissue disease, acute and chronic schistosomiasis, hepatitis and various types of cutaneous and systemic vasculitis, utilizing highly sensitive radioimmunoassays. We have developed a sensitive radioimmunoassay for the detection of IgA containing immune complexes. We have determined the antibody classes present in the immune complexes and examined the physiochemical characteristics of these complexes, as well as the reaction of these complexes with mediators of inflammation such as the complement system. We have examined the correlation between absolute levels of circulating immune complexes, the extent and severity of clinical disease, and reticuloendothelial system function. We have also examined the influence that certain genes of the major histocompatibility complex exert on immune function in vivo and in vitro in humans and demonstrated that individuals who possess the HLA-B8/DRw3 histocompatibility antigens have abnormal immunologic function both in vivo and in vitro. Since immune complexes may activate the complement system and since the complement fragments C5a and C3a are thought to be important in the pathogenesis of the inflammatory response in cutaneous and systemic diseases, we have purified C5a and studied its in vivo and in vitro reactivity. Its in vivo role was assessed by the first in-depth analysis of the cutaneous reactivity of this complement fragment in man. C3a has also been purified and is currently under study.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:

K.B. Yancey	Clinical Associate	Derm NCI
R.P. Hall	Expert	Derm NCI
J. Cason	Technician	Derm NCI

Project Description:

Objectives:

1) To further characterize circulating immune complexes in human diseases with regard to size and nature of the antigen, and subclass of antibody.

2) To develop reliable methods for specifically purifying immune complexes.

3) To continue clinical studies of immune complex diseases and reticuloendothelial system clearance function.

4) To examine the relationships between immunologic function and HLA antigen expression in patients and normal volunteers.

5) To continue to assess the relation of specific genetic phenotypes with the presence and function of immunologically relevant cell surface receptors in humans.

6) To characterize the role of intermediate filaments as antigens in the immune response.

7) To evaluate in vivo the role of human C5a in the inflammatory response of normals and individuals with immunologically mediated diseases, and conduct in vitro studies of C5a and C3a as modulators of the immune response.

Material:

Serum, red blood cells, white blood cells and skin biopsies from patients and controls will be used. Purified human Clq, Raji cells, and ^{125}I (Bolton-Hunter reagent) and immunospecific antisera are used in the immune complex assays. Purified IgG fraction of anti Rh(D) human antiserum and ^{51}Cr. Purified human C5a.

Methods Employed:

^{125}I-C1q binding assay, Raji cell IgG radioassay, Raji cell IgA radioassay, direct and indirect immunofluorescence, Fc specific reticuloendothelial system clearance assay, column chromatography, sucrose density gradient ultracentrifugation, monoclonal antibodies, fluorescein activated cell sorter, polyacrylamide gel electrophoresis, western blotting.

Major Findings:

1) We have developed monoclonal antibodies which react with a hitherto poorly defined structural protein of epidermal cells-profilaggrin.

2) The antigens which epidermal upper cytoplasmic antibodies are directed against have been identified and isolated. They are keratin intermediate filaments.

3) We have determined that all individuals studied, normal individuals as well as patients, have antibodies against keratin intermediate filaments.

4) Patients with the blistering skin disease, dermatitis herpetiformis have IgA deposits in their skin which are exclusively IgA_1.

5) Dermatitis herpetiformis patients have IgA circulating immune complexes. These soluble immune complexes contain both IgA_1 and IgA_2.

6) Human C5a is a potent mediator of cutaneous inflammation. It causes the rapid onset of a wheal and flare reaction when injected into normal skin.

7) Injections of human C5a into normal skin cause the accumulation of polymorphonuclear neutrophils around the blood vessels in the superficial and mid dermis.

8) The wheal and flare reaction associated with C5a skin tests can be decreased by pretreatment of the individual with antihistamines but not by pretreatment with systemic corticosteroids.

9) Normal individuals who possess the HLA-B8/DRw3 antigens have increased T-helper cell/T-suppressor cell ratios. This is due to a statistically significant decrease in the number of T-suppressor cells.

10) Patients with onchocerciasis have a high incidence of circulating immune complexes.

11) These levels are essentially unchanged by treatment with diethylcarbamazine over a two month treatment course.

12) Individuals with onchocerciasis who initially have high levels of immune complexes are at much higher risk to develop occular and systemic complica-

tions of diethylcarbamazine therapy.

Bibliography:

Hamburger, M.I., Lawley, T.J., Kimberly, R.P., Plotz, P.H., and Frank, M.M.: A serial study of reticuloendothelial system Fc receptor function in patients with systemic lupus erythematosus. Arth. Rheum. 25: 48-54, 1982.

Hall, R.P., Lawley, T.J., Smith, H., Katz, S.I.: Bullous lupus erythematosus: dramatic response to dapsone. Ann. Int. Med. 97: 165-170, 1982.

Lawley, T.J.: (1) Immune complexes-current methods of detection. (2) The relationship between Fc receptor-dependent clearance and HLA type. Ann. Int. Med. 93: 857-874, 1982.

Fries, L.F., Hall, R.P., Lawley, T.J., Crabtree, G.R., Frank, M.M.: Monocyte receptors for the Fc portion of IgG studies with monomeric IgG1: Normal in vitro expression of Fc receptors in HLA-B8/DRw3 subjects with defective Fc mediated in vivo clearance. J. Immunol. 129: 1041-1049, 1982.

Wei, N., Klippel, J.H., Huston, D.P., Hall, R.P., Lawley, T.J., Balow, J.E., Steinberg, A.D., Decker, T.J.: A randomized trial of plasmaphoresis in systemic lupus erythematous. Lancet. 1: 17-22, 1983.

Hall, R.P., Stachura, E., Cason, J., Whiteside, T., Lawley, T.J.: IgA containing immune complexes in patients with IgA nephropathy (Berger's disease). Am. J. Med. 74: 56-63, 1983.

Barral-Netto, M., Cheever, A.W., Lawley, T.J., Ottesen, E.A.: Cell-mediated and humoral immune responses in Capuchin monkeys infected with Schistosoma japonicum or Schistosoma mansoni. Am. J. Trop. Med. Hyg. (in press).

Greene, B.M., Taylor, H.R., Brown, E.J., Humphrey, R.L., Lawley, T.J.: Ocular and systemic complications of diethylcarbamazine therapy of onchocerciasis: Association with circulating immune complexes. J. Inf. Dis. (in press).

Hall, R.P., Peck, G., Lawley, T.J.: IgA immune complexes in psoriasis. J. Invest. Dermatol. (in press).

Yancey, K.B. and Lawley, T.J. Basic elements of immune complex disease and their role in several disorders of dermatologic interest. Am. J. Dermatopath. (in press).

Yancey, K.B., Cason, J.C., Hall, R.P., Lawley, T.J.: Dietary gluten does not influence the levels of circulating immune complexes in patients with dermatitis herpetiformis. J. Invest. Dermatol. (in press).

Lawley, T.J.: Methods of Detection of Circulating Immune Complexes. In Clinics in Immunology and Allergy. Fauci, A., (ed) W.B. Saunders Inc., pp. 383-396, 1982.

Lawley, T.J. and Yancey, K.B.: Dermatitis herpetiformis. In Dermatologic Clinics. Lazarus and Jegasothy (eds), W.B. Saunders Inc. (in press).

Hintner, H., Steinert, P., Lawley T.J.: Human upper epidermal cytoplasmic antibodies are directed again st keratin intermediate filament protein. J. Clin. Invest. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB03659-09 D

PERIOD COVERED

October 1, 1982 to September 30, 1983

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 on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

G.L. Peck, Senior Investigator, Dermatology Branch, NCI

COOPERATING UNITS *(if any)*

1) Clinical Chemistry Service, NIH, Bethesda, Maryland 20205
2) Molecular Disease Branch, NHLBI, NIH, Bethesda, Maryland 20205

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	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.)*

Oral 13-cis-retinoic acid was effective in the treatment of skin cancer, and a variety of disorders of keratinization (lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris), and cystic acne. An oral synthetic aromatic derivative of retinoic acid (RO-10-9359) was more effective and less toxic than 13-cis-retinoic acid in the treatment of the disorders of keratinization. A high initial followed by a low maintenance dosage of 13-cis-retinoic acid was comparably effective but less toxic than previously used continuous high-dosage schedules in the treatment of cystic acne. The high-low dosage schedule was superior to the high initial dose schedule used alone and to a continuous low dose schedule. 13-cis-retinoic acid led to small but significant elevations in plasma lipids and changes in lipoproteins during therapy. RO-10-9359 produced similar changes which were dose dependent and responsive to dietary management. Absorption of RO-10-9359 is greater with milk than with water. Etretinate is bound in plasma to beta-liproproteins. Administration of etretinate with milk vs. water yielded different ratios of drug to metabolite in the serum. One chronic toxicity, "Retinoid Hyperostosis" has been observed with long-term, high-dose isotretinoin: Anterior spinal ligament calcification and osteophyte formation of vertebrae.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:

G.L. Peck	Senior Investigator	Derm NCI
E.G. Gross		Cancer Prevention Branch, DRCCA, NCI
J.J. DiGiovanna	Senior Staff Fellow	Derm NCI
G. Gantt	Registered Nurse	Derm NCI
L. Zech	Senior Investigator	Molecular Dis. Br., NHLBI
D. Scarborough	Medical Staff Fellow	Derm NCI

Project Description:

Objectives:

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.

Agents:

1) 13-cis retinoic acid, 10, 20 and 40 mg capsules
2) An ethyl ester of a trimethylmethoxyphenyl derivative of retinoic acid (RO-10-9359) 10, 25 and 50 mg capsules.
3) An ethylamide of a tremethylmethoxyphenyl derivative of retinoic acid (RO-11-1430) in 0.1% cream and 0.3% gel.

Materials:

1) Keratinizing Dermatoses

   A Ichthyosis
      a) lamellar ichthyosis
      b) X-linked ichthyosis
      c) ichthyosis vulgaris
      d) epidermolytic hyperkeratosis
      e) non-bullous congenital ichthyosiform erythroderma
   B Darier's disease
   C Psoriasis
   D Keratoderma palmaris et plantaris
   E Pityriasis rubra pilaris
   F Nevus comedonicus
   G Erythrokeratodermia variabilis
   H Netherton's syndrome
   I Hailey-Hailey disease
   J Lichen planus
   K Pachyonychia congenita
   L Dyskeratosis congenita

2) Basal cell carcinoma

A Nevoid basal cell carcinoma syndrome
B Sunlight induced basal cell carcinomas
C Arsenical induced basal cell carcinomas
D X-ray induced carcinomas

3) Cystic and Conglobate acne and hidradenitis suppuritiva

Methods Employed:

1) Disorders of Keratinization

a) Oral 13-cis retinoic acid was given for 6 month courses of therapy at 1 mg/kg/day. The dose was gradually increased (up to 8 mg/kg/day) to tolerence.

b) Oral RO-10-9359 was also given for 6 month courses of therapy beginning at 0.5 mg/kg/day. The dose was increased up to 1.5 mg/kg day or to tolerence.

c) Topical RO-11-1430 cream, 0.1%, was given in a double-blind manner versus placebo to 9 patients with disorders of keratinization. Topical RO-11-1430 gel, 0.3% was similarly tested in 3 patients. Therapy was given twice daily for 4 to 8 weeks without occlusion.

2) Basal Cell Carcinoma

Oral 13-cis retinoic acid was given for 6 month courses of therapy at 1 mg/kg/day. The dose was gradually increased (up to 8 mg/kg/day) to tolerance.

3) Cystic acne

a) Patients, 16 years and older, with at least 10 cystic lesions were treated with oral 13-cis-retinoic acid in an initial pilot study (14 patients) or in a double-blind, randomized study against placebo (33 patients) or in a third study testing a high initial and low maintenance dosage schedule (40 patients) or in a fourth study testing the need for the low maintenance dose (72 patients).
b) In the pilot study oral 13-cis retinoic acid was given for 4 month courses of therapy beginning at 1 mg/kg/day. The dose was gradually increased to tolerance.
c) In the double-blind designed protocol, treatment was begun at a dosage of 0.5 mg/kg/day and was only increased if there was a significant worsening of the disease.
d) In the "high-low" study, 20 patients with predominantly facial acne, they were given a high initial dose of 1.0 mg/kg/day for either 2 weeks or 4 weeks and then were given a low maintenance dose of 0.25 mg/kg/day for the remainder of a 16 week treatment period. Similarly,

20 patients with predominantly trunk acne received a high initial dose of 2.0 mg/kg/day for either 2 or 4 weeks followed by a low maintenance dose of 0.5 mg/kg/day for the remainder of the 16 week course of therapy.

e) In the fourth study 3 groups of 24 patients received either a) a high-low dosage schedule consisting of 2.0 mg/kg/day for 2 weeks and 0.5 mg/kg/day for 14 weeks, b) a high-dosage schedule alone in which patients received 2.0 mg/kg/day for 2 weeks followed by placebo for 14 weeks, or c) a continuous low dosage schedule consisting of 0.5 mg/kg/day for 16 weeks.

Major Findings:

1. 14 patients with treatment-resistant cystic and conglobate acne was treated for 4 months with oral 13-cis retinoic acid, a synthetic isomer of naturally occurring all-trans-retinoic acid. The average maximum dose received was 2.0 mg/kg/day. 13 patients experienced complete clearing of their disease; the other had 89% improvement, as determined by the number of acne nodules and cysts present before and after therapy. Prolonged remissions, currently lasting an average of 6 years after discontinuation of therapy, have been observed in all 14 patients. One patient developed 2 lesions 12 months after discontinuation of therapy. Therapy was resumed in his case and he is once again free of disease. No other patient has required or been given additional therapy. The mechanism of action of 13-cis-RA in the therapy of acne probably involves a direct inhibitory effect of the drug on the sebaceous gland. Evidence for this inhibitory effect comes from 3 skin biopsies which revealed a marked decrease in size of the sebaceous gland, and from forehead skin surface lipid film analyses, which indicated sebaceous gland inhibition by significantly lower levels of squalene and wax esters during therapy. Additionally, there was an average 84% decrease in mean forehead sebum production as compared to pretreatment values. There was a complete return to pre-treatment values after discontinuation of therapy in the sebum composition studies but only a partial return to normal of sebum production.

2. A study comparing 13-cis-RA at initial dose of 0.5 mg/kg/day versus placebo in a double-blind format in the treatment of cystic and conglobate acne has been completed. 17 patients who initially received placebo worsened to the point where the double-blind code was broken and treatment with 13-cis-RA was begun. There was an overall 57% increase in the number of cystic lesions in this group. 16 of these 17 patients then received 13-cis-RA with a resultant 97% improvement. An additional 17 patients who had been randomly assigned to receive initial therapy with 13-cis-RA had an overall 96% reduction in number of nodules and cysts. There were an average of 46 nodules and cysts before treatment, and 2 afterwards. The average maximum dose of 13-cis-RA received by all 33 patients was 1.2 mg/kg/day, or 90 mg/day. 22 of the 33 patients have become completely free of lesions and only 3 patients

have more than 3 lesions remaining. Patients who typically responded rapidly to a low dose were female, with facial lesions, and with an average of 27 cysts pretreatment. Slower responders requiring higher doses were typically male, with chest and back lesions, and with an average of 67 cysts pretreatment. 21 patients received one 4-month course of therapy; 12 received 2 courses after a 2-month treatment-free interval. 4 patients who had cleared completely after one course of therapy with 13-cis-RA had mild relapses after 4 to 12 months post-discontinuation. 3 of these 4 patients had received only 0.5 mg/kg/day during their initial course of therapy, and all cleared completely with their second course. All other patients continue to have prolonged remissions ranging from 55 to 62 months, average of 58. These results indicate that the beneficial therapeutic response is not a placebo effect, that 0.5 mg/kg/day is an effective dose particularly for facial lesions and is also productive of the common side effects. Higher doses are frequently required for control of chest and back lesions. Continued healing after discontinuation of therapy indicates that lower doses or alternate dosage schedules may also be effective.

3. At the first post-treatment follow-up visit: 1.0 mg/kg/day for 2 weeks group there was an overall 70% reduction in cysts with an 85% reduction of facial cysts, in particular. In the 1.0 mg/kg/day for 4 weeks group there was an overall 82% improvement with an 89% response on the face. In the 2.0 mg/kg/day for 2 weeks group there was an overall 86% improvement with a 96% improvement on the face, 81% on chest, 83% on the back. In the 2.0 mg/kg/day for 4 weeks group there was an over all 77% improvement with 95% improvement on the face, 77% on the chest, and 63% on the back. Both 1.0 mg/kg/day groups had temporary, slight increases in numbers of observed cysts during the first 2 weeks of therapy, whereas the 2.0 mg/kg/day groups did not. We conclude that for most patients, especially for facial lesions, 2.0 mg/kg/day for 2 weeks is the optimal high dosage. In some patients with back acne, the high initial dose of 2.0 mg/kg/day may have to be prolonged beyond 2 or even 4 weeks. The observed toxicity during the initial high dose periods was similar to our other studies. However, once the low maintenance dose was begun there was a marked reduction initially in severity of toxicity and later in incidence. We conclude that except for a few patients with comparatively resistant back acne, the high-low dosage schedule is comparable in effectiveness to the continuous high dosage schedule of previous studies but is superior in minimizing the incidence and severity of observed toxicities.

4. In the fourth acne study, it was found that the high-low dosage schedule was superior to the initial high dose when used alone in the treatment of cystic acne. The high-low dosage schedule was also superior to the constant low dosage schedule in the treatment of cystic acne of the trunk. Cystic acne of the face responded well to both the high-low and the constant low dosage schedules. It was found that 12 of 21 patients receiving the high-low schedule, and 7 of 22 patients receiving only the high dose schedule, and 6 of 21 patients receiving the constant low dosage schedule had a 75% or greater reduction in total number of acne cysts at the end of the 16

week treatment period.

5. Prolonged partial suppression of quantitative sebum production was observed one year after discontinuation of a high initial, low maintenance dosage schedule for cystic acne. Twelve patients who responded partially to one 4 month course of isotretinoin were given a second course of therapy consisting of 2 mg/kg/day for 6 months. This group had a persistent 60% decrease in quantitative sebum production when measured one year or more after discontinuation of therapy. Patients who received and responded well to only one course of therapy had a persistent 39% decrease from their original values when measured one year later. Dose-dependent prolonged partial suppression of quantitative sebum production may be one mechanism by which prolonged remissions of cystic acne are induced by isotretinoin.

6. 12 patients with multiple basal cell carcinomal induced by sunlight, X-ray, arsenic, or the nevoid basal cell carcinoma syndrome were treated with oral 13-cis-retinoic acid. Of 270 tumors, 43 (16%) underwent complete clinical regression. Twenty-one of 35 of these tumors when biopsied after treatment were found to be gone microscopically as well. Correlation of therapeutic response with tumor size revealed that 19 of 83 (23%) tumors 3-5 mm in diameter and 18 of 99 (18%) tumors 6 to 10 mm in diameter underwent complete clinicl regression, whereas only 6 of 88 (7%) tumors 11 mm or greater in diameter responded completely. Of the remaining tumors, 173 (64%) decreased in size and 54 (20%) were unchanged. Average maximum dosage in this group of 12 patients was 4.6 mg/kg/day with a range of 1.5 to 8.2 or 370 mg/day with a range of 120 to 660 mg/day. Duration of treatment varied from 16 to 96 weeks with an average of 56. Four patients had been sensitized and treated with DNCB six years previously. Three of these 4 patients developed a marked inflammatory response in most tumors during therapy with oral 13-cis-retinoic acid. Two of the other 8 non-DNCB exposed patients developed inflammation in a few of their tumors. However, there was no difference in the final therapeutic response between these two subgroups with 21/109 (19%) tumors completely regressing in the DNCB treated group and 22/161 (14%) in the DNCB unexposed group. Most importantly, three patients in this group have received continuous courses of therapy for 4 1/2 to 51/2 years and have had no new tumors develop. The other patients who have either dropped out of the program or have had long intervals (greater than 8 months) between courses of therapy have had new lesions develop. We have concluded that isotretinoin may prevent or delay the development of tumors in this group of patients. Histologically, a dense small cell infiltrate was seen in tumors undergoing inflammation, suggesting that 13-cis-RA could either be enhancing a host immunologic response or be producing a direct cytotoxic effect on the tumor.

7. 57 patients with cutaneous disorders of keratinization were treated with oral 13-cis-retinoic acid, an oral synthetic retinoid from Hoffmann-LaRoche, Inc., Nutley, New Jersey. This retinoid is an ethyl ester of a trimethyl-methoxy-phenyl derivative of retinoic acid. Diseases included Darier's disease (DD) (9 patients), lamellar ichthyosis (LI) (10),

psoriasis (PSOR) (9), pityriasis rubra pilaris (PRP) (5), keratoderma palmaris et plantaris (KPP) (4), idermolytic hyperkeratosis (EHK) (4), non-bullous congenital ichthyosiform erythroderma (NBCIE) (3), x-linked ichthyosis (XLI) (3), Hailey-Hailey (HH) 2), and 1 each with a variant form of NBCIE, erythrokeratodermia variabilis (EKV), pachonychia congenita (PC), ichthyosis vulgaris (IV), Netherton's syndrome (NS), and porokeratosis (PORO). The patients ranged in age from 4 to 82 years. The dosage varied from 0.5 to 8.2 mg/kg/day, the duration of treatment varied from one week to over 5 years. The average maximum dosage was 160 mg/ kg/day or 2 mg/kg/day. Treatment was initially given in 16 week courses of therapy with intervening 8 week treatment-free intervals. Now it is given in 6 month courses with 1-4 week intervals. Good or excellent responses were seen in DD (7), LI (8), NBCIE (3), PSOR (4), KPP (2), EKV (1), and IV (1). Partial responses were obsered in EHK (3), PRP (2), LI (1), DD (2), KPP (1), PC (1, variant-NBCIE (1). Patients showing minimal or no response included: PSOR (5), XLI (3), LP (2), HH (2), NS (1), EHK (1), PRP (1), KPP (1). One patient with PORO and one with LI stopped treatment within one week before a therapeutic evaluation could be made. The mechanism by which this synthetic retinoid alters these disease states is not known but may be related to the observed ability of vitamin A to affect epithelial differentiation. The observed variation in therapeutic response could be related to the presence or persist after chronic administration, serum levels were corrected absence of specific retinoid binding proteins. Our results indicate that synthetic retinoids, such as 13-cis-RA may represent a potent new class of drugs in the treatment of cutaneous disorders of keratinization, several of which were previously treatment-resistant.

8. 75 patients with cutaneous disorders or keratinization were treated with RO-10-9359, an oral synthetic aromatic retinoid from Hoffman-La Roche, Inc., Nutley, New Jersey. This retinoid is an ethyl ester of a trimethyl-methoxy-phenyl derivative of retinoid acid. Diseases included DD (18), PSOR (23), PRP (10), LI (6), EHK (4), IV (3), KPP (2), variant-NBCIE, NBCIE (1), Kyrle's dis-(1), PC (1), HH (1), PORO (1), and LP (1). The average maximum dosage was 86 mg/day or 1.2 mg/kg/day. Duration of treatment varied from 1 week to over 3 years. Treatment was initially given in 16 week courses of therapy with 8 week treat-free intervals. Now it is given in 6 month courses with 1-4 week intervals. Good or excellent responses were seen in PSOR (8), DD (7), PRP (4), Kyrle's (1). Minimal responses were noted in one patient with PROP, the lingual lesions of PC lesions of PC (1), and the oral lesions of LP (1). Worsening was observed in HH (1). Response to therapy was initially variable in psoriasis. 12 patients worsened for 8, 12, or even 16 weeks of therapy and then improved; 11 other patients with psoriasis improved immediately. All other patients with responsive diseases improved immediately upon beginning therapy. RO-10-9359 is clearly superior to 13-cis-retinoic acid in the treatment of psoriasis, IV, KPP, and EHK.

9. 9 patients with disorders of keratinization were treated with topical RO-11-1430 cream, 0.1%, in a double-blind manner against placebo. Diseases included PSOR (3), KPP (2), EHK (1), and variant NBCIE (1). 7 patients received retinoid cream treated sites showed improvement indicating either a

beneficial effect of the vehicle or an overall seasonal improvement in the patients disease. No patient exhibited a preferential beneficial effect of the retinoid cream. No systematic or local toxicities were observed. These results indicate that either RO-11-1430 is ineffective or that the 0.1% concentration is inadequate. Similar testing with the 0.3% gel also revealed no therapeutic or toxic effects.

10. Common side effects in most patients treated with these retinoids were limited to the skin and mucous membranes and included cheilitis, facial dermatitis, conjunctivitis, xerosis, dryness of the nasal mucosa with mild nosebleeds, and easy peeling of the stratum corneum upon trauma termed "skin fragility". Aside from skin fragility, the above side effects were either more common or more severe during treatment with 13-cis-RA than with RO-10-9359. However, there were side effects that were present in higher incience in patients treated with RO-10-9359. These include: skin fragility, hair thinning (telogen), itching, palmar peeling, dry mouth with thirst, arthralgias, "stickiness" of the skin, and paronychia. Laboratory abnormalities during therapy are limited to elevations of the erythrocyte sedimentation rate, temporary low-grade elevations of the transaminases in approximately 10% of patients which return to normal values without discontinuation of therapy, and elevations of the alkaline phosphatase, LDH, and serum triglycerides in a few patients.

In the "High-Low" cystic acne study described in Methods employed, section 3d, and in Major Findings, section 3, analyses of serum lipids were performed. In those 20 patients in tially receiving 1.0 mg/kg/day of 13-cisRA, an increase of 20% in total plasma triglycerides and an increase of 6% in total plasma cholesterol was noted during therapy when compared to pretreatment values. In 9 of these 20 patients HDL-cholesterol was measured and found to be decreased by 12% during therapy. In 19 of the 20 patients initially receiving 2.0 mg/kg/day, the observed changes in serum lipids during therapy as compared to pretreatment values, were: 1) an increase of 24 mg/dl in HDL-cholesterol, 2) an increase of 5.4 mg/ dl in VLDL-cholesterol, 3) a decrease of 3.6 mg/dl of HDL-cholesterol, 5) an increase of 25.5 mg/dl of total plasma cholesterol, and 5) an increase of 44.2 mg/dl of total plasma triglycerides. All values were significant at levels of $p < 0.01$.

A third group of 9 patients were retreated with 2.0 mg/kg/day for 6 months. Data from each of these three different treatment schedules indicated no significant change in triglyceride or cholesterol values after the initial rise from base line values noted after the first week of treatment. Therefore 2 mg/kg/day of 13-cis-retinoic acid over 6 months did not lead to significantly higher triglyceride or cholesterol levels than those observed with shorter treatment periods. All values returned to base line within 4 weeks of stopping therapy.

Similar changes in serum lipids and lipoproteins have been observed in 9 patients with psoriasis treated with oral RO-10-9359 at a maximum dosage of 1.0 mg/kg/day. These changes returned towards normal with dietary management and with decreased dosage.

In general, these side effects are dose-dependent in incidence and severity, relieved by adjunctive bland therapies, well-tolerated by the paients, and totally reversible upon discontinuation of therapy.

11. Fifty patients with various skin disorders have been treated with the synthetic retinoids, isotretinoin and etretinate. Back and neck stiffness was a frequent symptom that we investigated with vertebral spine films. Seventy-two sex-matched controls were selected for comparison with all treated, regardless of skin diagnosis. The differences in frequencies between these two groups of the defined abnormality used in this study (anterior spinal ligament calcification and osteophyte at 2 or more vertebral levels without disc space narrowing) were not significant. However, when patients with basal cell nevus symdrome or basal cell carcinoma who had never received retinoid were compared with those who had received isotretinoin at a high dose for a minimum of 2 years the differences were significant ($p < 0.01$). This study suggests a correlation between long-term ingestion of high-dose (minimum = 1.5 mg/kg/day) isotretinoin and development of an ossifying diathesis, termed "retinoid hyperostosis". This is the first chronic toxicity observed with oral retinoid therapy.

12. To examine the association of synthetic retinoids within lipoprotein fractions, sera from 6 patients (psoriasis, Darier's disease, pityriasis rubra pilaris) receiving etretinate and from 6 receiving isotretinoin (basal cell carcinoma, acne) were separated into lipoprotein fractions by either ultracentrifugation or by heparin-manganese precipitation. Retinoid concentrations were measured with high pressure liquid chromatography. Sera were evaluated after a 12 hour fast and four hours after the patients ingested retinoid with whole milk. 80$\pm$ 1.7% of the serum etretinate but only 27 $\pm$ 1.5% of the serum isotretinoin was found with the beta-lipoproteins. The lipoprotein bound etretinate was found in both the very low density lipoprotein and the low density lipoprotein fractions. When the fasting and four hour specimens were compared, there were no differences in distribution of these retinoids within the betalipoprotein fraction or the albumin containing fraction, suggesting that these associations are independent of serum concentrations of the drug. No differences were observed between individuals despite differing ages and diseases. It is not known whether the beta-lipoproteins function to transport the retinoids to cell surface receptor sites or serve only as a circulating reservoir.

13. Since etretinate is lipid soluble and may be poorly absorbed in the absence of a fat load, we sought to determine whether diet affected its absorption. After an overnight fast, 5 Darier's disease and 4 psoriatic patients received a 1 mg/kg AM dose of etretinate with water or 1 pint of whole milk. Light protected serum samples were drawn at various times and analyzed for etretinate and its major metabolite (Ro 10-1670) by high pressure liquid chromatography. Since low levels of etretinate and Ro 10-1670 persist after chronic administration, serum levels were corrected for this baseline (zero time) value in each set of assays. At every time period, the mean corrected serum etretinate concentration after administration with milk was higher than after administration with water. The mean corrected peak serum

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB03630-13 D

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Effects of Vitamin A and Analogs on Chick, Mouse and Human Skin

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

G.L. Peck, Senior Investigator, Dermatology Branch, NCI

COOPERATING UNITS *(if any)*

Dept. Dermatology, UCSF
Lab of Vision Research, NEI

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

B

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

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 all-trans 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 mouse, normal human skin and 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 limbskin.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:

J.J. DiGiovanna	Senior Staff Fellow	Derm, NCI
D. Scarborough	Medical Staff Fellow	Derm, NCI
E.G. Gross	Senior Investigator	Cancer Prevention Branch, DRCCA, NCI
P.M. Elias	Professor, Dept. of Dermatology	Univ. of California Medical Center
G. Chader	Acting Chief, Lab of Vision Research	NEI

Project Description:

Objectives:

1) To investigate the mechanisms governing epidermal cell differentiation.

2) To morphologically investigate the mechanism by which vitamin A and its derivatives alter epidermal cell differentiation in normal adult skin and in benign and malignant lesions.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Material:

1) 0.2-0.4mm thick, Castroviejo keratotome slices of normal and diseased human skin. Skin from patients with Ichthyosis and Darier's Disease (NCI-3643) has been used to date.

2) 3-4 mm punch biopsies of normal and diseased human skin both treated and untreated with synthetic retinoids (NCI-3643).

Major Findings:

1) Freeze-fracture replicas and thin sections of cell membranes of: 1) 11 basal cell cancers (BCC) treated twice daily for two weeks with topical 1.0% all-trans retinoic acid (RA); 2) 21 BCC treated for 2 to 17 weeks with oral 13-cis retinoic acid (CRA) (1.0-8.0 mg/kg/day); and 3) 17 BCC prior to retinoid treatment and/or after applications of vehicle alone. Both thin sections and replicas were examined and photographed in a single-blind fashion, and the density and size distribution of gap junctions and desmosomes were computed planimetrically. Neither RA nor CRA treatment appeared to influence hemidesmosome or microfilament populations. Structural changes in both treatment groups did not correlate with either tumor regression or inflammation. This indicates that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms.

2) Sucrose density centrifugation was used to identify specific cytosol retinol (CRBP) and retinoic acid (CRABP) binding proteins in newborn mouse skin. A similar amount of retinol binding in skin was found after a 15 minute, 37°C incubation compared to the standard 2 hour, 4°C incubation. Stability of binding during frozen storage was demonstrated for periods up to 4 1/2 months. The ability to augment retinol binding by 75°C with the addition of a 24-hour pretreatment of the tissue with lyophilization allowed the detection of binding in smaller qualtities of tissue.

Epidermal-dermal separation of newborn mouse skin with trypsin and the amount of binding with 2 layers was determined. Equivalent amounts of retinoic acid binding and seven times more retinol binding were found in the dermis compared to epidermis.

Specific retinol and retinoic acid binding was also identified in adult human skin. Adult human skin separated with trypsin or EDTA revealed that the epidermis bound significant levels of retinol and retinoic acid while the dermis did not bind detectible levels. Adult human human epidermis bound 10 to 20 times more retinoic acid than retinol. The ratio of Retinol/ Retinoic Acid bound in adult human epidermis was similar to unborn mouse skin.

We now have identified CRBP in 0.3 mm keratomed normal adult human skin and examined the relative contribution of the epidermis and dermis to the total retinoid binding. CRBP and CRABP were measured as the binding of ^{3}H-retinol (^{3}H-ol) and ^{3}H-retinoic acid (^{3}H-RA) to cytosol preparations using sucrose gradient centrifugation. Saturability and specificity were determined by demonstrating the presence or absence of competition with an excess of nonradiolabeled retinoid. The mean specific activity of ^{3}H-ol and ^{3}H-RA binding (0.57±0.07, 3.43±0.57 pmol/mg protein) to to cytosol prepations from different specimens of adult human skin was determined. Skin obtained from one sample was assayed for cytosol ^{3}H-ol and ^{3}H-RA binding as full thickness skin (0.36±0.09, 2.11±0.38 pmol/mg protein), EDTA-separated epidermis (0.36±.03, 3.69±0.13 pmol/ mg protein) and subjacent dermis (neither detectable). The epidermis alone demonstrated binding for each retinoid at least as great as the full thickness skin. Adult human skin was keratomed at 0.1, 0.2 or 0.3 mm, and histology was obtained. Increasing the thickness from 0.1 to 0.2 mm (which added almost all of the lower epidermis) increased the specific activity for both retinoids. As thickness increased to 0.3 mm (adding significant dermal contamination), the specific activities decreased. These results suggest that CRBP and CRABP in adult human skin are predominantly located in the epidermis.

Specific Retinol and Retinoic acid binding was identified by the skin of a patient with Darier's disease. Specific retinol binding was found in basal cell carcinoma and psoriasis.

Significance to Cancer Research:

1) Since carcinogenesis is an instance of altered differentiation, studies of vitamin A effects on differentiation, may serve as an excellent model for investigations of cellular control mechanisms which relate to carcinogenesis. The fact that carcinogenesis is influenced very markedly by vitamin A deficiency directly links research on the epithelial effects of vitamin A to cancer research.

2) Furthermore retinoids are of value in the treatment of malignancy. The mechanisms of action of retinoids in affecting differentiation may be related to its antineoplastic activity.

Proposed Course of Project:

1) Continued ultrastructural examination of normal skin and benign and malignant lesions of skin both treated and untreated with synthetic retinoids.

2) Continued study of the specific mechanism of vitamin A and retinoid binding to normal and diseased skin with emphasis on elucidating the mechanism of action of these drugs on skin. The distribution and density of specific receptors for retinol and retinoic acid in normal and diseased skin will be studied.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB03638-14 D

PERIOD COVERED

October 1, 1982 to September 30, 1983

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 on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J.H. Robbins Senior Investigator Derm NCI

COOPERATING UNITS *(if any)*

Biometry Branch, DCCP, NCI.

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.8	4.0	2.8

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

B

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

Studies in this laboratory are designed to elucidate the role of DNA repair processes in human diseases and in carcinogenesis and in normal and abnormal aging. 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 ataxia telangiectasia and tuberous sclerosis, diseases with abnormal cell growth and differentiation, and from patients with primary neuronal, muscular and retinal degenerations are also being studied. These studies are designed to elucidate the pathogenesis of these disorders. We assess the biological effectiveness of DNA repair primarily by in vitro assays of cell survival after treatment of the cells with the DNA damaging agents.

Other Professional Personnel:

A.N. Moshell	Guest Worker	Derm NCI
R. Otsuka	Visiting Fellow	Derm NCI
L.R. Seguin-Spillman	Staff Fellow	Derm NCI
R.E. Tarone	Mathematical Statistician	B NCI
D.A. Scudiero	DNA Repair Lab	Chem. Carcin. Prog., FCRC
R. Polinsky	Expert	LCS, NIMH, NIH
C.D. Lytle	Research Biochemist. Experimental Studies, Br., Div. Biolg. Effects., FDA.	
L.E. Nee	Clinical Res. Soc. Worker	LCS, NIMH, NIH

Project Description:

Objectives:

To study DNA repair processes in normal cells, cells from patients with each of the nine genetic forms of xeroderma pigmentosum (XP), and cells from patients with other diseases in which defective DNA repair is claimed or suspected, including degenerative diseases involving the somatic and/or autonomic nervous systems, skeletal muscle, and the retina; to determine the relationship between the function of such repair processes and 1) carcinogenesis, mutagenesis, cytotoxicity and 2) the clinical findings of photosensitivity, premature aging, and UV-carcinogenesis.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Materials:

Cells are obtained from established cell repositories, from hospitalized patients and outpatients at the NIH and from the University of Minnesota Hospitals, Minneapolis, Minn. (Dr. Jonathan Wirtschafter). Cells currently under study are dermal fibroblasts and Epstein-Barr virus-transformed lymphocyte cell lines.

Methods Employed:

Fibroblast and lymphocyte lines are cultured in the absence of antibiotics in laminar flow hoods.

The survival of fibroblasts after irradiation with UV or X rays, or after treatment with chemicals, is performed by counting the number of colonies which form. The survival of lymphocytes is determined by their ability to exclude the vital dye trypan blue. Unscheduled DNA synthesis is determined by determining autoradiographically the UV or X-ray-induced incorporation of tritiated thymidine. Host-cell reactivation is determined by the

formation of plaques in fibroblast monolayers by irradiated Herpes simplex virus (Dr. Lytle).

Major Findings:

1. We have studied cells from a patient who has both Cockayne syndrome and XP. This combination of diseases has been seen in only one previous patient (our patient XP11BE in complementation group B). We have conducted UV-induced unscheduled DNA synthesis complementation group studies with the new patient's fibroblasts. His cells complemented those from the previous patient as well as those from XP patients representing each of the known complementation groups.

This new patient represents a new XP complementation group which we designate group H.

2. We have previously shown that hypersensitivity to X rays can be demonstrated in cultured lymphocyte cell lines from patients with ataxia telangiectasia and Huntington disease. The lines are irradiated in vitro with low doses of X rays and the survival of the lines is determined by their ability to exclude the vital dye trypon blue on the third postirradiation day. We have now demonstrated hypersensitivity to X rays in lymphocyte lines from patients with the following degenerative diseases: Duchenne muscular dystrophy, Usher syndrome (recessively inherited retinitis pigmentosa and sensorineural deafness), Alzheimer and Parkinson diseases, and in Down syndrome. It is possible that the degeneration of excitable tissue in these hypersensitive disorders may be caused by the accumulation of unrepaired DNA damage as the result of faulty DNA repair mechanisms.

3. With Dr. Dominic A. Scudiero we have previously detected hypersensitivity to the lethal effects of N-methy-N'-nitro-N-nitrosoguanidine (MNNG), a mutagenic and carcinogenic DNA-damaging chemical, in fibroblasts from patients with Huntington disease, familial dysautonomia, muscular dystrophy and Usher syndrome. We have found that fibroblasts from patients with Alzheimer disease are hypersensitive to MNNG. A group of fibroblasts from patients with spinal muscular atrophy had the same sensitivity to MNNG as normal fibroblasts. These results show that not all diseases with primary neuronal degeneration have a hypersensitivity to MNNG. Our results show also that hypersensitivity to MNNG is present in cells from patients with several types of primary degeneration of post-mitotic excitable tissue (e.g., nerves, photoreceptors, muscle). Elucidation of the molecular basis for the hypersensitivity may shed light on why most of these disorders, while manifesting hypersensivity to the lethal effects of MNNG, are not characterized by an abnormally increased incidence of cancer. Such studies may also determine whether the hypersensitivity reflects inherited defects in DNA repair mechanisms. Experiments are being conducted with DNA-damaging chemicals other than MNNG in an attempt to obtain greater degrees of hypersensitivity.

4. With Dr. David Lytle we have studied the host-cell reactivation capacity of fibroblasts from patients whose cells are hypersensitive to X rays (e.g., ataxia telangiectasia, Huntington and Alzheimer diseases, strain XP3BR of XP complementation group G). Their host-cell reactivation of X-irradiated Herpes simplex virus was the same as that of normal lines. These results indicate that it is not possible to demonstrate defective host cell reactivation of X-irradiated Herpes simplex virus in these radiosensitive cell lines. Similar results have previously been reported by others using adenovirus and SV40 virus.

5. Cytogenetic studies are being conducted to determine if DNA-damaging agents induce abnormal numbers of chromosome abberations in human cells which have a hypersensitivity to such agents. The development of such a cytogenetic test would make it possible to detect hypersensitivity to DNA-damaging agents within a few days, as opposed to the currently employed colony-forming test which requires two to three weeks. Cells from patients with tuberous sclerosis, Alzheimer and other diseases will also be studied.

6. With Dr. Tarone we have developed computer programs for the statistical analysis of fibroblast survival after treatment of the cells with DNA-damaging agents. Statistical analysis methods were developed for fitting a survival curve for a single cell line using data from a single experiment and from replicate experiments, for summarizing the survival curves of a particular group of cell lines, and for comparing the survival capabilities of two groups of cell lines (e.g., a patient group and a normal group). The method was illustrated using the MNNG survival experiments on a group of nine muscular dystrophy fibroblast lines.

7. In collaboration with Dr. Polinsky and Ms. Nee we are obtaining skin biopsies and blood samples from clinically well-characterized patients with premature death of nerve cells. Complete family pedigrees and neurological evaluations of the patients are also being obtained.

8. Under our contract with the Institute for Medical Research, Camden, NJ, lymphocyte and fibroblast lines were established from patients with degenerative diseases. Approximately 380 lines have been established, making it possible for investigators to study these diseases in tissue culture. This contract expired in November, 1982.

Significance to Biomedical Research and the Program of the Institute:

UV-radiation and ionizing radiation are causes of human cancer. Patients with XP are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor development in XP patients would, therefore, elucidate the role of DNA repair

in preventing in normal humans those cancers which may be due to certain chemical and physical carcinogens. Extension of this work to include study of diseases in which DNA repair may be defective, such as those with hypersensitivity to ionizing radiation, will increase our understanding of the relationships between DNA repair processes and carcinogenesis. XP patients' sun-exposed skin ages much more rapidly than normal humans' skin. Some XP patients develop neurological abnormalities that are due to the premature death of neurons. Our studies of the DNA repair defects are providing an understanding of the relationship of DNA repair processes to these aging phenomena in XP. Thus, our study of aging in XP organs is providing important knowledge as to how properly functioning DNA repair processes prevent such aging in normal humans. Our studies of other diseases with premature death of nerve and muscle cells are also providing an understanding of such abnormal aging phenomena and of the relationship between the ionizing-radiation-type of DNA damage and carcinogenesis.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Publications:

Kraemer, K.H. and Robbins, J.H.: Xeroderma pigmentosum. Current Dermatologic Therapy. W.B. Saunders, 1982, pp. 497-500.

Robbins, J.H., Otsuka, F., Tarone, R.E., Polinsky, R.J., Brumback, R.A., Moshell, A.N., Nee, L.E., Ganges, M.B., and Cayeux, S.J.: Radiosensitivity in Alzheimer Disease and Parkinson Disease. Lancet 1: 468-469, 1983, Letter.

Lytle, C.D., Tarone, R.E., Barrett, S.F., Wirtschafter, J.D., Dupuy, J.-M., and Robbins, J.H.: Host cell reactivation by fibroblasts from patients with pigmentary degeneration of the retina. Photochemistry and Photobiology 37, 503-508, 1983.

Moshell, A.N., Ganges, M.B., Lutzner, M.A., Coon, H.G., Barrett, S.F., Dupuy, J.-M., and Robbins, J.H.: A new patient with both xeroderma pigmentosum and Cockayne syndrome establishes the new xeroderma pigmentosum complementation group H. In Cellular Responses to DNA Damage. UCLA Symposia on Molecular and Cellular Biology, New Series Volume II. (Friedberg, E.C. and Bridges, B.R., Eds.). Alan R. Liss, Inc., 1983 (In press).

Robbins, J.H.: Hypersensitivity to DNA-damaging agents in primary degeneration of excitable tissue. In Cellular Responses to DNA Damage. UCLA Symposia on Molecular and Cellular Biology, New Series, Volume II. (Friedberg, E.C. and Bridges, B.R., eds.). Alan R. Liss, Inc., 1983 (In press).

Robbins, J.H., Polinsky, R.J., and Moshell, A.N. Evidence that lack of deoxyribonucleic acid repair causes death of neurons in xeroderma pigmentosum. Ann. Neurol. 13, 682-684, 1983.

Tarone, R.E., Scudiero, D.A., and Robbins, J.H. Statistical methods for in vitro survival assays. Mutation Res. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB03650-11 D

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Biochemical Characterization of Mammalian Melanosomes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

V.J. Hearing, Ph.D., Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(if any)*

Department of Chemistry, Howard University, Washington, D.C.
Department of Pathology, Georgetown University, Washington, D.C.
Department of Biochemistry, Med. Univ. of S. Carolina, Charleston, S.C.

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

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

The tumor specific proteins produced in malignant melanoma in vivo and in vitro are being studied: These studies are aimed at elucidating the mechanisms of formation of these abnormal proteins, as well as their importance to the immunology of melanoma tumor growth, and their possible applications to the immunoassay and/or immunotherapy of malignant melanoma.

Other Professional Personnel:

J.M. Nicholson	Professor	Howard University
D. Gersten	Assoc. Professor	Georgetown Univ.
J. Marchalonis	Professor	Medical Univ. of S. Carolina
Y. Tomita	Visiting Scientist	Derm. Br. NCI

Project Description:

Objectives:

To characterize the structural and enzymatic composition of normal and tumor specific membrane proteins of melanocytes throughout their development in vivo, both in normal and in abnormal pigmentary systems.

The objective of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Our research has been aimed primarily at characterizing membrane proteins in murine and human pigment systems, both in normal as well as in melanoma tissues. Our work has used both cells in vivo and in vitro. Tissues are excised and homogenized and subcellular fractions are then isolated and purified by means of an extensive series of differential centrifugations. Gel filtration and preparative polyacrylamide gel electrophoresis have enabled mg quantities of the proteins under study to be isolated in a pure state. This has allowed the characterization of these proteins with regard to their amino acid content, isoelectric point, molecular weight, prosthetic group content, end terminal sequences, and cyanogen bromide maps. In addition, antibodies to these purified proteins have been produced in both rabbits and goats, and immunochemical characterization of these proteins is underway.

Major Findings:

The melanosome in pigment cells in composed of multiple species of proteins, many of which are loosely bound and easily extracted; these constitute the proteins of the limiting membrane of the organelle. Other proteins are of lower molecular weight and are tightly bound to the granule, and probably constitute the structural, fibrillar proteins; they appear to be the proteins which complex with the melanin polymer. Melanosomal membrane proteins from melanoma tissues vary in structure from those of normal tissue. Several of the proteins seem to be missing completely from these granules, which are also structurally distinguishable from normal granules by electron microscopy. Perhaps more importantly, many of the proteins in melanoma melanosomal

membranes are unique and are not found in membranes of normal melanin granules. A comparison of analogous proteins from normal and melanoma melanin granules resolved a slight, but significant, difference of isoelectric points and a difference of 10,000 MW between the two proteins; both have amino acid contents which are identical with respect to 13 amino acids, but differ significantly with regard to four amino acids. The carboxy and amino terminals of these proteins are identical, while peptide mapping has revealed that amino acid sequences are deleted in 3 or more regions of the tumor specific protein. Other proteins in these tissues seem to differ in a similar manner. It has been found that tumor-specific proteins similar to these can be found in the serologic fluids of melanoma patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro. It has been recently shown that one of these shed proteins has structural homology to the albumins, and may represent a normally occurring gene which is abnormally expressed in neoplastic tissues. There are implications that these tumor-specific proteins may have a critical immunologic importance to the survival of the tumor in the host.

Significance to Cancer Research:

These observations concerning the aberrant biochemical characteristics of melanoma membrane proteins indicate that basic metabolic processes are in some manner affected by carcinogenesis. Melanomas are unusual among cancers since they are not completely dedifferentiated; the melanocyte's normal synthesis of pigment continues. However, our results indicate that although melanogenesis takes place, its metabolic patterns are grossly abnormal.

There are a wide range of implications of this research for possible immunotherapy and/or immunoassay of human malignant melanoma. An analogous situation of altered proteins has now been described in many other types of malignancies; thus this process of atypical formation of proteins may be common to neoplastic transformation. Our studies indicate that the unique proteins present in the malignant melanoma are an aberrant form of normally synthesized protein, although the mechanism effecting this has not yet been elucidated.

Proposed Course of Project:

Since there are indications that the metabolism of melanogenesis in melanoma is aberrant, our research is ultimately intended to more fully characterize the production of these tumor specific antigens in melanomas, and to attempt to determine the level on which these disorienting control mechanisms operate. To this end, we are producing monoclonal antibodies to assist in their biochemical analysis. It is hoped that further insight into such controls, be they at the level of replication, transcription, translation, or posttranslation, will provide clues as to the level at which carcinogenic information is expressed.

Publications:

Gersten, D.M., Hearing, V.J., and Marchalonis, J.J.: Surface antigens of murine melanoma cells. Comparative Pathology, Proc. Symp. Membranes and Recognition. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB03649-11 D

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Enzymatic Control Mechanisms in Melanogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Vincent J. Hearing, Ph.D., Dermatology Branch, DCBD, NCI

COOPERATING UNITS *(If any)*

Dermatology Branch, Yale Univ. School of Medicine, New Haven, Conn.

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

A

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)* This project has partially characterized the nature of tyrosinase, and determined the mechanism of normal latency of the enzyme, and its subsequent activation in vivo. The role of this enzyme in melanogenesis in normal tissue and the importance of the altered enzyme found in melanoma tissues is being studied. Further, control mechanisms involved in the regulation of melanin formation in the melanocytes of normal tissues and of pigment mutations are being investigated.

PHS 6040 (Rev. 1/83) GPO 895-100

Other Professional Personnel:

Yasushi Tomita	Visiting Scientist	Tokoyo Univ. Sch. of Med.
John Pawelek	Asst. Professor	Yale University

Project Description:

Objectives:

To investigate the control mechanisms involved in regulating melanogenesis in a variety of normal and malignant tissues.

The objective of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Tyrosinase and other enzymatic activities are investigated by histochemical, biochemical, spectrophotometric and radioactive assay methodology. Melanocytes are grown both in vivo and in vitro. Tissues are dissected, homogenized and fractionated into cellular fractions by means of density gradient centrifugation and differential centrifugation. After solubilization with detergents, enzymes are further purified by gel filtration and by preparative polyacrylamide gel electrophoresis. Samples are then incubated with the appropriate substrates and controls; the production of melanin and other reaction products can be followed spectrophotometrically, by liquid scintillation counting of the newly formed radioactive melanin or byproducts, or characterized by amino acid analysis.

Major Findings:

Since only one enzyme (tyrosinase) is essential for melanin biosynthesis, it is a unique system for the study of enzymatic control mechanisms in normal and malignant tissues. It has always been disputed how tyrosinase, which can be easily demonstrated to be present in an active configuration in the endoplasmic reticulum and Golgi apparatus, is inactivated in vivo in mammalian systems and subsequently activated once in situ in the melanosome. We have shown that L-DOPA is the natural activator of the enzyme in vivo; and that tyrosinase is extremely specific in its requirement for L-DOPA as a cofactor. It has been further shown that tyrosinase is under allosteric control and that phospholipids may play a part in the expression of the enzyme's activity. Our laboratory has provided evidence which supports the theory that the enzyme is additionally controlled by enzyme-associated factors which can further modify the production of pigment; these may prove to be critical to the control of pigment formation in mammals.

Significance to Cancer Research:

In view of the differences we have reported between the regulation of tyrosinase in normal and in melanoma tissues, we feel that at least one primary difference between the capabilities of normal and neoplastic melanocytes has been revealed. The fact that these controls are operational at the post-translational level is informative; whether other levels of cellular control mechanisms are affected by carcinogenesis remains to be investigated. It is hoped that further study of the cause of these enzymatic differences in the malignant tissues will provide insights into the nature of neoplastic transformation.

Proposed Course of Project:

Differences in the control mechanisms over melanogenesis in normal and in malignant melanoma tissues will continue to be investigated. The characteristics of enzymatic activity and control intracellularly are being studied. Monoclonal antibodies to tyrosinase isozymes are being produced to enable further analyses of intracellular processing pathways of these isozymes, and to allow for specific identification of melanocytes in tissues.

Publications:

Hearing, V.J., Korner, A.M., and Pawelek, J.: New regulators of melanogenesis are associated with purified tyrosinase isozymes. J. Invest. Derm. 79: 16-18, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB03663-07 D

PERIOD COVERED

October 1, 1982 to September 30, 1983

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

Tumor virus expression in vitro and in vivo

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Douglas R. Lowy, Deputy Chief, Dermatology Branch, NCI

COOPERATING UNITS *(if any)*

Laboratory of Tumor Virus Genetics, NCI; Laboratory of Molecular Virology, NCI; Laboratory of Viral Carcinogenesis, NCI; Center for Cancer Research, MIT (Drs. R. Weinberg and N. Hopkins).

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [ ] (b) Human tissues [ ] (c) Neither

[XX] (a1) Minors B

[ ] (a2) Interviews

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

We have cloned four different human p21 ras genes which are homologous to the p21 transforming genes of Harvey and Kirsten murine sarcoma viruses. Chromosomal mapping of these genes has demonstrated that they are dispersed to four different chromosomes. An activated form of one of these genes has been found to be a tumor oncogene. The highly oncogenic property of this activated gene has been localized by genetic studies and by DNA sequence analysis to a point mutation in the p21 coding region of the gene. Since elevated levels of the normal p21 protein encoded by this gene can also be oncogenic, these results indicate that p21 ras genes can be oncogenic by either of two mechanisms: increased levels of the normal gene product or normal levels of a structurally altered gene product.

Papillomavirus research has been both basic and applied. The capacity of Bovine papillomavirus (BPV) DNA to transform mouse cells has been studied. The sequences responsible for transformation by BPV are not identical to those required for its maintenance as a multiple copy extra-chromosomal element. A 2.3 kb segment of the viral DNA which cannot by itself induce transformation can be activated by a retroviral LTR to be transforming. Cells transformed by such a construction contain integrated copies of the viral DNA. The demonstration that cells transformed by Bovine papillomavirus can be cured of their viral DNA by long term treatment with interferon has been applied clinically to a trial of human leukocyte interferon in patients with epidermodysplasia verruciformis, a disease of chronic widespread wart virus infection. In short term studies, intralesional or systemic treatment with human leukocyte interferon resulted in a marked diminution in the size of warts and a decrease in the number of virus-positive cells in lesional skin.

Other Professional Personnel:

Dr. Sisir K. Chattopadhyay	Visiting Scientist
Dr. Elliot J. Androphy	Medical Staff Fellow
Dr. Pierre E. Tambourin	Guest Worker
Dr. Tim F. Kelly	Medical Staff Fellow
Dr. John Schuller	Guest Worker

Project Description:

Objectives:

1. To improve the biologic assay of DNA-mediated gene transfer (transfection).

2. To determine the effect of a variety of physical and chemical treatments on the biological activity of specific DNAs (viral and cellular).

3. To gain insight into how the state of differentiation of a cell regulates virus and gene expression.

4. To define the portions of sarcoma virus genomes which induce cellular transformation.

5. To study the structure, oncogenic potential and control of normal gene sequences which are homologous to the oncogenes of transforming retroviruses.

6. To analyze the normal function of genes homologous to the oncogenes of transforming retroviruses.

7. To study spontaneous human tumors for their expression of oncogenes and for the ability of tumor DNA to induce cell transformation.

8. To study the cellular origin of retrovirus components.

9. To study the evolution of retroviruses.

10. To study viral recombination, especially as it relates to the development of sarcoma viruses, leukemia viruses, and papillomaviruses.

11. To evaluate the effects of hormones and other chemicals on endogenous and exogenous virus expression.

12. To propagate and study papilloma viruses in tissue culture.

13. To define the functional organization of papillomavirus genomes especially with regard to their transforming and oncogenic activity.

14. To develop assays for wart viral proteins.

15. To adapt nucleic acid hybridization procedures for papillomavirus DNA.

16. To measure the relatedness of viruses in warts from different patients and species, including those from patients with epidermodysplasia verruciformis (EV), laryngeal papillomas, condyloma acuminata, flat warts, common warts, plantar warts, and warts associated with immunodeficiency.

17. To screen skin cancers of EV, kerato-acanthomas, and other tumors for papillomavirus or oncogenes.

18. To test reagents for their anti-tumor or anti-viral activity in vitro and in vivo.

Methods Employed:

1. Treatment of cells or animals with hormones, other chemicals, and tumor viruses.

2. Detection of retrovirus expression biologically by XC plaque test and focus induction.

3. Specific radioimmune, fluorescence, peroxidase techniques are used for antigen detection.

4. For the isolation of genomic DNA, DNA is extracted from tissue culture cells or tumors by the Marmur technique, except that proteinase K is used instead of pronase, since higher molecular weight DNA is obtained by this modification. Unintegrated viral DNAs are enriched by the Hirt procedure.

5. Transfection of DNA utilizes the calcium phosphate technique of Graham and Van der Eb as modified by Stow and Wilkie. Cell or viral DNAs are assayed for biologic activity in the DNA transfection assay. This activity is then correlated with the expression of the transfected genes in the cells.

6. Virus is grown in sensitive tissue culture cells and purified by sucrose density centrifugation in a zonal rotor. Isotopically labeled single stranded viral DNA probes are synthesized in an endogenous reverse transcriptase reaction carried out in the presence of antinomycin D or in exogenous reactions following purification of viral RNA on sucrose density gradients.

7. Nucleic acid hybridization between specific probes and cell or viral nucleic acids is carried out in liquid or by the Southern blotting technique.

8. Cellular and viral genes are molecularly cloned and amplified in prokaryotic systems. The cloned DNAs are then used as probes, for molecular hybridization, and for structure-function studies.

9. Specific deletions, mutations, or recombinations are introduced in the cloned

Major Findings

1. In collaboration with the Laboratory of Pathology, NCI (Dr. Peter Howley), we last year found that when Bovine papillomavirus (BPV) transformed mouse cells are treated long term with interferon, the average number of BPV DNA copies per cell decreases significantly. Following interferon treatment, DNAs to map biological and biochemical functions.

   It is possible to isolate cells which are no longer transformed, no longer form tumors in the mice, and have lost the BPV DNA genome. These results suggested that interferon treatment of papillomavirus induced lesions might represent a rational therapeutic approach. In order to test this hypothesis, we have now used human leukocyte interferon to treat a group of seven patients with Epidermodysplasia Verruciformis (EV), a disease of chronic widespread warts which can undergo malignant changes. After showing that intralesional interferon flattened the warts, six patients were treated systemically at 5×10^6 units per dose three times weekly for four weeks. In each patient there was a significant reduction in the size of their warts. This clinical improvement was correlated with histological improvement and a significant decrease in the number of virus positive cells in their lesions.

   We have also extended our studies of the molecular genetics of BPV induced cellular transformation. We have previously localized the transforming region to a 69% viral DNA fragment. We have provided evidence that a segment near the 5' end of this segment contains a control element while a 2.3 kb segment at the 3' end of this fragment encodes the transforming function. This function can be activated by placing a retroviral long-terminal repeat (LTR) upstream from this transforming region. Sequences located between these two discontinuous segments of the 69% fragment are apparently required for maintenance of the extrachromosomal integrated state of the viral DNA.

2. Significant progress has been made in viral and cellular oncogene studies. Last year we molecularly cloned from normal human DNA four different p21 ras genes. These genes were identified because of their homology to the p21 ras transforming segment of Harvey and Kirsten murine sarcoma viruses. In collaboration with Dr. Steven O'Brien, NCI, we have demonstrated that these four genes are dispersed to different chromosomes in the human genome. We showed last year that at least one of these human genes has the capacity to induce oncogenic transformation of mouse cells when the p21 encoded by this gene is expressed at high levels following the attachment of a retro-viral LTR in vitro. This same human p21 ras gene in a human bladder tumor cell line has been found capable by itself of transforming mouse cells. In collaboration with the laboratory of Robert Weinberg at MIT and that of Ravi Dhar at NCI, the lesion in this tumor oncogene has been localized

to a point mutation in the p21 coding region of the gene. This mutation leads to a structurally altered form of p21 protein which is highly oncogenic in a mouse cell transformation assay. Therefore, this p21 ras gene can transform mouse cells by either of two mechanisms: elevated levels of the normal gene product or structural alterations in the gene product.

3. Our studies of murine leukemia viruses have provided further insights into the recombinational events underlying the generation of leukemogenic MCF viruses. The 3' end of the viral genome is organized as gp70-p15E-LTR from left to right. In collaboration with the laboratory of Dr. Nancy Hopkins at MIT, we showed by DNA sequence analysis that the U3 region the LTR and the sequences encoding carboxy terminus of the p15E protein of the leukemogenic MCF virus 247 differ from this region in ecotropic virus genomes. However, the amino terminus of p15E in MCF 247 is indistinguishable from the same region in ecotropic viruses. Sequences to the left of p15E have also been shown to be different between these two virus types. Since we have previously shown that endogenous non-ecotropic viral genomes do not contain the ecotropic type of amino terminus to p15E, these results indicate that the leukemogenic virus MCF247 arose via at least two different independent recombinational events: one involving the viral gp70 region and the other the carboxy terminus of p15E and the LTR.

Significance to Cancer Research:

Papillomaviruses are a common cause of benign epithelial tumors in humans and other species. Some lesions induced by these viruses undergo malignant conversion. Little has been known about the functional organization of the genomes of these viruses or how lesions progress from a benign to a malignant state. The determination of the transforming sequences of BPV DNA represents a potentially important step towards understanding how these tumors are formed. Further studies will attempt to define the viral gene product(s) associated with transformation, to relate the BPV system directly to human papillomaviruses, and to elucidate the cellular genes with which the papillomavirus gene products interact. The finding that short term interferon treatment of patients with EV improves their lesions merits further study. Since papillomaviruses have recently been associated with a significant number of malignant tumors, this treatment may ultimately affect the development of these malignancies.

The p21 ras genes are the first cellular oncogenes shown to be part of a multigene family. The reasons underlying their multiplicity may be important both for understanding their normal function and their possible role in specific cancers. Activated forms and amplified numbers of these genes have been found in many animal and human tumors. Our data from one of the human p21 ras genes indicate that the product of this gene can induce tumorigenic transformation of cells by two different mechanisms: quantitative and qualitative changes in their protein product. It is likely that other tumor oncogenes will also contribute to the malignant phenotype by similar mechanisms.

The finding that a single amino acid substitution in the protein can profoundly alter the oncogenic activity of the protein should be interpreted broadly; it implies that other structural alterations, such as deletions of amino acids within a protein, may also enhance the transforming activity of an oncogene product.

C-type retroviruses have been implicated in tumors of a wide variety of species, including man. The murine viruses of this group represent the best studied mammalian retroviruses. Because retroviruses in mice are derived from endogenous gene sequences, understanding how their expression leads to oncogenic transformation may also be relevant to non-virus induced tumorigenesis. The finding of specific recombinational events in leukemogenic MCF viruses suggests that recombinational events between specific DNAs (either viral or non-viral) might be important to the pathogenesis of tumors, whether through the formation of a hybrid gene product or by increasing levels of specific genes. Such specific recombinatorial events have recently been described in malignant human and animal tumors.

Proposed Course:

Achieve stated goals.

Publications:

Chang, E.H., Furth, M., Scolnick, E.M., and Lowy, D.R.: Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature 297: 479-483, 1982.

Chang, E.H., Gonda, M.A., Ellis, R.W., Scolnick, E.M., and Lowy, D.R.: The human genome contains four genes homolgous to the transforming genes of Harvey and Kirsten murine sarcoma viruses. Proc. Natl. Acad. Sci. USA 79: 4848-4852 (1982).

Dhar, R., Ellis, R. W., Shih, T. Y., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D., and Scolnick, E. M.: Nucleotide sequence of the p21 transforming protein of Harvey murine sarcoma virus. Science 217: 934-937 (1982).

Ellis, R.W., Lowy, D.R., and Scolnick, E.M.: The viral and cellular p21 ras gene family. In Oncogene Studies, Advances in Viral Oncology (vol. 1), edited by Klein, G., Raven Press, New York, pp. 107-126 (1982).

Papageorge, A., Lowy, D.R., and Scolnick, E.M.: Comparative biochemical properties of p21 ras molecules coded for by viral and cellular ras genes. J. Virol. 44:509-519 (1982).

Tabin, C.J., Bradley, S.M., Bargman, C.I., Weinberg, R.A., Papageorge, A.G., Scolnick, E.M., Dhar, R., Lowy, D.R., and Chang, E.H.: Mechanism of activation of a human oncogene. Nature 300: 143-149 (1982).

Turek, L.P., Byrne, J.C., Lowy, D.R., Dvoretzky, I., Friedman, R.M., and Howley, P.M.: Interferon induces morphologic reversion with elimination of extrachromosomal viral genomes in bovine papillomavirus-transformed mouse cells. Proc. Natl. Acad. Sci. USA 79: 7914-7918 (1982).

Kelly, M., Holland, C. A., Lung, M. L., Chattopadhyay, S. K., Lowy D. R., and Hopkins, N. H.: Nucleotide sequence of the 3' end of MCF 247 murine leukemia virus. J. Virol. 45: 291-298 (1983).

McCoy, M. S., Toole, J. J., Cunningham, J. M., Chang, E. H., Lowy, D. R., and Weinberg, R. A.: Characterization of a human colon/lung carcinoma oncogene. Nature 302: 79-81 (1983).

O'Brien, S. J., Nash, W. G., Goodwin, J. L., Lowy, D. R., and Chang, E. H.: Dispersion of the ras family of transforming genes in man to four different chromosomes. Nature 302: 839-842 (1983).

Even, J., Anderson, S.J., Hampe, A., Galibert, F., Lowy, D.R., Khoury, G., and Sherr, C.J.: Mutant feline sarcoma proviruses containing the viral oncogene (v-fes) and either feline or murine control elements. J. Virol. 45: 1004-1016 (1983).

Lowy, D.R., Gonda M.A., Furth, M.E., Ellis, R.W., Scolnick E.M., and Chang, E.H.: The human genes homologous to p21 ras viral oncogenes. In Tumor Viruses and Differentiation, edited by Levine, A.J. and Scolnick, E.M., Academic Press, New York, in press.

Chang, E.H., Gonda, M.A., Furth, M.E., Goodwin, J.L., Yu, S.S., Ellis, R.W., Scolnick, E.M., and Lowy, D.R.: Characterization of four members of the p21 gene family isolated from normal human genomic DNA and demonstration of their oncogenic potential. In Gene Transfer and Cancer, Raven Press, New York, in press.

Turek, L.P., Byrne, J.C., Lowy, D.R., Dvoretzky, I., Friedman, R.M., and Howley, P.M: Interferon inhibits bovine papillomavirus transformation of mouse cells and induces reversion of established transformants. In Chemistry and Biology of Interferons: Relationship to Therapeutics, edited by Merigan, T.C. and Friedman, R.M., Academic Press, New York, in press.

Nakabayashi, Y., Chattopadhyay, S. K., and Lowy, D. R.: Organization of the transforming function of bovine papillomavirus DNA. Proc. Natl. Acad. Sci. USA, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB03656-10 D

PERIOD COVERED

October 1, 1982 to September 30, 1983

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 on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

P.M. Steinert, Visiting Scientist, Dermatology Branch, NCI

COOPERATING UNITS *(if any)*

Experimental Pathology Branch, DCCP, NCI; Laboratory of Molecular Biology, DCBD, NCI; Laboratory of Physical Biology, NIAMDD

LAB/BRANCH

Dermatology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.75	4.25	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 polypeptide chains which comprise the subunits of the keratin filaments of normal human, murine and bovine epidermis have been isolated and characterized. The subunits polymerize in vitro into native-type filaments. The details of filament ultrastructure are being investigated using image analysis procedures of filaments examined by transmission electron microscopic and scanning transmission electron microscopic techniques. Model structures generated from these methods will be computationally tested for compatibility with other physio-physico chemical data and amino acid sequence studies of individual filament subunits. cDNA cloned probes that encode human and mouse epidermal keratins have been isolated and are being used to determine the amino acid sequences of the proteins, and to study the structure and expression of keratin genes. The 10nm filaments of fibroblasts, muscle cells and neuronal tissues have been shown to be structurally similar to, but immunologically different from keratin filaments. A histidine-rich basic protein isolated from human epidermis and the slightly different protein of mouse epidermis specifically aggregate keratin filaments and other 10nm filaments in a manner suggestive of an interfilamentous matrix component. cDNA cloned probes will be isolated to study their structure, expression and amino acid sequence.

Other Professional Personnel:

L.D. Johnson	Senior Staff Fellow	Derm. NCI
W.W. Idler	Chemist	Derm. NCI
S.H. Yuspa	Branch Chief	Lab. of Chemical Carcinogenesis and Tumor Promotion, DCCP, NCI
D. R. Roop	Expert	Lab. of Chemical Carcinogenesis and Tumor Promotion, DCCP, NCI
A.C. Steven	Visiting Scientist	Lab. of Phys. Biol., NIAMDD
R.D. Goldman	Professor	Dept. Antmy & Cell Biol. Northwestern Univ.
D.A.D. Parry	Professor	Dept. of Biochemistry and Biophysics, Massey Univ., Palmerston North, New Zealand
B.L. Trus	Senior Investigator	Division of Computer Research and Technology

Project Description:

Objectives:

1) To characterize the ultrastucture of keratin filaments polymerized in vitro from the subunits of the filaments isolated form normal human, murine and bovine epidermis.

2) To characterize the polypeptide subunits of keratin filaments isolated from various benign and malignant human and mouse epidermal diseases.

3) To investigate the biosynthesis of the keratin filament polypeptide subunits in cultures of normal mouse epidermal cells and to investigate the effects of various drugs and carcinogens on the cells.

4) To study the structure and expression of human and mouse epidermal keratin genes by use of cloned cDNA probes complementary to keratin mRNAs.

5) To investigate the nature of the highly-specific interaction between epidermal keratin filaments and filoggrin isolated from the epidermis.

6) To study the structure and expression of mouse and human filaggrin and their precursors by use of cloned cDNA probes complementary to filaggrin in RNAs.

7) To determine the amino acid sequences of keratins and filaggrins by DNA sequencing of the respective cDNA clones.

8) To investigate the detailed secondary, tertiary and possible quaternary

structure of keratins and filaggrins by use of computational analyses of the primary amino acid sequence data.

9) To investigate the chemical, immunological and structural similarities between epidermal keratin filaments and 10nm filaments isolated from a variety of cell types, such as BHK-21, CHO, HeLa, and PtK1 cells grown in culture, and of muscle and neuronal tissues.

Major Findings and Methods Employed:

1. The polypeptide chains which comprise the subunits of the keratin filaments of normal bovine and murine epidermis have been isolated and individually characterized by standard protein chemical techniques. The unfractionated mixture of polypeptides or combinations of two of the purified polypeptides spontaneously polymerize in vitro in dilute salt solution into filaments that are uniformally 80 A wide and up to 40 μm long. The polypeptide composition of these filaments, and their structure, based on electron microscopy and X-ray diffraction, are the same as the in situ keratin filaments. The stoichiometry of the recombination experiments reveals that the polypeptides are present in the filaments in the precise molar ratios of 1:2.

2. The X-ray diffraction and stoichiometric data implies that the filaments contain regions of three-chain coiled-coil α-helix; that is, the filament may consist of a three-chain building block unit. This structural concept has been supported by partial proteolytic digestion of filaments and the subsequent isolation and characterization of α-helix-enriched fragments. The structural unit is about 20A wide by about 500 A long and consists of three subunits aligned side-by-side with two coiled-coil α-helical segments each about 180 A long, interspersed by regions of non α-helix.

3. In collaboration with Dr. R.D. Goldman (Dept of Anatomy & Cell Biology, Northwestern University, Chicago, IL) it has been shown that the 10nm filaments of a variety of epithelial and mesenchymal cell-types grown in culture are morphologically very similar to epidermal keratin filaments, and interestingly, may also possess a three-chain unit that is structurally identical to that of keratin filaments. Partial specific cleavage of purified 10 nm filament subunits and bovine epidermal keratin subunits indicate that the subunits are all distinctly different, but they are structurally very similar. Such studies have permitted the construction of subunit domain maps that show two regions of α-helix of M_r 13,000 on each subunit, interspersed by regions of a non α-helix that vary in size between different subunits. Most of these studies have been done with the 10nm filaments of BHK-21 and CHO cells since these are readily available in large quantities, but similar comparative work is also or underway with the filaments of HeLa cells, and the neurofilaments isolated from cattle brain and squid giant axons. The single protein vimentin and desmin, the principal intermediate filament subunits of fibroblasts and muscle cells, respectively,

are capable of filament assembly by themselves in vitro; that is, they form homopolymer filaments. All keratin filaments, in contrast contain at least two demonstrably different subunits; that is they are obligate copolymer filaments. Preliminary studies suggest that certain neurofilament subunits are capable of homopolymer and/or copolymer filament assembly in vitro. Since all of these filaments are basically very similar (although subtlely different), it is perhaps not surprising that combinations from different sources also form native-type filaments in vitro. Hybrid filaments containing subunits from epidermis + fibroblasts, epidermis + smooth muscle, bovine epidermis + mouse epidermis, etc, have been formed. Such filaments are termed heterologous copolymers. A preliminary conclusion from these observations is that perhaps cells can modulate their 10nm filament composition with regard to specific functions. In support of this idea, BHK-21 cells contain two types of filament subunits, that characteristic of fibroblasts and that of muscle cells, which apparently copolymerize in situ to form a filament of properties intermediate between the two. The presence of 10 nm filaments in cells is obviously extremely important and the structural studies described below should provide insights into their function in normal and transformed cells.

4. In collaboration with Drs. D.R. Roop (LCCTP, DCCP, NCI), cDNA species complementary to mouse and human epidermal keratin subunits have been produced and cloned in the E coli plasmid victor pBr322. Cloned probes which respectively encode mouse keratins of 67, 60, 59, 55 and 50 kdaltons and a human keratin of 67 kdaltons in size have been identified. These cDNA species are being subjected to DNA sequence analyses by use of the Maxam-Gilbert procedures. Assignment of amino acid sequences to the DNA sequences is then possible by application of the genetic code. Preliminary results suggest that the keratin subunits do indeed contain two long stretches of α-helical regions that are separated by a region of non- α-helical sequences, and contain regions that are rich in glycine and serine residues at the amino and carboxyl-terminal ends of the proteins.

These amino acid sequences will be analyzed to determine the higher orders of structure that the proteins may assume. This work will be done in collaboration with Dr. B.L. Trus (Computer Systems Lab, DCRT), and Dr. D.A.D. Parry (Dept. of Biochemistry and Biophysics, Massey University, Palmerston North, New Zealand).

Dr. Johnson in this laboratory is using these cDNA probes to isolate from a phage Charon library the genomic versions of these keratin genes. Experience in other enkaryote gene systems indicates the coding information in genomic DNA ('exons') is interspersed by regions of noncoding information ('introns').

Dr. Johnson will characterize the isolated gDNAs with respect to the size, number, location and sequence of splice-points of the expected non-coding regions. This work will include extensive restriction endonuclease mapping and Southern (and Northern) hybridization techniques, as well as D-looping of the electron microscope level and further DNA sequencing.

5. In collaboration with Dr. A.C. Steven, (Laboratory of Physical Biology, NIAMDD) attempts are underway to understand the higher orders of filament structure. Good negative-stained images of filaments can be subjected to optical diffraction analysis to obtain information of prominently repeating structural elements. Many filaments have to be computationally straightened in order to eliminate "noise" introduced by the curvilinear shape of the negatively-stained filaments. Diffraction images are then computationally averaged to identify the prominent repeats, which should then provide clues as to how the proposed three-chain units are assembled into the filament. Basic structural information on filaments is also derived from scanning transmission electron microscopy (STEM) of filaments. This technique will be performed at the N.I.H. STEM facility located in the Department of Biology of the Brookhaven National Laboratory (Dr. J. Wall, Director) in Upton, New York. This technique provides information on the mass of the filaments and clues on the numbers of protofilamentous units in relation to length. Preliminary data suggests that (1) there are 7-11 three-chain units per unit length of about 500 A of filament; and (2) at least filaments assembled in vitro are polymorphic; that is, some filaments differ in the number of units they contain per unit length. The significance of this observation is unclear but may have enormously important implications in terms of the structure and function of the filaments in cells. In addition, STEM technology will provide information on the shape of intact filaments and of the three-chain units. It is expected that the application of this new technology to the study of keratin (and other related intermediate filaments will for the first time enable the construction of working models for the filaments. Optical diffraction and STEM analysis will be performed on (1) intact filaments from a variety of keratin sources as well as on the intermediate filaments of several cell types; (2) protofilamentous forms of these filaments, obtained by dissociation in low salt or in high concentrations of sodium citrate buffer, pH 2.6; (3) particles of intermediate size obtained during various early stages of filament assembly in vitro; and (4) native filaments obtained from various types of cells where possible to provide a direct comparison of in vitro and in vivo filament structural forms.

6. Comparisons of the polyacrylamide-gel electrophoretic profiles of the keratin subunits obtained from abnormal human epidermis such as Darier's disease and lamellar ichthyosis with those of normal epidermis show prominent differences in numbers and mobilities of bands. Also, the abnormal polypeptides show limited facility for polymerization in vitro. Therefore, there may be differences in the chemical structures of the proteins. Attempts are underway to identify such differences by comparisons of two-dimensional gel electrophoretic maps of the CNBr and NBS peptides of the proteins obtained from normal and abnormal epidermis. Filaments assembled in vitro from psoriatic epidermis are also abnormal, and interestingly, form 'paracrystalline' structures consisting of several filaments associated side-by-side in an apparently ordered manner. This may be due to the presence of an additional protein that perhaps functions like the basic protein or may be a feature characteristic of the filament proteins of psoriatic epidermis. Studies are underway to characterize this phenomenon further. Such studies may provide important clues on how the subunits are arranged in normal intact keratin and other types of intermediate filaments.

7. Normal mouse epidermal cells grown in monolayer culture can be made to synthesize the normal complement of keratin polypeptides. These proteins have been isolated and characterized by standard protein chemical techniques as done earlier with the bovine proteins. The reason why the mouse keratins are also being studied is that this cell culture system is currently being used for studies of in vitro carcinogenisis. Since the keratins are the principal synthetic products of the cells, they will be used as specific markers for the studies on carcinogenesis. To this end, a specific radioimmune assay has been developed to follow the changges occurring during carcinogenesis. Preliminary experiments have revealed marked alterations in the synthesis of the keratin proteins during treatment with carcinogens such as TPA and other growth effectors such as vitamin A.

8. We have shown that a histidine-rich protein isolated from rat epidermis specifically aggretates epidermal keratin filaments from several species in vitro to form a highly-ordered fiber. Electron microscopy of such fibers reveals a pattern of filaments 70-80 A in diameter embedded in a darker-staining background, or matrix. This structure is typical of the "keratin pattern" seen in the fully-differentiated stratum corneum of the epidermis. This suggests strongly that the basic protein is the matrix protein of epidermis. Our work constitutes the first real evidence for and demonstration of the role of a matrix protein in the epidermis. The interaction between the basic protein and filaments is highly specific since other fibrous proteins do not form the ordered structures. Therefore, there are structural features unique to keratin filaments which recognize the basic protein. One practical limitation of such studies has been the difficulty in isolation of the basic protein. In an effort to resolve this, we have developed a very simple method for isolating large quantities of the similar protein from mouse epidermal stratum corneum which functions in the same way as the rat protein. Its chemical and functional properties will now be studied in detail. Studies on the interaction between the basic protein and defined fragments of filaments and filament subunits are underway to characterize the nature and specificity of the associations between these two components in the epidermis. Interestingly, in certain diseases of the epidermis involving abnormal keratinization, such as psoriasis, the amount of the basic protein is greatly diminished from normal. There may be a relationship between the absence of the basic protein and presence of abnormal keratin filaments which could provide important information on the disease itself. Since this basic protein also aggregates the intermediate filaments from all sources so far examined we have chosen for it a new functionally-specific name, filaggrin. Presumably the filaggrin recognises structural features common to keratin and intermediate filaments. Studies are underway to determine the physiological significance of this result; for example it is not yet known whether filaggrin-like proteins are present in fibroblasts etc.

Attempts will also be made to study the biosynthesis of the filaggrin in intact epidermis and epidermal cells in culture. Preliminary studies suggest that filaggrin is initially synthesized on a very high molecular weight protein (300,000) which is subsequently processed to give filaggrin (26,000). This suggests that there are multiple copies of 26,000 filaggrin in the large precursor. Further studies to characterize this will be to prepare cDNA cloned probes

from human and mouse epidermal mRNA, followed by DNA sequencing of the clones.

9. Intermediate filament subunits are phosphorylated in vivo by cyclic nucleotide dependent protein kinases. Presumably cells regulate the structure and/or function of the filaments in this way. Attempts to characterize this process will initially involve estimation of the amount of phosphate bound to subunits, its location and possible function in terms of filament assembly in vitro. Filament subunits of mouse and bovine epidermis, CHO and BHK-21 cells, smooth muscle and various neuronal tissues will be examined. The serine-phosphate content will be estimated by reaction of subunits with methylamine.

Significance to Cancer Research:

The epidermis offers a unique opportunity for the study of tumors not only because of the prevelance of tumors in this tissue but also because of its accessibility. One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. The keratin filaments and filaggrin are the most prominent intracellular components of all epidermal cells and therefore a study of their chemistry, structure and biosynthesis in both normal and abnormal epidermis will be of profound importance in studying tumors in this tissue. The production of these protein in a well defined cell culture system will facilitate studies of carcinogenesis in vivo and in vitro.

Proposed Course of Project:

The DNA cloning and related experiments will be done by Dr. Johnson and in collaboration with Dr. Roop. The biochemical, biophysical and electron microscopic studies of the structure of normal epidermal keratin filaments will be done in this laboratory. The computational image analysis studies of filaments will be done in collaboration with Dr. A.C. Steven. The cell culture studies will be done in the Experimental Pathology Branch in collaboration with Dr. Yuspa. Collaborative efforts with Drs. B. Trus, R.D. Goldman and D.A.D. Parry will continue in the areas described above.

Publications:

Steinert, P.M., Rice R.H., Roop D.R., Trus B.L. and Steven A.C.: Complete amino acid sequence of a mouse epidermal keratin subunit: Implications for the structure of intermediate filaments. Nature 302 794-800, 1983.

Steinert, P.M., Steven, A.C. and Roop, D.R.: Structure features of epidermal keratin filaments reassembled in vitro. J. Invest. Dermatol. (in press), 1983.

Steven, A.C., Hainfeld, J.F., Trus, B.L., Wall, J.S. and Steinert, P.M.: The distribution of mass in heteropolymer intermediate filaments assembled in vitro: STEM analysis of vimenti/desmin and bovine epidermal keratin. J. Biol. Chem. (in press), 1983.

Whitman-Anyandi, M., Steinert, P.M. and Goldman, R.D.: Human epithelial (HeLa) intermediate filaments: isolation, purification and characterization. J. Cell Biol. (in press), 1983.

Zackroff, R.V., Goldman, A.E., Jones, J.C.R., Steinert, P.M. and Goldman, R.D.: The isolation and characterization of keratin-like proteins from cultured fibroblasts. J. Cell. Biol. (in press), 1983.

SUMMARY REPORT
LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE
October 1, 1982 to September 30, 1983

The Laboratory of Pathology is responsible for all the diagnostic services in Pathological Anatomy for the Clinical Center of the NIH and has research programs in various areas of experimental pathology. The Laboratory is divided into 9 sections:

A. Surgical Pathology and Postmortem Section (Dr. Ernest E. Lack, Chief)
B. Cytopathology Section (Dr. Elizabeth W. Chu, Chief)
C. Ultrastructural Pathology Section (Dr. Timothy J. Triche, Chief)
D. Biochemical Pathology Section (Dr. David A. Zopf, Chief)
E. Tumor Invasion & Metastases Section (Dr. Lance A. Liotta, Chief)
F. Viral Oncology & Molecular Pathology Section (Dr. Peter M. Howley, Chief)
G. Hematopathology Section (Dr. Elaine S. Jaffe, Chief)
H. Pathological Technology Section (Ms. Barbara Coolidge, Chief)
I. Image Processing Section (Dr. Lewis E. Lipkin, 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.

A. Surgical Pathology and Postmortem Section

5,666 surgical specimens or biopsies 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 tissue procurement nurse works closely with the staff and helps in the distribution of specimens to scientists. Clinicopathological studies in pulmonary vasculitis, breast cancer, pancreatic carcinoma, esophageal carcinoma, recurrent hyperparathyroidism and soft tissue sarcoma, are in progress. Dr. Cheryl Reichert, Chief of the Autopsy Service, has completed a number of clinicopathologic case studies in a variety of subjects ranging from hemorrhage following liver biopsy to pneumocystis pneumonia.

B. Cytopathology Section

This section provides diagnostic services in cytology (both exfoliative and fine needle aspiration) and medical genetics (service and research). The chromosomal analysis includes conventional and special banding techniques (C-, G-, and R-bands) for the examination of individual chromosome pairs. During the year, approximately 3,800 cytology specimens were accessioned.

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. H.-Z. Zhang, a Visiting Fellow from the People's Republic of China, and Dr. Sue Ellen Martin have been studying the feasibility of applying immunoperoxidase techniques to cytological materials. Dr. Chu has been a pioneer in the development of the fine needle aspiration technique. Dr. Chu also serves on the staff of Tutorial on Clinical Cytology, sponsored by IAC, ASC, and AARM. She gives lectures and workshops on Fine Needle Aspiration Cytology.

C. 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 326 cases were accessioned; over 300 were processed and diagnosed. This facility provides diagnostic training and clinical research opportunities for residents and fellows. Dr. William Green, a Visiting Fellow from the University of North Carolina, has been learning diagnostic EM while investigating the production of matrix proteins and tissue-specific proteins such as Factor VIII by Ewing's sarcoma and other childhood tumors. Dr. Andrea Modesti, a Fogarty Fellow from the University of Rome, Italy, has been employing investigative ultrastructure, including EM immunocytochemistry, on several projects, including a study of the basic organization of basement membranes, using the amniotic membrane model employed by Dr. Lance Liotta in conjunction with monospecific antisera for various basement membrane components.

Dr. Triche has continued to collaborate with Drs. Henry Metzger and Chaviva Isersky-Carter of NIAMDD. An extensive study of the disposition of the IgE Fc receptor in the basophil membrane, employing monomeric ferritin-labelled IgE and basophil membrane ghosts, has been completed. Related collaborative work with Dr. Isersky-Carter on the biological fate of cross-linked IgE (as in allergen binding) is complete. These studies have demonstrated that only cross-linked IgE with its associated receptors is internalized; this is followed by rapid lysosomal degradation and exocytosis. Both IgE and its receptor are degraded; no surface re-expression of either was found, unlike most other systems documented in the literature. The biological fate of both IgE and its receptor thus appears unique.

Drs. Modesti and Triche have completed a collaborative study on cystinosis with Dr. Joe Schulman and associates of NICHHD. Correlative morphological and biochemical studies on homo- and heterozygous cystinotics and normal controls have demonstrated that heterozygotes, though clinically normal, have a diminished capacity to lysosomally process cystine; such impaired lysosomes are detectable in peripheral blood neutrophils and the defect is exacerbated by pretreatment with cystine (dimethyl cystine). Normals show no abnormality; homozygotes display an even more conspicuous defect. This may allow detection of suspected or unknown carriers by examination of peripheral blood neutrophils.

Studies of matrix synthesis by Ewing's tumor have been completed by Dr. Dickman. These studies have clearly established that this tumor is a sarcoma, since it synthesizes types I and III (stromal) collagens, but it is also very primitive, since it also synthesizes type IV collagen, on "epithelial" collagen.

Dr. Triche's chapter on Pediatric Pathology in "Cancer in the Young" and review on Round-cell Tumors of Childhood in "Perspectives in Pediatric Pathology" were both published in May, 1982. A review on round-cell tumors by Drs. Triche and Askin has been solicited for a special symposium on pediatric pathology in Human Pathology, to be published in spring, 1983.

☆ U.S. GOVERNMENT PRINTING OFFICE: 1983-381-132:3172