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National Cancer Institute

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October 1, 1982 through September 30, 1983

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DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE

SUMMARY REPORT OF THE DIRECTOR

October 1, 1982 through September 30, 1983

INTRODUCTION

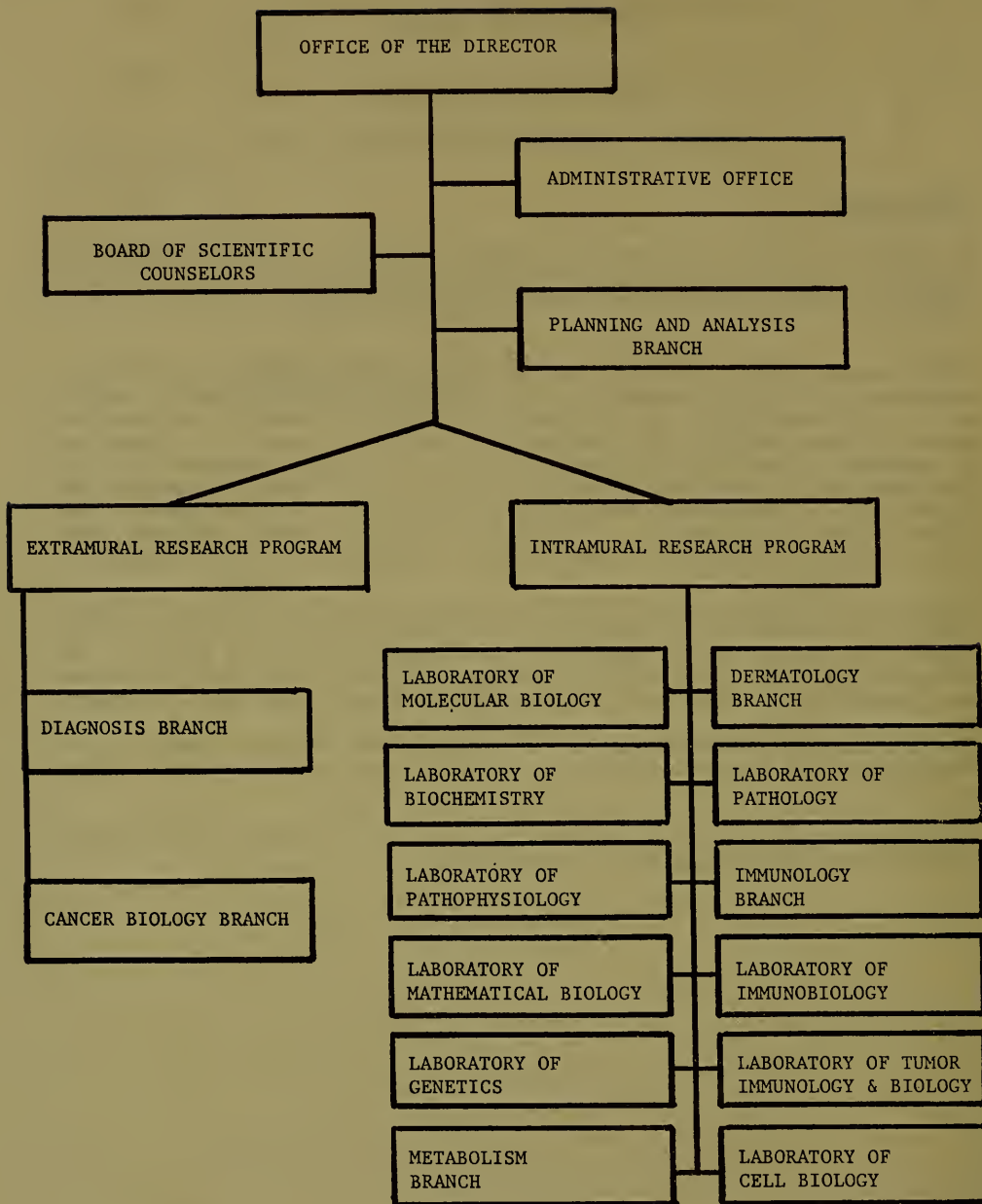
The Division of Cancer Biology and Diagnosis consists of both intramural and extramural research programs in the biology and diagnosis of cancer. A close coordination is maintained with other divisions of NCI in areas of common interest.

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 Extrsmural Program is given in the second volume, which includes both contract and grant funded research.



D. Biochemical Pathology Section

The Biochemical Pathology Section is carrying on research on immunochemistry of complex carbohydrates. Current approaches include 1) determination of carbohydrate structures of glycoproteins and analysis of oligosaccharide mixtures by gas chromatography/mass spectrometry (GC/MS), 2) development of hybridoma antibodies against oligosaccharide haptens, and 3) studies on the origin, metabolism, and excretion of a urinary oligosaccharide [(Glc)₄] derived from glycogen.

1) Structural studies on the carbohydrate chains of human haptoglobin 1-1 and hCG have been completed by Dr. Nilsson and Ms. Cashel. Methods for separating and analyzing oligosaccharides by GC/MS have been developed 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 anti-human colorectal carcinoma hybridoma has been solved.

2) A hybridoma antibody useful as a reagent for radioimmunoassay of a urinary tetrasaccharide (G)₄ has been prepared by Dr. Schroer and Dr. Lundblad. In addition, a hybridoma antibody that binds the oligosaccharide lacto-N-fucopentaose III has been prepared by immunization with a synthetic glycoconjugate. A glycolipid antigen isolated from a human colorectal carcinoma, is the tissue antigen for several hybridoma antibodies that bind human carcinomas.

3) Studies on urinary excretion of (Glc)₄ in patients with soft tissue sarcomas are being conducted by Dr. Zopf, Dr. Ugorski, and Mr. Fernandez in collaboration with Dr. Pizzo of Pediatric Oncology. 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.

E. Tumor Invasion and Metastases Section

The Section of Tumor Invasion and Metastases is studying the biochemical mechanisms which play a role in tumor cell invasion and metastases formation. This group has identified two biochemical factors: a) specific new types of proteases and b) a new type of matrix receptor. Both factors are quantitatively enhanced in actively invading tumor cells. Antibodies against the protease react with actively invading breast carcinoma cells in tissue sections, and are therefore of diagnostic usefulness. Blocking the proteases inhibits tumor cell invasion in vitro. Blocking the matrix receptor abolishes experimental metastases in mice. This receptor can also be readily measured in human breast carcinoma tissue. Attempts to correlate receptor content with clinical stage of disease are in progress.

The basement membrane is a ubiquitous extracellular matrix which separates organ parenchymal cells from the connective tissue stroma. Metastasizing tumor cells penetrate basement membrane barriers when they invade across tissue boundaries or traverse vessel walls. Histologic studies demonstrate that basement membranes are disrupted at the point of tumor cell invasion. Dr. Liotta

observed that tumor cells could degrade whole basement membranes. Therefore, this group searched for tumor cell derived proteases which could degrade purified basement membrane components.

Highly metastatic tumor cells elaborate a family of previously undiscovered metalloproteinases which can degrade components of the basement membranes. One of the enzymes selectively degrades type IV collagen. The collagen is found only in the basement membrane. A separate metalloprotease degrades type V collagen, a collagen found at the basement membrane-stromal interface. Both of these enzymes have been purified more than 1000-fold and characterized. Classic mammalian collagenase failed to degrade collagens IV and V, under conditions where collagens I, II and III were totally degraded. It was proposed that the different types of collagen are markedly different in their protease susceptibility. Different classes of proteases degrade the different types of collagen and this may be physiologically significant in both neoplastic and non-neoplastic situations. Metastatic tumor cells of different histologic origins consistently elaborate metalloproteinases which degrade basement membranes. For a certain group of mouse tumors the level of type IV collagenolytic activity can be correlated with metastatic behavior. Physiologic serine proteases have been identified which degrade the glycoprotein components of basement membrane laminin and fibronectin. The fragments of laminin produced by thrombin and plasmin have been purified and used to study structure of the laminin molecule. Metalloproteinases which degrade basement membrane are not unique to tumor cells but may be present in normal tissues during certain physiologic conditions. A unique class of membrane-bound enzymes was identified by Dr. Tea Kalebic. Antibodies made against these tumor metalloproteinases consistently block metastases formation from intravenously injected tumor cells. These same antibodies react with metastasizing human tumor cells by immunohistology.

The biochemical mechanism of tumor invasion have been poorly understood because a routine quantitative in vitro assay of this process has not existed. Therefore, Dr. Thorgeirsson originated an in vitro assay using human amnion membrane. This tissue consists of only three defined layers: epithelium, basement membrane, and collagenous stroma. At least 50 highly reproducible assays can be performed with the amnion from one human placenta. 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 are as follows. Tumor cell invasion of host basement membranes and collagen stroma does not absolutely require a) living host cells or b) tumor cell proliferation. Purified inhibitors of metalloproteinases inhibit tumor cell invasion of amnion in vitro. These same inhibitors are not cytotoxic and do not inhibit cell motility or chemotaxis. Chemotactic agents stimulate tumor cell invasion of amnion connective tissue in vitro.

Using purified monospecific antibodies to basement membrane components and antibodies to different collagen types, Dr. Sanford Barsky, in Dr. Liotta's group, performed immunohistology studies of human breast cancer. Carcinoma in situ and all other 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. Some breast carcinoma cells continue to produce basement membrane components which can be identified in their cytoplasm (extracellular basement membranes are absent).

This cytoplasmic staining can be used to aid identification of micro-metastases staining can be used to aid identification of micro-metastases of breast cancer in lymph nodes. As a natural extension of the above observations, the production of collagen in human breast carcinoma desmoplasia was also studied by Dr. C.N. Rao and Dr. Barsky. By collagen typing, the source of the collagen in desmoplasia is the host, not the carcinoma cell. Desmoplastic tissue is elevated in absolute and relative type V collagen content. The host cells responsible for collagen and elastin synthesis in desmoplasia are probably myofibroblasts. Human breast cancer desmoplasia may be a stromal host response to invading tumor cells.

In order to further study the interaction of tumor cells with laminin, a glycoprotein of basement membranes, Dr. Liotta's group identified the domain of the laminin molecule which attached to the tumor cell. In addition, a specific cell membrane receptor for laminin was identified by Dr. Liotta's group for the first time. By rotary shadowing electron microscopy, performed by Ms. Margulies, laminin has the configuration of a cross with 3 short arms and one long arm. The cell binding site resides near the intersection of the short arms. The type IV collagen binding domain of laminin is associated with the globular end regions of the short arms but not the long arm. The long arm contains a major binding site for basement membrane proteoglycan. The laminin receptor has a K_d of 2×10^{-9} , 10-100,000 receptors per cell. The receptor is present on isolated cell membranes and is destroyed by trypsin. The receptor is isolated from cell membranes by detergent treatment, HPLC, nitrocellulose blotting and/or laminin affinity chromatography. The laminin receptor molecular weight is 67,000. A fragment of the laminin molecule which binds to the receptor and blocks attachment will inhibit metastases formation in animal models. Laminin receptors can be readily measured in surgical specimens of human breast cancer tissue.

F. Viral Oncology and Molecular Pathology Section

The investigative studies ongoing in the section are primarily involved with animal viruses with an emphasis on malignant transformation. Concerns are with the mechanisms by which these viruses interact with cells susceptible to lytic productive infection as well as the non-productive malignant transformation of cells.

Dr. Costa, with Ms. Yee, has continued to study the Fc binding receptor induced by herpes virus. New studies strongly suggest that a receptor for Protein A IgG complexes can be discriminated from a receptor for aggregated IgG. Biochemical studies indicate that the receptor for IgG-Prot. A complexes present in the HSV-infected cell is a glycoprotein with an approximate molecular weight of 120,000 daltons.

Dr. P. Howley, in collaboration with Dr. S. Schlegel-Banks, has transformed human epidermal cells with the cloned DNA fragments containing the entire early regions of the wild-type SV40 and of a tsA mutant of SV40 (tsA209). In parallel experiments, they were unable to obtain transformants using the cloned DNA of a tsA288 virus which is similar to tsA209, except for an additional deletion which renders it incapable of making a small t antigen. These data suggest that a functional small t antigen as well as a functional large T antigen are required for transformation of primary cells.

Drs. Howley, Law, Sarver, Yang, and Rosenbaum have continued their studies with the human and bovine papillomaviruses. Studying BPV transformation, Dr. Law has shown that the BPV-1 genome remains exclusively extrachromosomal in transformed mouse cells. Dr. Heilman, with Ms. Engel, has mapped the viral transcripts present in BPV-transformed cells as well as those present in productive fibropapillomas of cattle. All the transcripts are derived from a single strand. There appears to be only one coding strand in the papillomaviruses. Dr. Howley, in collaboration with Drs. Chen, Seeburg, and Levinson of Genentech Corp., have completed the sequence of the bovine papillomavirus and only one strand has open-reading frames. Dr. Heilman and Ms. Engel have shown that there are two additional transcripts present in productively-infected cells which are not present in the transformed cells. They have demonstrated that one of these messages encodes the major capsid protein, VP1, by DNA filter selection of the mRNA from a fibropapilloma and translating this into VP-1 in vitro.

Drs. Howley, Sarver, Law, and Rosenbaum have continued their studies utilizing papillomavirus DNAs as eukaryotic cloning vectors. The ability of BPV-1 to be used as a cloning vector was demonstrated in collaboration with Drs. Khoury and Gruss (Laboratory of Molecular Virology, NCI) in which the transforming region of the bovine papillomavirus was cloned together with the rat preproinsulin 1 gene and the hybrid DNA was used to transform susceptible mouse cells.

Drs. Howley and Turek, with Ms. Byrne, with the collaborative assistance of Dr. Lowy and Dr. Robert Friedman, 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. They have found that mouse cell interferon markedly reduces the level of transformation by BPV on mouse cells (approximately 20 fold). Continued treatment of established transformed lines with mouse L-cell interferon, leads to an overall reduction in the number of plasmid BPV copy numbers in the ID13 and ID14 cell lines.

G. Hematopathology Section

Drs. Jaffe, Cossman, Crabtree, Kant, Neckers, Fornace, Brazier and Hsu have major programs in diagnostic and experimental studies of neoplastic and non-neoplastic lesions of the lymphoreticular and hematopoietic system. Drs. Jaffe, Cossman, Crabtree, Kant, Fornace and Brazier are Board Certified Pathologists who take responsibility for the diagnostic material on cases of malignant lymphoma admitted to the various clinical services in the Clinical Center. Drs. Jaffe and Cossman also receive several hundred cases submitted for diagnostic consultation from pathologists in the regional medical community as well as throughout the United States.

The Hematopathology Section has an active research program in several areas. The section has continued its work on the immunologic, cytochemical, biochemical, and functional aspects of human malignant lymphoma. This program has been exceptionally active with over 185 fresh biopsy specimens analyzed during the past year from patients with malignant lymphoma admitted to the Clinical Center. Areas which are receiving particular emphasis include the

clinicopathologic correlations of this immunologic data. A study on the correlation of immunologic phenotype with conventional morphology, showing that immunologic phenotype cannot be predicted by morphologic criteria alone, is published. They have recently completed a study in collaboration with the Medicine Branch, DCT, evaluating the clinical importance of immunologic phenotype in patients with diffuse, aggressive non-Hodgkin's lymphomas. This study represents the most extensive one to date analyzing a clinically homogeneous group of patients in a prospective fashion prior to therapy. 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 subset of peripheral T-cell lymphomas including morphologic, immunologic, and clinical aspects. Peripheral T-cell lymphomas represent a broad spectrum of histologic categories, most of which are included in the diffuse, aggressive subtypes of non-Hodgkin's lymphoma. Using a battery of monoclonal antibodies, most of these cases display a helper/inducer phenotype, but other T-cell associated antigens are inconsistently demonstrated. IA antigens can also be demonstrated on most peripheral T-cell lymphomas. The transferrin receptor, OKT9, is variably expressed and appears to correlate with rapidity of growth of the malignant lymphoma. Clinical features frequently identified in this patient population include hypercalcemia and polyclonal hypergammaglobulinemia.

The section is in collaboration with Dr. Robert C. Gallo, Division of Cancer Treatment, investigating the role of HTLV in peripheral T-cell lymphomas. Approximately 10% of patients with T-cell malignancies have antibodies to HTLV and express viral-associated antigens on the neoplastic cells.

It has previously been shown that hypercalcemia occurs in certain patients with peripheral T-cell lymphomas and that this hypercalcemia may be due to the production of osteoclast-activating factor (OAF) by the neoplastic cells. Further studies in progress include the use of a monoclonal antibody directed against OAF to analyze neoplastic cells directly for this important biologic material. These studies are being done primarily by Dr. Rita Brazier in the Hematopathology Section.

The section has previously reported on the syndrome which simulates malignant histiocytosis seen in certain patients with peripheral T-cell lymphomas. It had been postulated that this syndrome might be due to the production of a lymphokine by the neoplastic T cells which could stimulate the phagocytic cells of the reticuloendothelial system. One of our residents, Dr. Charles Simrell, completed a study which is in press demonstrating that both normal and neoplastic T cells can elaborate factors which induce phagocytic activity in U937 cells, a cell line of true histiocytic origin. The factor produced by these cells can act independently of an effect on 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.

Using a battery of monoclonal antibodies and the fluorescence-activated cell sorter, the expression of various antigens has been investigated in non-Hodgkin's lymphomas. Dr. Jeffrey Cossman has taken primary responsibility for these studies in collaboration with other members of the Section. 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 reflect different stages in normal intrathymic differentiation, particularly late thymic differentiation. This contrasts with published reports of T-ALL, which normally correspond to an earlier stage of T-cell differentiation. Unlike T-ALL, the helper and suppressor phenotypes are mutually exclusive. Furthermore, the thymocyte antigen, OKT6, is present in less than half of cases. Two cases of lymphoblastic lymphoma expressing the common ALL phenotype were identified, and by immunoglobulin gene rearrangement studies done in collaboration with Dr. Korsmeyer, these cases were shown to be committed to the B-cell lineage. In addition, two cases with a pre-B phenotype demonstrating cytoplasmic immunoglobulin were also identified. Furthermore, it was shown that all of these cases share a convoluted nuclear morphology and that convoluted nuclei are not useful in predicting either T- or B-cell markers.

Dr. Neckers and Dr. Cossman have shown that one can quantify the expression of the transferrin receptor during PHA activation of normal peripheral blood lymphocytes. They are also investigating the expression of the transferrin receptor as it correlates with the receptor for T-cell growth factor as identified by TAC binding. The expression of both the transferrin receptor and the TCGF receptor are normally both dependent upon prior exposure to lymphocyte activating factor or interleukin I. However, TPA can be used to bypass this normal physiological requirement.

Additional studies conducted by Dr. Cossman 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 is normally not identified. While TPA induced immunoglobulin secretion, TDT, a marker of lymphoblasts, decreased in these cells, further indicating induction of differentiation. While it had previously been postulated that immunoglobulin synthesis was not seen in these cases because of defective gene rearrangement, Dr. Cossman's observation indicates that this is not the case.

Dr. Gerald R. Crabtree and his associates, Drs. Jeffrey Kant, Al Fornace, and Dana Fowlkes, have been involved in the development of a model to study developmentally- and hormonally-controlled gene expression. They have chosen to study the small family of genes which code for the three polypeptide chains of fibrinogen. To date, they have concentrated on the regulation of fibrinogen mRNA levels as well as the molecular cloning and structure of the rat and human fibrinogen genes. They have begun by obtaining cDNA clones for each of the three chains of fibrinogen. This was done by constructing a library of cDNA clones from maximally-induced rat liver mRNA, using Malayan pit viper venom, as they have previously described. Clones corresponding to fibrinogen chains were identified from the *in vivo* translation products of mRNA selected by cloned DNAs. The identification of each clone was confirmed by determining the nucleic acid sequence and aligning the predicted amino acid sequence with the known human amino acid sequence. Thus far, the sequence

of these clones has provided two new pieces of information which have been useful in understanding the molecular biology of fibrinogen. First they have found that the gamma chain of fibrinogen is encoded by two mRNAs which arise from a single gene by alternate splicing patterns. An intervening sequence at the 3' end of the gene is either removed or not removed to produce two gamma chain proteins which differ in length by 8 amino acids and have different carboxy termini. These two fibrinogen gamma chains are present in a wide variety of species including man and each chain is incorporated into fibrin polymers during coagulation.

They have explored the structure of the rat fibrinogen genes by Southern blotting and examination of genomic DNA clones for the three fibrinogen chains. They have found that each of the fibrinogen chains is encoded by a single gene per cell and that the alpha and gamma chains are tightly linked within 12 kb of each other. Future aims include a complete description of the structure of the rat fibrinogen genes, regulation of the rat fibrinogen genes, and exploration of the structure of the human fibrinogen genes and defects in the afibrinogenemias and dysfibrinogenemias.

H. Pathological Technology Section

The technology staff, under Mrs. Barbara Coolidge, is located in Ft. Detrick in Frederick, Maryland. They provided all types of histological services and staining procedures for scientists in DCBD and other divisions of NCI. During FY 82 they processed more than 35,000 pieces of animal tissue, cut and stained more than 45,000 H & E slides and prepared over 2,000 special stained slides. They also prepared over 20,000 unstained for immunoperoxidase studies and other special techniques. All types of animal tissue were accepted in their laboratory and the more routine work is sent to private contractors for sectioning and staining. There was a total of 86 NCI investigators using the services of the section. The quality and performance of the Pathological Technology staff continues to be outstanding and they represent a major technical resource for the research of scientists in the NCI. The section also prepares study sets for the NCI Tumor Registry which are used by scientists all over the world as well as NCI.

I. Image Processing Section

Computer Aided 2 Dimensional Electrophoretic Gel Analysis (GELLAB). Bio-medical research involving 2D gels produces multiple (3 or 4 to as many as hundreds of) gels each one of which may show from several hundred to more than 1000 spots each. The problem of analysis of such multiple gels is establishing correspondences among spots across gels and evaluating qualitative and/or quantitative differences in subsets of spots corresponding to gels resulting from differing experimental treatment. The huge size and formidable complexity of the data bases generated by even simple experiments is obvious.

GELLAB was one of the first systems to be successfully applied to aid in the analysis of such massive and complex data structures. It is in very heavy use in

our laboratory, as the major tool in a large series of substantive biomedical research problems, and at other installations where DECsystem-10 or -20 computers have installed our export versions. These sites currently include: a) University of Chicago (Dr. Eric Lester); b) Christian-Albrechts-University in West Germany (Dr. H.G. Busse); c) Biochemistry Department, University of Zurich (Dr. Peter Sonderegger, currently at NIH but returning to Zurich with GELLAB software to set up a system on their DECsystem-10.

There are six collaborative projects using GELLAB active during this past year. Four of these are ongoing while two users in Dr. Thorgeirsson's (NCI) lab have finished analytic work on our system. Dr. Thorgeirsson now has a production 2D gel analysis system. However, collaboration in areas of algorithm design will be continued with his group.

Research in nucleic acid secondary structure has continued. Our collaboration with Dr. Jacob Maizel, Chief of the Molecular Structure Section of the National Institute of Child Health and Human Development has remained quite close. Collaboration with Dr. Kathleen Currey in the Molecular Structure Section has also strengthened.

The two drawing algorithms that were previously reported have been enhanced. The polygonal algorithm now allows the searching for specific base patterns and allows the superposition of these base patterns onto the secondary structure to determine in what types of structures these patterns reside. The ability to label base positions has also been added. Over 150 molecules have been drawn using this algorithm. The size molecule that the algorithm can accommodate has also been increased to permit sizes up to 1500 bases.

In order to analyze secondary structures and their significance, we have compared the predicted structure(s) of natural sequences with the same sequence which had been randomly shuffled. In sequences known to have high secondary structure such as viroids and 5srna, we found the natural sequence to have a more stable predicted structure and agree better with the chemical data. This approach is being pursued on selected adenovirus sequences. We tentatively observed that the natural sequence not only has regions of greater stability than the shuffled one but also that some regions have lower stability, suggesting that the potential both for forming and avoiding secondary structures has evolved in genomes. Further analysis of the natural and shuffled sequence stability data led to the observation that stability (or energy of the structure) is linear with G+C content. Thence, from these data it is possible to predict a stability for a given region or molecule and assess the likelihood of the presence or absence of significant secondary structure for that sequence. In addition, artificially created, shuffled sequences of varying length and specific base composition were examined and the energy (stability) was found to be directly proportional to length. These properties are currently being analyzed in finer detail with regard to how varying the base composition influences the stability (energy) of single strand RNAs produced by genomes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00853-30 LP
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.) Surgical Pathology		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Ernest E. Lack, Chief, Surgical Pathol. & Postmortem Section, Lab. of Path., NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Surgical Pathology & Postmortem Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 20	PROFESSIONAL: 20	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The Surgical Pathology and Postmortem Section, together with the Cytopathology Section, Ultrastructural Pathology Section and Hematopathology Section provide complete service in anatomic pathology for the Clinical Center patients and collaborate with the research staff of all institutes in those investigations which involve the use and study of human pathological material. A new frozen section and surgical pathology processing area has been constructed adjacent to the new operating rooms and became operational on April 18, 1983. This new facility will enhance processing of specimens and communication of diagnostic findings.</p> <p>The staff is engaged in a variety of projects involving clinicopathological correlation and pathologic characterization of disease studied at the Clinical Center. Immunocytochemical techniques have been applied to the characterization and study of tumors and other non-neoplastic diseases. The use of immunohistochemical staining has greatly facilitated more precise diagnosis in selected difficult cases and with the increasing number of monoclonal antibodies available this technique should have even greater value in diagnostic and research pathology.</p>		

Project Description

Other Professional Personnel:

Dr. Cheryl M. Reichert, Chief, Autopsy Pathology
 Dr. Sanford H. Barsky, Expert
 †Dr. David L. Levens
 †Dr. Christine B. Thomas
 †Dr. Carl C. Baker
 †Dr. Gail J. Bryant
 †Dr. Ilona Linnoila
 †Dr. Charles R. Simrell
 †Dr. Carl W. Rettenmier
 †Dr. Alice Ahlgren
 †Dr. Maria Tsokos
 Dr. Sue Ellen Martin
 Dr. Elaine S. Jaffe, Chief, Hematopathology Section
 Dr. Peter M. Howley, Chief, Viral Oncology & Molecular Pathology Section
 Dr. Timothy J. Triche, Chief, Ultrastructural Pathology Section
 Dr. Jeffrey Cossman
 *Dr. Lance A. Liotta, Laboratory of Pathology, NCI
 *Dr. Paul E. McKeever, Laboratory of Microbial Immunity, NIAID
 *Dr. Kurt J. Stromberg, Division of Cancer Cause and Prevention, NCI
 *Dr. Marius Valsamis, Associate Professor of Pathology, New York
 Medical College, Center for Aging and Chronic Diseases
 *Dr. William C. Roberts, Pathology Branch, NHLBI

The objectives of the Surgical Pathology and Postmortem Section are:

- (1) to provide diagnostic services in pathologic anatomy to the clinical research projects conducted at NIH; (2) to carry out independent research;
- (3) to conduct a residency training program in anatomic pathology; and
- (4) to collaborate with investigators in research involving the use and study of human materials.

The service functions of the section during the past year included:

(a) 158 autopsy examinations. The residents perform nearly all of the postmortem dissections under supervision of various staff members. A conference is held weekly by the staff and residents where gross organs are reviewed and clinical pathologic correlations are made. The residents review the microscopic slides from each autopsy they perform with one of the staff before completing the autopsy protocol.

The staff assists the residents in preparing for the numerous clinical conferences in which the section participates.

†These physicians are full-time Residents in the Laboratory of Pathology.

*These Associate Pathologists spend part time in the activities of the Surgical Pathology and Postmortem Section.

(b) Slightly more than 5500 specimens were accessioned in the Surgical Pathology and Postmortem Sections. The surgical pathology specimens are initially examined and prosected by a resident and their reports are checked by the staff. Associate pathologists from other laboratories or sections are frequently consulted about diagnostic problems in surgical pathology and/or assume responsibility for handling certain tissue removed for specific research projects. Approximately 600 pre-operative consults were rendered.

(c) Histological preparation and special procedures: The functions are carried out under the direction of Mrs. Ruby Howard. More than 53,000 slides were prepared. Of these, 4/5 were routine H&E stained and 1/5 were specially stained.

(d) Photographic services of the department: A photographic record is made of the large amount of pathological material which is handled and studied in the department. The photography is done by the staff and residents with the technical assistance of Mr. A. Calhoun and Mr. J. Banks who also maintain the equipment and carry out much of the photographic processing. Gross photographs and photomicrographs of the pathologic material are provided to the clinical staff on request, and are used extensively for conferences, seminars and many are prepared for publication. Over 325 black and white negatives and over 640 black and white prints have been prepared from autopsy and surgical pathology material. A total of 40 35 mm roles have been prepared from gross specimens in the surgical and autopsy service.

A large proportion of the photomicrography is done under the direction of Mr. Ralph Isenberg, who provides professional assistance and facilities for the entire staff of the Laboratory of Pathology.

Conferences. The staff takes part in the following inter-departmental conferences:

- Pediatric Oncology Tumor Board (weekly)
- Sarcoma Staging Conference (weekly)
- Surgical Morbidity and Mortality Conference (monthly)
- Medicine-Radiation Conference (weekly)
- Surgical Pathology Conference (weekly)
- Lymphoma Staging Conference (weekly)
- Testicular Tumor Staging Conference (weekly)

In addition, the staff participates in numerous clinicopathological conferences discussing specific patients.

Data retrieval system. In conjunction with DCRT, a program has been created for storing and retrieving the surgical pathology and autopsy material and to automatically encode all the diagnoses. The language used is a modification of SNOP and a dictionary is being constantly improved in order to accommodate a maximum of currently used terms.

Proposed Course: (a) Continue to provide the services described. (b) Increase the interaction with the clinical branches in the design and evaluation of protocols. (c) Improve the opportunities for the resident staff to participate in teaching, conferences, and seminars, and provide elective periods to be spent accomplishing research projects with the senior staff. (d) Implement data retrieval programs.

Publications:

Lack, E.E., Perez-Atayde, A., and McGill, T.J.: Gingival granular cell tumor of the newborn (congenital "Epulis"). Ultrastructural observations relating to histogenesis. Hum. Pathol. 13: 686-689, 1982.

Abramson, S.J., Lack, E.E., and Teele, R.L.: Ultrasonic appearance of benign vascular tumors of the liver in infancy. Am. J. Roentgenol. 138: 629-632, 1982.

Lack, E.E., Eraklis, A.J., Harris, C.B., and Vawter, G.F.: Primary bronchial tumors in childhood. A clinical and pathologic study of six cases. Cancer 51: 492-497, 1983.

Lack, E.E., Neave, C., and Vawter, G.F.: Hepatoblastoma. A clinical and pathologic study of 54 cases. Am. J. Surg. Pathol. 6: 693-705, 1982.

Sayed, A.K., Miller, B.A., Lack, E.E., and Sallan, S.E.: Heterochromia iridis and Horner's syndrome in a child with paravertebral neurilemmoma. J. Surg. Oncol. 22: 15-16, 1983.

Gallivan, M.V.E., Lack, E.E., Chun, B., and Ishak, K.G.: Undifferentiated ("embryonal") sarcoma of the liver with presentation as an intracardiac tumor. Ped. Pathol. (in press)

Perez-Atayde, A.R., Lack, E.E., Katz, A.J., and Geha, R.S.: Intestinal malakoplakia in childhood. Ped. Pathol. (in press)

Lack, E.E., Neave, C., and Vawter, G.F.: Hepatocellular carcinoma. Review of 32 cases in children and adolescents. Cancer (in press)

Lack, E.E., Cassady, R.J., Levey, R., and Vawter, G.F.: Tumors of the exocrine pancreas in children and adolescents. A clinical and pathological study of seven cases. Am. J. Surg. Pathol. (in press)

Mostoufizadeh, M., Lack, E.E., Gang, D.L., Vawter, G.F., and Driscoll, S.G.: Postmortem manifestations of echovirus II sepsis in newborn infants. Hum. Pathol. (in press)

Erisman, M.D., Linnoila, R.I., Hernandez, O., DiAugustine, R.P., and Lazarus, L.H.: Human lung small-cell carcinoma contains bombesin. Proc. Natl. Acad. Sci. USA 79: 2379-2383, 1982.

Linnoila, R.I.: Effects of diethylnitrosamine on lung neuroendocrine cells. Exp. Lung Res. (in press)

Petrusz, P., and Linnoila, R.I.: Applications of immunohistochemistry on lung. Review. In Hook, G.E.R. (Ed.): Pulmonary Toxicology. Raven Press. (in press)

Gould, V.E., and Linnoila, R.I.: Pulmonary neuroepithelial bodies, neuroendocrine cells, and pulmonary tumors. Hum. Pathol. 13: 1064-1066, 1982.

Gould, V.E., Linnoila, R.I., Memoli, V.A., and Warren, W.H.: Neuroendocrine cells and neuroendocrine neoplasms of the lung. Pathology Annual (in press)

Barsky, S.H., Linnoila, R.I., Triche, T.J., and Costa, J.C.: Hepatocellular carcinomas with carcinoid components. Hepatology (in press)

Tsokos, M., Howard, R.M., and Costa, J.C.: Immunohistochemical study of alveolar and embryonal rhabdomyosarcoma. Lab. Invest. 48: 148-155, 1983.

Jaffe, E.S., Costa, J.C., Fauci, A.S., Cossman, J., and Tsokos, M.: Malignant lymphoma and erythrophagocytosis simulating malignant histiocytosis. Am. J. Med. (in press)

Tsokos, G., and Tsokos, M.: Monoclonal antibodies. Basic and clinical aspects. Min. Med. Greca (in press)

Kiritsis, A., and Tsokos, M.: Neurotransmitters in the retina of the eye. Min. Med. Greca (in press)

Simrell, C.R., Crabtree, G.R., Cossman, J., Fauci, A.S., and Jaffe, E.S.: Stimulation of phagocytosis by a T cell lymphoma derived lymphokine. In Vitetta, E., and Fox, C.F. (Eds.): B and T Cell Tumors: Biological and Clinical Aspects. UCLA Symposia on Molecular and Cellular Biology, Vol. XXIV, New York, Academic Press, 1982, pp. 247-252.

Seibert, K.A., Rettermier, C.W., Waller, B.F., Battle, W.E., Levine, A.S., and Roberts, W.C.: Osteogenic sarcoma metastatic to the heart. Am. J. Med. 73: 136-141, 1982.

Levens, D., Christianson, T., Edwards, J., Lustig, A., Ticho, B., Locker, J., and Rabinowitz, M.: Transcriptional initiation of yeast mitochondrial RNA and the regulation of mitochondrial RNA polymerase biosynthesis. In Attardi, G., Borst, P., and Slonimski, P. (Eds.): Mitochondrial Genes. Cold Spring Harbor, Cold Spring Harbor Laboratory (in press)

Bryant, G.J., Lavielle, F., and Levin, I.W.: Effects of membrane bilayer reorganizations on the 2103 cm^{-1} Raman spectral C-D stretching mode line-widths in dimyristoyl phosphatidylcholine- d_{54} liposomes. J. Raman Spectroscopy 12: No. 2, 1982.

Macher, A.M., Palestine, A., Masur, H., Bryant, G., Chan, C., and Rodrigues, M.: Palpebral conjunctival Kaposi's sarcoma in a male homosexual with the new acquired immunodeficiency syndrome. Ophthalmol. (in press)

Fraser, N., Baker, C., Moore, M., and Ziff, E.: Poly A sites of adenovirus type 2 transcription units. J. Mol. Biol. 155: 207-233, 1982.

Pretorius, H.T., Katikineni, M., Kinsella, T.J., Barsky, S.H., Brennan, M.F., Chu, E.W., and Robbins, J.: Thyroid nodules after high-dose external radiotherapy. Fine-needle aspiration cytology in diagnosis and management. J.A.M.A. 247: 3217-3220, 1982.

Barsky, S.H., Martin, S.E., Mattlien, M., Gazdar, A., and Costa, J.C.: Low-grade mucoepidermoid carcinoma of the bronchus with high-grade biological behavior. Cancer (in press)

Barsky, S.H., Linnoila, I., Triche, T.J., and Costa, J.C.: Hepatocellular carcinomas with neuroendocrine components. Cancer (in press)

Barsky, S.H., Baker, A., Siegal, G.P., Togo, S., and Liotta, L.A.: Use of anti-basement membrane antibodies to distinguish blood vessel capillaries from lymphatic capillaries. Am. J. Surg. Pathol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00872-01 LP
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.) Autopsy Service		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Cheryl M. Reichert, Chief, Autopsy Service, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Surgical Pathology and Postmortem Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 4	PROFESSIONAL: 2	OTHER: 2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews A		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The Autopsy Service of the Laboratory of Pathology provides complete service in autopsy pathology for the Clinical Center patients and collaborates with the research staff of all institutes in those investigations which involve the use and study of human pathological material.</p> <p>The staff is engaged in several projects involving clinicopathological correlation and pathologic characterization of disease studied at the Clinical Center. Immunocytochemical techniques have been applied to the characterization and study of tumors and other non-neoplastic diseases.</p>		

Project Description

Other Professional Personnel:

+Dr. Gail J. Bryant
 +Dr. R. Ilona Linnoila
 +Dr. Charles R. Simrell
 +Dr. David L. Levens
 +Dr. Carl C. Baker
 +Dr. Christine B. Thomas
 Dr. Timothy J. O'Leary
 *Dr. Kurt J. Stromberg, Division of Cancer Cause and Prevention, NCI
 *Dr. William C. Roberts, Pathology Branch, NHLBI
 Dr. Lance A. Liotta, Chief, Laboratory of Pathology
 Dr. Peter M. Howley, Chief, Viral Oncology & Molecular Pathology Section,
 Laboratory of Pathology
 Dr. C. Richard Schlegel, Laboratory of Pathology
 Dr. William R. Green, Laboratory of Pathology
 Dr. David A. Zopf, Chief, Biochemical Pathology Section, Laboratory of
 Pathology
 Dr. Carlos Restrepo, Laboratory of Pathology
 Dr. Unnur P. Thorgeirsson, Laboratory of Pathology

The objectives of the Autopsy Service are: (1) to provide diagnostic services in pathologic anatomy to the clinical research projects conducted at NIH; (2) to carry out independent research; (3) to conduct a residency training program in pathologic anatomy; and (4) to collaborate with investigators in research involving the use and study of human material.

1. The service and teaching functions of the section during the past calendar year included:

a) 141 autopsy examinations as follows: 121 NIH patients, 2 patients without NIH records, 18 cases submitted as slides or paraffin blocks. (During the 1982 calendar year, there were 146 deaths among hospitalized Clinical Center patients). The residents perform the postmortem dissections with the assistance of one of two full-time dieners and under the supervision of a senior pathologist. A gross review is held weekly by the staff and residents, and is attended by a radiologist who reviews pertinent x-rays and scans, and by clinicians. The residents review the microscopic slides of each autopsy with one of the staff before completing the autopsy protocol. The final reports are returned to the primary care physicians within 90 days after expiration of the patient.

b) The staff assists the residents in preparing for various morbidity/mortality conferences.

+These physicians are full-time Residents in the Laboratory of Pathology.

*These Associate Pathologists spend part time in the activities of the Autopsy Service.

2. The research functions of the Autopsy Service included:

- a) The distribution of human tissues to more than 75 authorized scientists with approved research protocols.
- b) The review and approval of requests for patient-related material as part of a formal tissue procurement policy.
- c) Independent and collaborative research projects of the Autopsy Service:

I. Projects recently completed:

- A. Reichert, C.M., Weisenthal, L.M., and Klein, H.G.: Delayed hemorrhage following percutaneous liver biopsy. J. Clin. Gastroenterol. (in press)
- B. Reichert, C.M., Everett, D.F., Jr., Nadler, P.I., and Papadopoulos, N.M.: High-resolution zone electrophoresis, combined with immunofixation, in the detection of an occult myeloma paraprotein. Clin. Chem. 28: 2312-2313, 1982.

II. Projects currently underway:

- A. Lumpectomy with radiation therapy vs. mastectomy for local control of Stage I-II breast cancer. (Lichter, Lippman, Danforth, Reichert, et al). An ongoing clinical protocol.
- B. Monoclonal antibodies - application to breast cancer.
 - 1. Detection of breast antigens in the axillary nodes of patients with primary breast cancer - a comparison of biochemical vs immunohistochemical and routine pathologic approaches (Reichert, Claysmith, Schlom)
 - 2. Heterogeneity of breast cancer antigens: a comparison of the primary tumor with regional lymph nodes and metastatic diseases (Bowen, Reichert, Schlom)
 - 3. Antigenic phenotyping in inflammatory breast cancer (Reichert, DeMatos, Levine, Schlom et al.)
 - 4. Antigenic phenotyping in advanced breast cancer (Reichert, Lippman, Schlom, Bagby et al.)
- C. Complete regression of abdominal wall sarcomas in mice treated with adoptive immunotherapy: a histologic and immunohistochemical assessment (Reichert, von Glatz, Hsu, Rosenstein, Rosenberg).
- D. ABO(H) cell surface antigens in parathyroid adenoma and hyperplasia (Woltering, Brennan, Marx, Reichert, and others).

- E. Anti-glycophorin antibodies in nucleoside phosphorylase deficiency (Wasniowska, McGuiness, Zopf, Strauss, and Reichert).
 - F. Vascular transformation of lymph nodes secondary to retroperitoneal fibromatosis (Ahlgren, Triche, and Reichert).
 - G. Bony invasion by soft tissue sarcomas (Reichert, Anderson, Dwyer, and others).
 - H. Acute capillary leak syndrome, correlation of pathology with cardiac monitoring (Parrillo, Parker, DeMatos, Reichert).
 - I. Cis-platinum neurotoxicity (Reichert, Triche, Thomas, Claysmith and others).
 - J. Angiosarcoma of the skull: a case report (Tsokos, Stromberg).
 - K. Acquired immunodeficiency syndrome: An autopsy review (Reichert).
 - L. Pachydermodactyly (Reichert, Costa, Barsky, Claysmith, Liotta, Enzinger, Triche). (manuscript submitted)
 - M. Pulmonary infiltrates in metastatic malignant melanoma (Dwyer, Woltering, Flye, Reichert). (manuscript under revision)
 - N. Cystic ovaries in cystic fibrosis: an ultrasound and autopsy study (Shawker, Hubbard, Reichert, McCarthy, DeMatos). (manuscript submitted)
 - O. Pneumocystis pneumonia: a pathologist's approach. (Macher, Weinstein, Reichert). (manuscript in preparation)
 - P. Pulmonary infiltrates in metastatic malignant melanoma. (manuscript under revision)
- d) Generation of a data base to facilitate clinical research by other NIH departments.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00876-01 LP
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.) Breast cancer: role of monoclonal antibodies in the detection of nodal metastases		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Cheryl M. Reichert, Chief, Autopsy Service, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any) Dr. Jeffrey Schlom, Chief, Laboratory of Tumor Immunology and Biology		
LAB/BRANCH Laboratory of Pathology		
SECTION Surgical Pathology and Postmortem Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1	PROFESSIONAL: 1/4	OTHER: 3/4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews A		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The detection of metastatic breast cancer within the draining axillary lymph nodes of patients with primary breast cancer serves as an important prognostic indicator and provides a rationale for the administration of adjuvant chemotherapy. Currently, the assessment of nodal involvement is based upon a pathologist's histologic evaluation of a 6 micron section through each palpable lymph node. Hence, the accuracy of this crucial information is limited by (1) the inability of the pathologist to detect all lymphoid tissue within the fibroadipose tissue and (2) the sampling error inherent in examining only one histologic section from each node. Mathematical models have shown that the examination of a single lymph node section gives rise to a 30% probability of a false negative assessment. The validity of this concept has been borne out in studies where serial sections through lymph nodes results in the detection of "occult" metastases in 15-30% of cases which were "node negative" by conventional criteria. Therefore, we pose the question, "Can metastatic disease within the axilla more accurately or more easily be determined by an Eliza or a radioimmunoassay"?</p>		

Project Description

Other Professional Personnel: A. Claysmith, Bio. Lab. Tech., LP, NCI

Objectives: This project represents one facet of the collaborative research currently in progress with the Laboratory of Tumor Immunology and Biology. Using monoclonal antibodies directed against breast cancer antigens, we are studying the stability and heterogeneity of expression of these antigens. In addition, we are involved in studies which explore the potential clinical applications of the monoclonal antibody reagents.

Methods Employed: Immunohistochemistry, radioimmunoassay, ELIZA, biochemical extraction techniques.

Major Findings: Antigenic reactivity appears conserved over a wide range of conditions of fixation and handling. Preliminary studies reveal a relative stability of antigenic phenotype between the primary breast cancer, the axillary nodes, and systemic metastases. The antibodies exhibit preferential reactivity with neoplastic breast epithelial cells over their normal counterparts. There is some evidence that antigenic phenotyping may be useful in identifying node negative women at high risk for recurrence.

Significance to Biomedical Research and the Program of the Institute: These studies have the potential of improving the clinical approach to the treatment of patients with breast cancer. In addition, if successful, these studies may alter the manner in which axillary lymph nodes are handled by pathologists.

Proposed Course: Halves of axillary nodal dissections for breast cancer will be systematically frozen and stored at -80°C . Homogenates of these nodes will be analyzed via RIA/ELIZA techniques employing monoclonal antibodies. The findings will be compared with both routine histologic evaluation and immunohistochemical stains of the paraffin-embedded tissue sections for each patient. Approximately thirty cases will be studied in this manner.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01CB00852-30 LP
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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.)
Exfoliative cytology applied to human diagnostic problems and research problems

PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.)
(Name, title, laboratory, and institute affiliation)
Elizabeth W. Chu, Chief, Cytopathology Section, Laboratory of Pathology, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Cytopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 8	PROFESSIONAL: 4	OTHER: 4
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CHECK APPROPRIATE BOX(ES)

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

(a1) Minors

(a2) Interviews

A

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

The Cytopathology Section provides complete diagnostic service in exfoliative cytology, medical cytogenetics, and fine needle aspiration cytology. In addition, the section collaborates in various clinical research projects utilizing special techniques including special staining and tissue culture techniques. The section also investigates and applies new immunocytochemistry techniques to improve and enhance cytological diagnostic efficacy, and investigates chromosomal hybridization techniques in mapping genes.

Project Description

Other Professional Personnel: S.E. Martin, Staff Pathologist, LP, NCI; H.-Z. Zhang, Visiting Fellow, LP, NCI; T.A. Wood, Biologist, LP, NCI; L. Galito, Biologist, LP, NCI; A.M. Honig, Biologist, LP, NCI; K. Nakahara, Biologist, LP, NCI; E. Sanders, Bio. Lab. Tech., LP, NCI

Objectives:

1. To provide the staff of the Clinical Center with an accurate and complete exfoliative and aspiration cytology service as well as diagnostic cytogenetics (karyotyping and in situ hybridization).
2. To collaborate in various clinical research projects evaluating cancer therapy, the hormonal status of the cancer patients, the course and natural history of the cancer lesions and the anatomical and physiological changes in the human body associated with various pathologic conditions.
3. To collaborate with the Dental Research Institute in the evaluation of intra-oral fluoride releasing device by examination of oral epithelial cells.
4. Development of better diagnostic techniques.

The specific objectives include:

A) Cytology

1. Continued improvement in cytologic techniques in establishing definitive diagnosis.
2. Improvement in fine needle aspiration cytologic technique.
3. Establishing cytologic characteristics of cell types seen only in fine needle aspirations.
4. Setting up special stain techniques which may be used for general cytology or special research problems.
5. To use immunoperoxidase technique on cytological material to assist in the establishment of a definitive diagnosis.
6. To use Pap stain on oral smears from patients who are fitted with the intra-oral fluoride device.
7. Maturation index in precocious children.

B) Diagnostic Cytogenetics

1. Improvement in short-term culture system to assure of successful harvesting of in vitro cells.
2. Improvement in staining techniques.
3. Improvement in special chromosomal analytical techniques such as banding by various methods.
4. Gene mapping by chromosome in situ hybridization.
5. Chromosomal characterization of human and mouse cell lines after application of butyrate.

Methods Employed:

1. Special procedures in addition to the standard Papanicolaou stain are used:

- A. Millipore filtration of all fluids
- B. Special stains
 - 1. Feulgan reaction for sex chromatin bodies
 - 2. Acid fast, Pneumocystis, Methenamine silver, Brown Brenn for various organisms and fungi
 - 3. PAS, Wright Giemsa, iron stain, melanin stain, congo-red, oil-red-0, mucicarmin for various specific reactions

2. Short-term cultures of peripheral blood, bone marrow cells and tumor cells found in body fluids are the standard diagnostic methods.

In addition, special techniques involving trypsin digestion followed by special stains such as G-banding, Q-banding, and C-banding, as well as fluorescence staining whenever indicated. Also, gene mapping by chromosome in situ hybridization and autoradiography.

Major Findings: Major contributions of the Cytopathology Section:

- 1. Providing prompt services and early diagnosis in cytological materials.
- 2. Providing cytologic evaluation and estimated ranges of either relapse or remission in the continual management of meningeal leukemia.
- 3. Providing cytological evaluation of therapeutic effects on cells seen in urinary specimens, vaginal-cervical smears, sputum and effusions.
- 4. Providing chromosomal analysis for clinical studies as well as in the establishment of definitive malignancies.
- 5. By using chromosomal hybridization in situ techniques, certain immunoglobulin genes were mapped to sites of non-random chromosomal translocations in malignant B lymphoma.
- 6. Providing cytologic methods to research projects; specifically, fine needle aspiration in thyroid nodules.
- 7. Immunohistochemical technique often offers means to differentiate mesothelial cells from cancer cells in body fluids by using suitable antisera.

Significance to Biomedical Research and the Program of the Institute:

- 1. The diagnostic value of exfoliative cytology in the clinical management of various disease states is an established fact.
- 2. Aspiration cytologic techniques are useful in establishing diagnosis on metastatic diseases and/or recurrent malignancies. In such instances, the fine needle aspiration technique eliminates the more involved incisional biopsy and therefore is more economical to the clinicians as well as to the patients.
- 3. Cytochemical techniques may offer early resolutions to equivocal cases.
- 4. Chromosomal analysis is a way of establishing definitive diagnoses in selected patients with endocrine disorders, congenital defects and also in some malignancies.

5. Chromosomal hybridization in situ technique makes it possible to map immunoglobulin genes to their chromosomal bands with precision. This has great implication in certain human B cell lymphomas and leukemias in light of induction and/or maintaining the malignant state.

6. Chromosomal hybridization in situ technique will eventually open the way for extensive experimental tests involving other genes.

7. Immunoperoxidase technique can support the clinical diagnosis of malignancy with specific monoclonal antibodies.

Proposed Course:

1. Continue to provide a complete cytodiagnostic service for the entire staff of the Clinical Center in various research projects.

2. Continual improvement and development of established as well as new techniques in cytological field.

3. Continual work with gene mapping by chromosomal hybridization technique.

4. Develop and improve prophase banding technique.

5. Intensive pursuit of cytochemical techniques to improve and abet diagnostic cytology.

Activities:

1. Invited by the Formosan Medical Association to participate in the 75th annual meeting as a panelist in November 1982 to discuss the chemotherapeutic effects observed in fluids.

2. Invited by USAF at Keesler Air Force Base, MS, to present a seminar on thyroid aspiration cytology, October 4-5, 1982.

3. Lecture and workshop on fine needle aspiration, Buckeye Society of Cytology, Cincinnati, Ohio, September 24-25, 1982.

Publications:

El-Bolkainy, M.N., Chu, E.W., Ghoneim, M.A., and Ibrahim, A.S.: Cytologic detection of bladder carcinoma in a rural Egyptian population infested with schistosomiasis. Acta Cytol. 25: 303-310, 1982.

Martin, S.E., Dwyer, A., Kissane, J.M., and Costa, J.C.: Small cell osteosarcoma. Cancer 50: 990-996, 1982.

Barsky, S.H., Martin, S.E., Mathews, M., Gazder, A., and Costa, J.C.: Low grade mucoepidermoid carcinoma of the bronchus with high-grade biological behavior. Cancer 51: 1505, 1983.

Wright, W.E., Davis, M.C., Geffen, D.B., Martin, S.E., Nelson, M.J., and Strauss, S.E.: Alveolar bone necrosis and tooth loss: A rare complication associated with herpes zoster infection of the fifth cranial nerve. Oral Surg. Oral Med. Oral Path. (in press)

Kirsch, I.R., Morton, C.C., Nakahara, K., and Leder, P.: Human immunoglobulin heavy chain genes map to a region of translocation in malignant B lymphocytes. Science 216: 301-303, 1982.

Pretorius, H.T., Katikineni, M., Kinsella, T.J., Barsky, S.H., Brennan, M.F., Chu, E.W., and Robbins, J.: Thyroid nodules after high-dose external radiotherapy. Fine-needle aspiration cytology in the diagnosis and management. J.A.M.A. 247: 3217-3220, 1982.

Chu, E.W., and Martin, S.W.: Fine Needle Aspiration Cytology of Metastases. In Liotta, L.A., and Hart, I.R. (Eds.): Tumor Invasion and Metastasis. The Hague, Martinus Nijhoff Publ., 1982, pp. 495-510.

Chu, E.W., and El-Bolkainy, M.N.: Cytology and Screening of Bladder Cancer. In Bladder Cancer, Vol. I (in press)

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

PROJECT NUMBER

201CB00518-05 LP

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.)

Fate of IgE bound to mast cells

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

(Name, title, laboratory, and institute affiliation)

Chaviva Isersky, Senior Investigator, A&R, NIAMDD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

12

PROFESSIONAL:

6

OTHER:

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.)

IgE bound to the surface of mast cells and/or basophils is responsible for the immediate hypersensitivity reaction. This response once established, persists for prolonged periods of time. We have recently shown that the mechanism is not due to internalization (J. Immunol. 122: 1926-1936, 1979). Cross linking of the IgE by allergen (or other means) is normally necessary to elicit cell degranulation, which results in histamine release. We wish to determine if analogous, chemically induced cross linking affects the fate of IgE compared to monomeric IgE. The possible effect of oligomerized IgE binding to the recently described IgG Fc of basophils is also being investigated.

Project Description

Other Professional Personnel: T.J. Triche, Chief, Ultrastructural Pathology Section, LP, NCI; S.J. Mims, Biologist, LP, NCI; J. Rivera, Biologist, A&R, NIAMDD

Objectives: 1. To study the effect of IgE cross linking on its fate after cell surface binding; 2. To study internalization, catabolism, disposition, and receptor regeneration.

Methods Employed: 1. Radiolabelled IgE binding assays. 2. Electron microscopic autoradiography. 3. Statistical analysis using Salpeter's technique. 4. Digital analysis (computer assisted). 5. Protein A/gold labelling.

Major Findings: 1. Oligomerization of IgE with a chemical cross linker (dimethyl suberimidate) and subsequent binding of defined oligomers ($n \approx 2,3$, etc.) to cultured basophil leukemia cells results in time dependent internalization of these oligomers; suberimidate treated monomeric IgE, like untreated IgE, is not. 2. This internalization is temporally independent of the histamine release or exocytosis induced by these oligomers ($n \approx 3$). 3. Internalization and exocytosis are independent events, since dimerized IgE is internalized but does not elicit exocytosis. 4. Although IgE normally binds only to its receptor, oligomerized IgE displays an increased affinity for the $Fc\gamma$ receptor; oligomerized IgE also binds to the $Fc\epsilon$ receptor. Despite this, the observed internalization of oligomerized IgE is not due to $Fc\gamma$ binding, since saturation of this receptor with IgG has no effect on IgE internalization and minimal effect on IgE binding.

Significance to Biomedical Research and the Program of the Institute: These findings will help clarify the mechanism by which hypersensitivity reactions, mediated by cell bound IgE reactive with specific allergens, can be provoked after prolonged periods of time.

Publications:

Iserky, C., Rivera, J., Triche, T.J., and Metzger, H.: Characterization of the receptors for IgE on membranes isolated from rat basophilic leukemia cells. Molec. Immunol. 19: 925-941, 1982.

Iserky, C., Rivera, J., Segal, D.M., and Triche, T.J.: The fate of IgE bound to rat basophilic leukemia cells. II. Endocytosis of IgE oligomers and effect on receptor turnover. J. Immunol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00520-05 LP
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.) Surface disposition of cell bound IgE with time		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Timothy J. Triche, Chief, Ultrastructural Pathology Section, Lab. of Path., NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Ultrastructural Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 4	PROFESSIONAL: 2	OTHER: 2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Antigenic responsiveness to allergens is imparted to mast cells and basophils by specific membrane binding of allergen binding IgE. Other cells have been shown to bind ligands non-randomly, especially to microvilli (dePetris, Nature 272: 66-68, 1978). Further, cell bound IgE has been shown to survive for prolonged periods of time on the cell surface (Istersky, Rivera, Mims, and Triche, J. Immunol. 122: 1926-1936, 1979). This study attempts to determine whether the prolonged survival is related to cellular re-distribution away from sites of active endocytosis; i.e., onto microvilli.</p>		

Project Description

Other Professional Personnel: C. Isersky, Senior Investigator, A&R, NIAMDD; S.J. Mims, Biologist, LP, NCI; J. Rivera, Biologist, A&R, NIAMDD

Objectives: To determine the mechanism whereby mast cells and basophils retain cell bound IgE for prolonged periods of time.

Methods Employed: 1. Radiolabelled IgE binding studies. 2. Electron microscopic autoradiography. 3. Statistical analysis using Salpeter's technique.

Major Findings: Preliminary manual analysis indicates no preferential binding to microvilli when values are corrected for actual membrane surface area; uncorrected values suggest binding to microvilli only because of their greater membrane area. Despite this, the greater membrane area accounted for by these structures does result in greater IgE binding to microvilli.

A more detailed, computer-assisted analysis of these findings is underway. Initial results demonstrate a nearly two-fold greater density of IgE receptors on microvilli than cell surface using this more sensitive and reliable technique. A more comprehensive investigation is underway.

The time course of this binding is also being investigated.

Significance to Biomedical Research and the Program of the Institute: Understanding of the mechanism by which individuals retain immediate hypersensitivity to various haptens such as pollen and insect venom for exceedingly prolonged periods of time is hampered by an incomplete understanding of the cellular mechanisms by which IgE is retained on the cells mediating this response. This study is designed to clarify the mechanisms involved.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00545-05 LP
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.) Extracellular matrix synthesis by human tumors in vitro		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and Institute affiliation) Timothy J. Triche, Chief, Ultrastructural Pathology Section, Lab. of Path., NCI		
COOPERATING UNITS (if any) National Institute of Dental Research		
LAB/BRANCH Laboratory of Pathology		
SECTION Ultrastructural Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3	PROFESSIONAL: 3	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The type and amount of matrix proteins synthesized by human tumor cells <u>in vitro</u> appears to parallel that of cultured normal cell counterparts to some extent. We have broadened these observations to a variety of human tumors to determine whether these patterns might allow more precise categorization of the tumor's origins. In addition, we are characterizing a new matrix protein synthesized by some of these tumors. The identity, function, and molecular organization within the extracellular matrix of this component is currently unknown.</p>		

Project Description

Other Professional Personnel: A. Modesti, Visiting Fellow, LP, NCI; S. Scarpa, Visiting Fellow, LP, NCI

Objectives: We wish to study matrix protein synthesis by normal and tumor cells in vitro, in cultures established by us from patients with known disease and in lines obtained from commercial sources.

Methods Employed: These lines will be carefully evaluated for tumor cell growth, using all available techniques (chromosome analysis, density dependent growth, tumor production in nude mice, etc.). Matrix protein synthesis will be evaluated by ion exchange chromatography, slab gel electrophoresis, autoradiography of radiolabelled cells, immunoprecipitation and SDS-PAGE, and electrophoretic transfer with immunodetection.

Major Findings: 1. Five Ewing's sarcoma cell lines have been investigated and have shown a unique collagen synthetic profile. These cells produce types I, III, and IV collagen, as demonstrated by immunofluorescence and gel electrophoresis techniques. This pattern has not been observed in any other tumors or tumor cell lines studied or reported. 2. Preliminary data from osteosarcoma, chondrosarcoma, fibrosarcoma, and carcinoma cultures and, in some cases, tumors, indicated characteristic collagen type synthesis - types I, II, III, and IV, respectively. 3. Laminin and fibronectin have been detected in some tumors but not others, in patterns which reflect ectodermal or mesodermal origin of various childhood tumors. 4. A high molecular weight protein of unknown identity has been detected in several tumor lines. Its identity and function are currently being investigated.

Significance to Biomedical Research and the Program of the Institute: Study of in vitro synthesis of matrix components by human tumors will substantiate the applicability of similar studies in animal model systems. These results will 1) have bearing both on our understanding of the origin of these tumors (with obvious therapeutic implications) as well as 2) provide valuable models for the study of the synthesis and assembly of the extracellular matrix by human tissues, as well as providing isolated, purified components of human origin, whose structure and function can then be studied in detail.

Proposed Course: An appropriate array of tumors has been established in culture (sarcomas, neuroblastomas, Ewing's sarcomas, and lymphomas). Their patterns of matrix protein synthesis have been in large part determined. Some unusual constituents have been identified and these are being further characterized biochemically, immunologically, and immunoelectron microscopically. It appears that some constituents such as laminin are similar to but uniformly distinct from their counterparts in animal tumors, and even from one human tumor to another. The biological activity of these constituents will be evaluated.

Publications:

Dickman, P.S., Liotta, L.A., and Triche, T.J.: Ewing's sarcoma: Characterization in established cultures and evidence of its histogenesis. Lab. Invest. 47: 375-382, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00874-01 LP
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.) Neuron-specific enolase in childhood tumors		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Timothy J. Triche, Chief, Ultrastructural Pathology Section, Lab. of Path., NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Ultrastructural Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3	PROFESSIONAL: 3	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews A		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The diagnosis, and thus therapy, of solid tumors of childhood is often difficult due to lack of distinguishing characteristics. This is especially true of Ewing's sarcoma, neuroblastoma, primitive soft tissue sarcomas, and (occasionally) lymphoma. We have evaluated the presence of a specific neural enzyme, neuron-specific enolase (NSE), in paraffin-embedded sections of a diverse group of solid childhood tumors, including previously unrecognized variants of neural tumors, employing immunocytochemistry with antisera to NSE. We find uniform reactivity of all neural tumors with this antibody. No cross reactivity with non-neural tumors, save a rare example of differentiated rhabdomyosarcoma, was found. We conclude that NSE is a reliable, readily detected marker in even primitive childhood tumors of neural origin. Since these tumors, in particular, appear to have an ominous prognosis outside the neonatal age group, recognition is especially important for therapeutic and prognostic purposes.</p>		

Project Description

Other Professional Personnel: M. Tsokos, Visiting Associate, LP, NCI; R.I. Linnoila, Medical Staff Fellow, LP, NCI; R. Chandra, Children's Hospital, Washington, D.C.

Objectives: To evaluate the use of a neural marker (NSE) in the diagnosis of primitive solid childhood tumor diagnosis.

Methods Employed: Paraffin sections of various childhood tumors are incubated with monospecific, anti-neuron specific enolase raised against purified rat brain enolase ($\gamma\gamma$ isomer), and detected by the Petrusz immunoenzyme bridge technique.

Major Findings: 1. Only tumors of neural crest origin are reactive with the antibody, save for an isolated instance of reactivity with only the differentiated myoblasts of a rhabdomyosarcoma. 2. Normal skeletal muscle, some smooth muscle, nerve, and liver are also reactive. This may reflect cross-reactivity with the α or β isomer subunits, found in liver and muscle, respectively. 3. All neural crest tumors evaluated so far (>20) have been reactive.

Significance to Biomedical Research and the Program of the Institute: A reliable means of identifying all neural crest tumors in ordinary tissue sections is, to date, unavailable. This technique appears to provide such means.

Proposed Course: We will evaluate more tumors and especially several tumors of unknown or disputed histogenesis.

Publications:

Tsokos, M., Linnoila, R.I., Chandra, R., and Triche, T.J.: Neuron-specific enolase in the diagnosis of neuroblastoma of other small, round-cell tumors of childhood. Hum. Pathol. (in press)

Triche, T.J., and Askin, F.: Neuroblastoma and the differential diagnosis of small, blue, round-cell tumors of childhood. Hum. Pathol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00875-01 LP
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.) Differentiation, matrix proteins, & in vitro invasiveness of human neuroblastoma		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Maria Tsokos, Visiting Associate, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Ultrastructural Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3	PROFESSIONAL: 3	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Neuroblastoma is a neoplasm known to show spontaneous histologic maturation <u>in vivo</u> which correlates with a better biologic behavior and prognosis. Extracellular matrix (ECM) proteins, on the other hand, have been shown to influence tumor invasion and metastasis. Production of ECM proteins has been previously reported only for the C1300 murine neuroblastoma cell line. We have studied ECM synthesis in relation to differentiation of human neuroblastoma <u>in vitro</u>. Differentiation was induced by dibutyl-cyclic AMP and retinoic acid. Morphologic differentiation has also been studied by electron microscopy and will be correlated with differences in biochemical expression of neurotransmitter enzymes.</p> <p>In the present project, we will assess qualitative and quantitative differences in ECM protein synthesis by neuroblastomas <u>in vitro</u> before and after differentiation by immunofluorescence and polyacrylamide gel electrophoresis. The differences will then be correlated with matrix degradation and invasion <u>in vitro</u>, employing a human amnion invasion assay. Preliminary data show that differentiating neuroblastoma is associated with increased fibronectin synthesis and decreased <u>in vitro</u> invasiveness.</p> <p>Neuroblastoma is an especially useful model of biological control of malignancy, since it has the highest rate of spontaneous regression of any tumor, and this is correlated with differentiation. Study of the expression of ECM proteins and their degradation with <u>in vitro</u> invasiveness will hopefully elucidate various aspects of the biologic control of invasion and metastasis.</p>		

Project Description

Other Professional Personnel: S. Scarpa, Visiting Fellow, LP, NCI; U.P. Thorgeirsson, Visiting Scientist, LP, NCI; L.A. Liotta, Chief, Laboratory of Pathology, NCI; T.J. Triche, Chief, Ultrastructural Pathology Section, LP, NCI

Objectives: To study biologic behavior of neuroblastomas in relation to maturation and extracellular matrix protein synthesis.

Methods Employed: Immunofluorescence in tissue cultures with antisera to matrix proteins, polyacrylamide gel electrophoresis of biosynthetically radiolabelled matrix proteins, and electron microscopy

Major Findings: 1. Human neuroblastoma produces various extracellular matrix proteins in vitro. 2. Differentiating neuroblastomas seem to produce a larger amount of fibronectin and show decreased in vitro invasiveness.

Significance to Biomedical Research and the Program of the Institute: These studies should provide insight into the biological factors involved in neuroblastoma invasion. By experimentally manipulating differentiation, the role of various matrix proteins and their degradation products, as well as cell receptors for each, can be explored.

Proposed Course: Precise quantitation of matrix protein synthesis, binding, and degradation will be correlated with in vitro invasiveness, to determine which factors are important in tumor cell invasion.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CB00884-02 LP

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.)

Ultrastructural organization of basal lamina

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

(Name, title, laboratory, and institute affiliation)

Timothy J. Triche, Chief, Ultrastructural Pathology Section, Lab. of Path., NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Ultrastructural Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

4

PROFESSIONAL:

4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

B

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

The basal lamina has been ultrastructurally characterized as a continuous electron lucent layer (the lamina lucida) adjacent to the cell surface with an overlying electron dense layer (lamina densa), which interfaces with the mesenchymal stroma (collagens and other matrix proteins). Biochemically, the basal lamina is known to contain type IV collagen, laminin, and basement membrane proteoglycan. The actual disposition of these constituents in the l. lucida-l. densa, cell surface, and matrix is uncertain, various conflicting ultrastructural studies notwithstanding. Also, the relationship of type V collagen, a so-called cell surface collagen, to the basal lamina, is unknown. We are employing high resolution (ca. 5 nm) immunoelectron microscopy on tissue sections with purified antisera to laminin, type IV collagen, and type V collagen, using appropriate controls, to precisely localize these constituents of the basal lamina and neighboring extracellular matrix.

Project Description

Other Professional Personnel: L.A. Liotta, Chief, Laboratory of Pathology, NCI; A. Modesti, Visiting Fellow, LP, NCI; S. Scarpa, Visiting Fellow, LP, NCI; T. Kalebic, Visiting Fellow, LP, NCI; S. Togo, Guest Worker, LP, NCI

Objectives: To determine the molecular organization of the basal lamina and neighboring extracellular matrix.

Methods Employed: Human amniotic membrane. 2. Antisera against lamina, type IV collagen, type V collagen, keratin (positive technique control), non-immune serum (negative control), and affinity column eluates (positive and negative controls). 3. Sized protein A-Gold complexes (4 to 12 nm range; \pm .8 nm variation in a given preparation), for regular and multiple label experiments. 4. In vitro and embedded, sectioned tissue incubations with reagents. 5. Immunoperoxidase EM studies to complement (4), above.

Major Findings: Initial data indicate that laminin is found at the cell surface and in the proximal l. densa, and to a lesser extent in the l. lucida. Type IV collagen is found in the distal l. densa. Type V collagen appears to be the fine, poorly banded fibrils between the l. densa and the large collagen fibres (types I and III), with possibly some large fibre staining as well.

Significance to Biomedical Research and the Program of the Institute: Understanding of the biological function of constituents of the extracellular matrix such as laminin, type IV collagen, and type V collagen depends on a knowledge of the molecular organization within tissues. This information is also essential to an understanding of the basic mechanism of cancer cell invasion and metastasis.

Publications:

Modesti, A., Kalebic, T., Scarpa, S., Togo, S., Liotta, L.A., and Triche, T.J.: Type V collagen in human amnion is a 12 nm fibrillar component of the basement membrane reticulum. J. Cell Biol. (in press, tentative)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00508-06 LP
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.) Immune response of CBA/N mice to oligosaccharides coupled to protein carriers		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) David A. Zopf, Chief, Biochemical Pathology Section, Lab. of Pathology, NCI		
COOPERATING UNITS (if any) Aftab Ahmed, Merck Institute, Rahway, New Jersey		
LAB/BRANCH Laboratory of Pathology		
SECTION Biochemical Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>CBA/N mice are an inbred strain of animals that exhibit an X-linked deficiency in immune responsiveness to certain carbohydrate antigens including dextrans. Isomaltodextrins derived by partial enzymatic or acid hydrolysis of dextran were coupled as haptens to the protein carrier keyhole-limpet hemocyanin and were used as immunogens. These glycoconjugates were used to study formation of antibodies that bind dextran in normal adult and neonatal mice and in mice with the CBA/N defect. Of particular interest are studies of the size requirements for an oligosaccharide hapten to elicit a cross-reactive antibody response to the native polysaccharide and the ontogeny of the response to the polysaccharide following immunization with a glycoconjugate.</p>		

Project Description

Other Professional Personnel: K. Stein, Senior Staff Fellow, DBP, BOB, FDA

Objectives: To investigate immune responsiveness of CBA/N and normal mice to isomaltodextrin oligosaccharide determinants coupled to a protein carrier.

Methods Employed: Isomaltodextrins are prepared by partial enzymatic or acid hydrolysis of *Leuconostoc mensenteroides* strain 512. Oligosaccharides are derivatized with β -(p-aminophenyl) ethylamine and coupled to KLH after activation to the isothiocyanate. Immune responses are monitored by iso-electric focusing of mouse sera or hemolytic plaque assays of spleen cells, and radioimmunoassays for various IgG subclasses.

Major Findings: Early studies of the anti-Dex response in mice with the CBA/N defect following immunization with IM6-KLH demonstrated that defective mice could make an anti-Dex response but that it was lower in magnitude than that of control animals. This suggested that the subset of B lymphocytes missing in the defective animals, the Lyb5⁺ cells, might be important for dextran responses even when they are stimulated by a glycoconjugate. Previous studies by others had demonstrated that this B cell subset was required for the response to dextran immunization. To test this possibility, two types of experiments were performed: 1) neonatal mice which lack Lyb5⁺ cells were immunized at various times after birth with IM6-KLH and the anti-dextran antibodies were measured and 2) a secondary adoptive transfer experiment was performed in normal adult mice in which IMG-KLH primed B cells, with or without anti-Lyb5⁺ and complement treatment, were transferred to irradiated recipients and challenged with IM6-KLH and then the anti-Dex plaque-forming cell (PFC) response was measured.

The results of these experiments demonstrated that up to one week of age mice immunized with IM6-KLH do not make anti-Dex antibodies although they do produce anti-IM6 antibodies as detected with IM6-BSA. The anti-Dex cross reactive antibodies developed in parallel with the development of Lyb5⁺ cells and reached a maximum at 3-4 weeks of age. This is considerably earlier than the response to dextran itself which does not reach a maximum until twelve weeks of age. These experiments reinforce the hypothesis that Lyb5⁺ cells are required for the response to polysaccharides even when a glycoconjugate is used to stimulate that response. They also demonstrate, however, that once Lyb5⁺ cells are present, glycoconjugates can stimulate an anti-polysaccharide response considerably earlier and of greater magnitude (100-1000 times higher titers) than polysaccharides themselves.

The adoptive transfer experiments were performed to directly assess the requirement for Lyb5⁺ cells. In those experiments spleen cells from normal adult mice, primed to IM6-KLH, were treated *in vitro* with anti-Lyb5⁺ and complement (performed in collaboration with Aftab Ahmed, Merck Institute, Rahway, N.J.) to remove the Lyb5⁺ cells or were treated with a control antiserum and complement. These cells were transferred to irradiated recipients and boosted with IM6-KLH. Seven days later the spleens were removed from the recipients and the anti-Dex PFC response was measured. These

experiments demonstrated that following removal of the Lyb5⁺ cells, the primed cells were no longer capable of making an anti-Dex response. Taken together these experiments clearly demonstrate that anti-polysaccharide responses depend on the presence of the Lyb5⁺ cells whether the response is stimulated in a thymus independent or on a thymus dependent fashion.

Significance to Biomedical Research and the Program of the Institute: The immune deficiency of CBA/N mice for response to polysaccharide antigens apparently can be circumvented when polysaccharide determinants are presented via a "T cell dependent" route. This finding opens a possible route to vaccination against bacterial organisms (e.g. pneumococcus) with surface polysaccharides that contain abundant repeating carbohydrate sequences but which may be poorly immunogenic in their native state. These studies also demonstrate that glycoconjugates stimulate 100-1000 times higher titers of antibody than the polysaccharide and significantly earlier in ontogeny than obtained using the polysaccharide. They suggest that glycoconjugates would be ideal for use as vaccines in infants that are unresponsive to polysaccharides.

Proposed Course: The above findings open the question of whether larger molecular weight dextran fragments, or even the intact molecule, might be converted to T-dependent antigens. Dextrans of various molecular weights will be conjugated to KLH and tested for immunogenicity in xid and normal mice.

Publications:

Stein, K., and Zopf, D.A., Miller, C.B., Johnson, B.M., Mongini, P.K.A., Ahmed, A., and Paul, W.E.: Immune response to a thymus-dependent form of B512 dextran requires the presence of Lyb-5⁺ lymphocytes. J. Exp. Med. 157: 657-666, 1983.

Project Description

Other Professional Personnel: M. Ugorski, Visiting Fellow, LP, NCI; P.A. Pizzo, Surgeon, PO, NCI

Objectives: 1) To determine the relationship between urinary excretion of a specific glucose-containing tetrasaccharide $(G)_4$ and altered states of glycogen metabolism. 2) To determine whether the urinary excretion rate of $(G)_4$ can be used as an indicator of tumor burden in patients with tumors that accumulate glycogen.

Methods Employed: A hybridoma antibody was raised against $(Glc)_4$ and a solid phase microtiter plate RIA developed for rapid screening of many samples.

Major Findings: 1) Patients affected with types II and III glycogenoses excrete $(G)_4$ at rates 20 to 50 times normal. Unaffected parents, who are heterozygous for the recessive trait, excrete $(G)_4$ at approximately 10 times the normal rate. Some unaffected sibs of patients excrete $(G)_4$ at the same rate as their parents while others excrete normal amounts. 2) Urinary excretion of $(Glc)_4$ was elevated in 7 of 7 patients with Ewing's sarcoma, 9 of 12 with rhabdomyosarcoma, 3 of 4 with ALL, 1 of 4 with AML, and 2 of 7 with Burkitt's lymphoma. Of 21 hospitalized non-cancer patients, 3 who had chronic urinary tract infections had elevated urinary $(Glc)_4$ while the other 18 were in the normal range.

Prolonged treatment of 25 mg rabbit liver glycogen with human salivary or pancreatic amylase gives limit dextrans that include $(G)_4$ (0.7 mg). Treatment of the higher limit dextrans (11 mg) with human plasma releases additional $(G)_4$ (0.8 mg). It appears likely that $(G)_4$ excreted in urine represent the product of the combined actions of α amylase and α glucosidase in plasma on glycogen released from cells into the circulation. The amount of $(G)_4$ normally excreted could arise from this pathway by degradation of about 0.01% of total body glycogen per 24 h.

Significance to Biomedical Research and the Program of the Institute: The radioimmunoassay for $(G)_4$ in human urine has replaced previously used direct chemical analysis which required several days workup for each sample to be analyzed by gas chromatography/mass spectrometry.

Determination of urinary excretion of $(G)_4$ by RIA is easy, noninvasive, and inexpensive. $(G)_4$ excretion appears useful as an adjunct to other conventional methods for screening family populations suspected as carriers of recessive traits related to abnormal glycogen metabolism. Elevated excretion of $(G)_4$ in patients with tumors that accumulate glycogen appears promising as an indicator of tumor burden. As there is currently no biochemical marker for sarcomas, similar to the CEA and alpha-fetoprotein markers for some carcinomas, the excretion of $(G)_4$ offers a possible biochemical assay for monitoring tumor burden in selected patients.

Proposed Course: Studies on the metabolic origin of (G)₄ have been completed. Preliminary clinical studies also have been completed. The next phase of clinical trials has been delayed during administrative reorganization of POB. A new series of prospective studies will be undertaken if and when logistically feasible.

Publications:

Ugorski, M., Seder, A., Lundblad, A., and Zopf, D.A.: Studies on the metabolic origin of a glucose-containing tetrasaccharide in human urine. J. Exp. Path. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00511-05 LP
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.) Carbohydrate heterogeneity in alpha subunits of human polypeptide hormones		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Bo Nilsson, Visiting Scientist, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any) Clinical Endocrinology Branch, NIAMDD		
LAB/BRANCH Laboratory of Pathology		
SECTION Biochemical Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Purified alpha subunits from human chorionic gonadotropin, TSH, FSH, and LH will be treated with neuraminidase and then subjected to alkaline borohydride degradation followed by trifluoroacetolysis. Oligosaccharides released by the alkaline borohydride step will be studied by gel filtration, methylation analysis and mass spectrometry of the permethylated oligosaccharide derivatives. Conditions for trifluoroacetolysis will be adjusted so as to destroy reducing amino sugars after release of oligosaccharides from chitobiosyl-asparagine linkages. Following removal of N-trifluoroacetyl groups from any remaining amino sugars in the mixture, oligosaccharides will be subjected to ion exchange chromatography to separate "high mannose" from "complex" type chains. The oligosaccharides obtained will be subjected to gel filtration chromatography, high voltage electrophoresis in borate buffer, and paper chromatography to investigate possible heterogeneity of carbohydrate chains. Fractions will be monitored by sugar analysis at each step.		
642		

Project Description

Other Professional Personnel: D.A. Zopf, LP, NCI; S.W. Rosen, Senior Investigator, CE, NIAMDD; B. Weintraub, Senior Investigator, CE, NIAMDD

Objectives: To investigate possible differences in structure of carbohydrate chains among alpha subunits of human polypeptide hormones.

Methods Employed: We are applying a recently developed method - trifluoro-acetolysis - that hydrolyses the polypeptide portion of glycoproteins but leaves carbohydrate chains intact. Conditions can be adjusted so as to selectively destroy certain sugar residues such as reducing amino sugars. Heterogeneity of sugar chains will be investigated by gel filtration, high voltage paper electrophoresis in borate buffers, and analysis of monosaccharide components by gas chromatography and mass spectrometry after conversion to their alditol acetates. Further structural analysis will be carried out if sufficient material is available. A procedure for performing methylation analysis on sugars attached to intact glycoproteins has been worked out. Using the GC/MS in a selected ion mode, the monosaccharide compositions of the human pituitary hormones TSH, FSH, and LH have been determined on 0.2 mg of starting material. Methylation analysis was carried out on 0.5 mg of starting material for each hormone.

Major Findings: 1) Each hormone contains between 20 and 35% carbohydrate by weight. 2) All carbohydrate is carried on asparagine-linked chains. 3) None of the hormones contains high mannose type oligosaccharide chains. 4) Virtually all galactosyl residues are sialylated. 5) Some chains contain terminal GlcNAc and/or GalNAc. 6) A small amount of terminal fucose is present.

Significance to Biomedical Research and the Program of the Institute:

If our examination of alpha subunits derived from different polypeptide hormones reveals differences in their patterns of glycosylation, these differences in carbohydrate structure may serve as a marker for the cell of origin for endocrine tumors that secrete alpha subunits. At present radioimmunoassays used to classify endocrine tumors on the basis of the hormones they produce identify beta subunits but cannot distinguish alpha subunits produced by different endocrine cell types. The differences in carbohydrate structures among pituitary glycoprotein hormones suggests a mechanism by which alpha chains of the various hormones could be distinguished, i.e. by specific structural features of their carbohydrate chains. These differences in glycosylation may serve as markers for the cells of origin for endocrine tumors that secrete alpha subunits. The differences in glycosylation of these hormones poses a fundamental problem: how do polypeptides synthesized within a single cell of the pituitary gland acquire different carbohydrate chains. The pituitary hormone system is the first human system in which differential glycosylation of similar glycopeptides produced by the same cell has been observed.

Proposed Course: The project is completed and a manuscript describing results is in preparation.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CB00523-04 LP

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.)

Complex carbohydrate released from mammalian cells by trifluoroacetolysis

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

(Name, title, laboratory, and institute affiliation)

David A. Zopf, Chief, Biochemical Pathology Section, Lab. of Pathology, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Pathology

SECTION

Biochemical Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

B

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

Trifluoroacetolysis is a recently-developed method that releases oligosaccharides intact from glycoproteins and glycolipids. Carbohydrate chains released as a mixture from whole tissues, tissue fractions, or cells grown in culture, are easily recovered in nearly quantitative yield and reconstituted to their native form. Analysis of the majority of oligosaccharides containing six or fewer monosaccharide units is performed by combined gas chromatography and mass spectrometry of permethylated, N-trifluoroacetylated oligosaccharide derivatives. Analysis for certain specific oligosaccharides is carried out by radioimmunoassay using antibodies produced against purified oligosaccharides coupled to polypeptide carriers. It is anticipated that the repertoire of oligosaccharide chains produced by cells or tissues will reflect states of cellular differentiation and reveal potential cell surface markers.

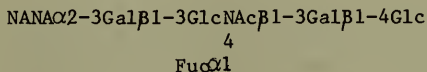
Project Description

Other Professional Personnel: B. Nilsson, Visiting Scientist, MTRB, NIDR; M. Ugorski, Visiting Fellow, LP, NCI; J. Cashel, Biologist, LP, NCI; V. Ginsburg, Senior Investigator, LBP, NIADDK

Objectives: To separate and identify the major oligosaccharide chains present in mammalian cells and to correlate the occurrence of specific oligosaccharide structures with states of cellular differentiation.

Methods Employed: Oligosaccharides are released by trifluoroacetyolysis from whole mammalian tissues, tissue fractions, or cells grown in vitro. An aliquot of the mixture of sugar chains is reconstituted and fractionated by gel filtration chromatography. Fractions are analyzed directly by radioimmunoassay. The remainder of the mixture is treated so as to leave nitrogen atoms of amino sugars trifluoroacetylated and is reduced and permethylated. This preparation is analyzed by combined gas chromatography-mass spectrometry.

Major Findings: A ganglioside identified as the target antigen of a hybridoma antibody (19.9) that specifically binds human colorectal carcinoma cells was treated with neuraminidase and subjected to trifluoroacetyolysis. The desialylated oligosaccharide was analyzed by GC/MS. In conjunction with sugar and methylation analysis, the GC/MS analysis established the following novel carbohydrate structure for the ganglioside:



Significance to Biomedical Research and the Program of the Institute: Many laboratories seeking to produce tumor-specific hybridoma antibodies for diagnostic and therapeutic trials have found that the antibodies bind complex carbohydrate determinants. Determination of the chemical structure of the 19.9 antigen provides a clear rationale for the observed fact that this antigen is lacking in individuals of blood group Le(a⁻b⁻). The 19.9 antibody is being marketed as a clinical assay for detecting antigen shed by gastrointestinal tumors into blood plasma.

Proposed Course: Investigations of oligosaccharides released from human tissues will continue using GC/MS methodology developed for this purpose. Human colorectal carcinoma cell lines will be grown to mass cultures and their glycolipids compared with the single case studied to date. An attempt will be made to segregate human chromosomes in human-mouse somatic cell hybrids in order to determine which chromosomes are required for expression of glycolipids elevated in tumor cells.

Publications:

Magnani, J.L., Nilsson, B., Brockhaus, M., Zopf, D.A., Steplewski, Z., Koprowski, H., and Ginsburg, V.: A monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-N-fucopentaose II. J. Biol. Chem. 257: 14365-14369, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00525-04 LP
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.) Analysis of oligosaccharides by combined gas chromatography-mass spectrometry		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) David A. Zopf, Chief, Biochemical Pathology Section, Lab. of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Biochemical Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.2	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Separation of reduced and permethylated oligosaccharides by gas chromatography can be facilitated by the use of a fused silica capillary column 100 meters long, coated with methyl silicon. The presence of N-acetylhexosamines in oligosaccharides increases their retention time and interferes with efficient GC separation. Transamidation of hexosamines by trifluoroacetolysis followed by reduction, removal of O-trifluoroacetyl groups and permethylation, dramatically reduces the retention time of hexosamine-containing oligosaccharides and permits separation of oligosaccharides containing up to six monosaccharide units, regardless of how many of these are hexosamines. The mass spectra of permethylated oligosaccharides with N-trifluoroacetylated amino sugars show unexpectedly high abundances of mass ions containing the N-trifluoroacetyl group. As many of these ions are large, they provide useful information regarding oligosaccharide structure.</p>		
646		

Significance to Biomedical Research and the Program of the Institute:

Structural analysis of oligosaccharides released from biological glycoconjugates usually requires purification and multiple analytical procedures to establish sugar sequence, linkage positions, and anomeric configuration. The gas chromatography/mass spectrometry method under development will permit a direct estimate of structural diversity in oligosaccharide mixtures and, in many cases, identification of oligosaccharides according to retention time and mass spectrum by comparison with standards. This approach has enabled structural analysis of compounds present in mixtures that cannot be resolved by any known alternate method. Complex carbohydrates are constituents of many biologically active molecules and play a role in many biochemical recognition events. Rapid structural analysis of these molecules is vital to obtaining an understanding of their role in biological processes.

Proposed Course: Studies have been carried out on more than thirty standard oligosaccharides derived from human milk, glycolipids, asparagine-linked chains of glycoproteins, and other sources. Additional oligosaccharides derived from human blood group substances, proteoglycans, and urine are under study.

Publications:

Nilsson, B., and Zopf, D.A.: Oligosaccharides released from glycolipids by trifluoroacetylation can be analyzed by gas chromatography-mass spectrometry. Arch. Biochem. Biophys. 222: 628-648, 1983.

Rao, A.S., Kabat, E.A., Whittaker, N.F., Nilsson, B., Zopf, D.A., and Nimmich, W.: Isolation and characterization of 3,4 pyruvic acetalated-D-galactose-containing oligosaccharide obtained from the capsular polysaccharide of *Klebsiella* K33 by Smith degradation. Carbohydr. Res. (in press)

Rao, A.S., Kabat, E.A., Nilsson, B., Zopf, D.A., Nimmich, W.: Isolation and characterization of 4-O-(3,4-O-carboxyethylidene)B-D-galactopyranosyl-erythritol. Carbohydr. Res. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00549-03 LP
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.) Hybridoma antibodies to oligosaccharide haptens		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) <i>(Name, title, laboratory, and institute affiliation)</i> Kenneth R. Schroer; Sr. Asst. Surgeon, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any) 		
LAB/BRANCH Laboratory of Pathology		
SECTION Biochemical Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">2.0</div>	PROFESSIONAL: <div style="text-align: center;">1</div>	OTHER: <div style="text-align: center;">1</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Hybridoma antibodies that bind oligosaccharides are valuable reagents for analysis, localization, and purification of free oligosaccharides and glycoconjugates. We have developed immunization protocols and screening procedures, for producing hybridomas against oligosaccharides purified from human milk and urine. Many of these oligosaccharides are structurally identical with carbohydrate chains found in naturally-occurring glycolipids and glycoproteins. Hybridoma antibodies against a glucose-containing tetrasaccharide (G)₄ with the structure Glc₁-6Glc₁-4Glc₁-4Glc have been used in a radioimmunoassay to study the metabolic origin of the tetrasaccharide. The same anti (G)₄ hybridoma antibodies have been used for affinity purification of the free oligosaccharide. We also have prepared hybridomas against the milk oligosaccharide lacto-N-tetraose whose structure is identical to the Type I blood group precursor chain. Lacto-N-tetraosyl ceramide is a precursor to an "oncofetal" ganglioside. We are investigating the possibility that hybridomas against LNT may be useful for detecting products of gastrointestinal carcinoma cells which may be enriched in Type I precursor chains.</p>		

Project Description

Other Professional Personnel: D.A. Zopf, Surgeon, LP, NCI; K. Wasniowska, Visiting Fellow, LP, NCI; and R.E. Levinson, Biologist, LP, NCI

Objectives: To examine the diversity and binding characteristics of hybridoma proteins (HP) constructed against the carbohydrate portions of glycoconjugates.

Methods Employed: A glucose-containing urinary tetrasaccharide, (G)₄, been purified, phenethylamine derivatized and conjugated to keyhole-limpet hemocyanin for use as an immunogen in mice. Synthetic glycoconjugates containing lacto-N-tetraose (LNT), lacto-N-fucopentaose III (LNFIII) and a urinary octasaccharide from patients with GM₁ gangliosidosis (GM₁ Octa) also have been employed. Following hyperimmunization, spleen and lymph node cells were fused with PEG to a murine myeloma cell line and hybrids selected with HAT medium. Hybridomas were screened for desired antibody production, cloned in vitro and passaged in vivo to generate monoclonal antibodies.

Major Findings: 1) More than 20 hybridoma antibodies that bind (G)₄ have been characterized. Two antibodies bind (G)₄ with sufficiently high affinity to permit their use as reagents in hapten displacement RIA. One of these also can be adsorbed to sepharose-staphylococcal protein A and used for affinity purification of (G)₄.

2) Hybridoma antibodies that bind either LNT or LNFIII coupled to polypeptides have been identified. Detailed characterization of their specificities is in progress.

3) Ten hybridomas that bind GM₁ Octa have been identified. These bind to asialotransferrin but not native transferrin. Some, but not all of these, also bind asialoorosomucoid and asialofetuin but not the native glycoproteins. Detailed characterization of the specificities of these hybridomas is in progress.

Significance to Biomedical Research and the Program of the Institute:

Structural analysis of important cell surface carbohydrates is greatly facilitated by use of antibodies as reagents that identify, purify and assay for specific structural determinants. Homogeneous antibodies prepared against synthetic immunogens bearing specific carbohydrate determinants provide a means to generate these reagents. Hybridoma antibodies against (G)₄ will be sought to replace currently-used rabbit serum antibodies for RIA analyses of urinary excretion of this oligosaccharide. Antibodies against LNT, LNFIII, and GM₁ Octa may prove useful for detecting "carcinoembryonic" carbohydrate chains as each of these chains has been detected in embryonic and/or tumor tissues.

Proposed Course: We will continue to evaluate the utility of hybridomas against defined oligosaccharides as tools for biochemical analysis and for identifying carbohydrate markers of cellular differentiation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01CB00556-01 LP
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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 glycolipids in lymphocyte subpopulations

PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.)
(Name, title, laboratory, and institute affiliation)
David A. Zopf, Chief, Biochemical Pathology Section, Lab. of Pathology, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0.5
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CHECK APPROPRIATE BOX(ES)
 (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews B

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

Neutral glycolipids are differentially expressed in functionally distinct subpopulations of murine lymphocytes. Subpopulations of B cells can be studied by examining hybridoma lines derived from fusion of splenic B lymphocytes with the mouse myeloma SP2/0. We are analyzing total neutral glycolipids from hybridomas by thin layer chromatography and by GC/MS analysis of oligosaccharides after trifluoroacetylation. Hybridomas from Balb/c splenocytes express glycolipids containing from two to five simple sugars. These include globoside and its precursors as well as asialo-GM₂ and 2' fucosyllactosyl ceramide. The goal of this project is to correlate expression of oligosaccharide chains of glycolipids with functional parameters of B cell subsets such as responsiveness to Type I and Type II antigens.

Project Description

Other Professional Personnel: K. Schroer, Sr. Asst. Surgeon, LP, NCI; M. Ugorski, Visiting Fellow, LP, NCI; R. Levinson, Biologist, LP, NCI; J. Cashel, Biologist, LP, NCI; J. Fernandez, Biologist, LP, NCI

Objectives: To examine the major glycolipids of hybridomas derived under conditions selective for early and late maturing B cell subsets.

Methods Employed: A mouse myeloma SP2/0 that expresses only mono- and di-hexosylceramides is fused to mouse splenocytes. The fusion products express neutral glycolipids of both the myeloma and lymphocyte parent cells. Thus, glycolipids larger than dihexosyl ceramide are contributed by the B cell parent lymphocyte. Hybridomas (10^7 cells) are labeled in vitro for 48 h with [^{14}C]Gal and [^{14}C]GlcNAc and purified total neutral glycolipids are separated by thin layer chromatography and detected by autoradiography. Selected clones are expanded to 10^9 cells and the purified total neutral glycolipids are subjected to trifluoroacetylation to release the oligosaccharide chains. The oligosaccharide mixture is reduced, permethylated, and analyzed by GC/MS.

Major Findings: 1) For all cell lines studied thus far the results of TLC analysis and GC/MS analysis agree. 2) The majority of hybridomas derived from Balb/c splenic B cells express ceramide trihexoside and globoside in addition to ceramide dihexosides. Some lines also express asialo GM₂. 3) A few hybridomas express 2'fucosyllactosyl ceramide, a glycolipid previously unknown in murine cells. 4) Hybridomas randomly selected from Con A-stimulated CBA/N splenocytes express only very small amounts of glycolipids containing more than two sugars.

Significance to Biomedical Research and the Program of the Institute: The complex interactions between cells of the immune system are mediated by numerous recognition events at cell surfaces. We are studying the major glycolipids expressed by lymphocyte subpopulations in order to determine whether any correlations can be made between the carbohydrate chains displayed on the cell surface and the functional state of the lymphocyte. Thus far, a novel glycolipid has been detected in IgM-secreting hybridomas that is not seen in hybridomas secreting antibodies of other isotypes. In addition, preliminary results suggest that B cells from xid mice (i.e. mice with an X-linked immunodeficiency in responsiveness to carbohydrate antigens) fail to express long chain glycolipids. If additional studies confirm these correlations, then specific glycolipids can be used as markers for stages of cellular differentiation and their possible roles in immunoregulatory pathways should be explored. Immunoregulation of anticarbohydrate responses will be critically important for understanding immune surveillance against tumors since many recent attempts to define oncofetal antigens by hybridoma antibodies have yielded antibodies that react with cell surface carbohydrates.

Proposed Course: Comparison of hybridomas from newborn, adult, and xid mice will be pursued. Hybridomas made against Type I and Type II antigens early and late in development will be studied. Antibodies that bind 2'fucosyllactosyl ceramide will be compared with anti μ antibodies by FACS to study the possible correlation between surface expression of these two molecules on B cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CB00559-01 LP

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 matrix receptors role in metastases

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

(Name, title, laboratory, and institute affiliation)

Lance A. Liotta, Chief, Tumor Invasion & Metastases Section, Lab. of Path., NCI

COOPERATING UNITS (if any)

Laboratory of Developmental Biology and Anomalies, NIDR

LAB/BRANCH

Laboratory of Pathology

SECTION

Tumor Invasion and Metastases Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2

PROFESSIONAL:

1.5

OTHER:

.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.)

Laminin, a major glycoprotein of basement membranes, has the molecular shape of an assymetric cross with three short arms (3 x 200 Kd) and one long arm (400 Kd), all bearing globular end domains. By enzymatic digestion, we have produced a series of laminin fragments retaining one or more molecular domains. The fragments were used to study the biologic properties of laminin relative to tumor cell attachment and metastases. Laminin was found to bind to a high affinity tumor cell surface receptor (Kd = 2×10^{-9}). The receptor is identified on both human and murine tumor cells, can be isolated from plasma membrane extracts, has a molecular weight in the range of 70 Kd and will not bind fibronectin or other serum components. A laminin fragment retaining the intact short arms, but not the long arm, binds to the receptor and mediates attachment of human and murine tumor cells to type IV collagen. A laminin fragment (C₁), which retains the intersection region of the three short arms, but not the globular end regions, binds to the receptor and blocks cell attachment *in vitro*. The laminin C₁ fragment purified from syngeneic mice when pre-incubated with BL6 melanoma cells prior to I.V. injection, markedly inhibited or abolished lung metastases formation (compared to untreated controls or cells treated with whole laminin) in a dose dependent manner in 7 separate experiments.

Project Description

Other Professional Personnel: C.N. Rao, Visiting Fellow, LP, NCI; S.H. Barsky, Expert, LP, NCI; V.P. Terranova, Research Fellow, NIDR

Objectives: a) to isolate and chemically characterize the laminin receptor; b) to produce polyclonal and monoclonal antibodies to the laminin receptor; c) to study the genetics and regulation of the receptor; and d) to identify receptors for other matrix components.

Methods Employed: Plasma membranes were isolated from cells in log phase of growth or from human breast carcinoma biopsy samples. The plasma membrane homogenate was solubilized in 0.1% Triton X 100, 1.0 - 2.0 mg protein/ml. After centrifugation at 30,000 g for 45 min, the supernate was collected and incubated with SM2 Biobeads (Bio-Rad) to remove the Triton. Iodination of the laminin ligand and the plasma membrane extract was performed using the lactoperoxidase method. Laminin receptors were measured on living cells in suspension. After trypsinization the cells were incubated in complete media under constant agitation at 37°C for 2 hr. The labeled ligand plus 250 fold excess unlabeled ligand was added and the incubation was continued for 2 hr at 25°C. The cell-bound and free ligand was separated by centrifugation. Binding assays on plasma membrane extracts were performed using one member of the ligand or receptor pair bound to solid phase nitrocellulose Millipore SCWP circles or cyanogen bromide activated Sepharose 4B. In the latter case, 25 µg of laminin or plasma membrane extract protein bound to cyanogen bromide activated Sepharose 4B (100 µl) was mixed with an equal amount of 25 mM Tris, 5 mM MgCl₂ and CaCl₂, pH 7.4 and 100 µl of this buffer containing 0.1% BSA. ¹²⁵I-labeled plasma membrane extract (10⁸ cpm/mg) or ¹²⁵I-labeled laminin (10⁹ cpm/mg) was added in a total volume of 100 µl, diluted with the buffer. Competition was performed with various concentrations of unlabeled laminin in solution or unlabeled plasma membrane extracts (2 to 20 µg). The binding assay mixture was incubated at 4°C overnight. The laminin-Sepharose beads were collected by centrifugation at 5,000 rpm for 30 min and the pellet was washed twice with 2.0 ml of the buffer containing 0.1 BSA. The proteins in the first spin supernate and the pellet were identified by electrophoresis on 7% slab gels by the method of Laemmli followed by autoradiography. Laminin affinity chromatography was performed using purified laminin cross linked to Sepharose 4B. ¹²⁵I-labeled plasma membrane extract was incubated 15 hr in the laminin-Sepharose affinity column (1 x 15 cm) at 4°C. The unbound radioactivity was washed with 40 ml of 25 mM Tris, 5 mM CaCl₂, 5 mM MgCl₂, 0.9% NaCl, pH 7.4. The bound activity was eluted with 0.2 M glycine HCl, pH 3.5, immediately neutralized with 1.0 M Tris saline, and lyophilized. The proteins were identified by slab gel electrophoresis and autoradiography. The number of laminin receptor sites and the Kd were calculated by Scatchard analysis.

Major Findings: BL6 melanoma cells, human breast carcinoma, and human squamous carcinoma exhibited saturable binding for laminin. Scatchard analysis demonstrated approximately 110,000 binding sites per cell with a high affinity: Kd = 2.2 nm. Laminin binding to the tumor cells was abolished by trypsinization. The receptor regenerated after 2 hr of cell incubation in serum free or serum containing media. Collagen, denatured laminin, fibronectin or serum did not compete for binding. Binding of ¹²⁵I laminin to isolated cell plasma membranes

also showed a high affinity: $K_d = 1.5 \text{ nm}$. Excess unbound laminin competed for binding of the solubilized membrane receptor to laminin immobilized to a solid phase. Gel electrophoresis of the solubilized membrane proteins bound to laminin before and after competition demonstrated a single molecular weight class for the receptor. Laminin affinity chromatography was therefore used to isolate the receptor with a 900-fold purification relative to the crude membrane extract. The receptor molecular weight was 67,000 after reduction by polyacrylamide gel electrophoresis. The isolated receptor retained a high binding affinity for laminin: $K_d = 2 \text{ nm}$. The syngeneic ligand fragment which inhibited binding of the tumor cell laminin receptor was shown to consistently inhibit metastases in animal models.

Significance to Biomedical Research and the Program of the Institute: a) A pharmacologic agent which blocks the laminin receptor could be clinically useful in treatment of metastatic disease; b) Measurement of laminin receptors in biopsy specimens of human breast cancer could be of diagnostic usefulness.

Proposed Course: a) to develop monoclonal antibodies to the isolated receptor; b) to correlate laminin receptor content with disease stage; and c) to sequence the receptor binding domain on laminin.

Publications:

Terranova, V.P., Liotta, L.A., Russo, R.G., and Martin, G.P.: Role of laminin in the attachment and metastasis of murine tumor cells. Cancer Res. 42: 2265-2269, 1982.

Giavazzi, R., Liotta, L.A., and Hart, I.: Laminin inhibits the adhesion of a murine tumor of macrophage origin. Exp. Cell Res. 140: 315-322, 1982.

Rao, C.N., Margulies, I.M.K., Tralka, T.S., Terranova, V.P., Madri, J.A., and Liotta, L.A.: Isolation of a subunit of laminin and its role in molecular structure and tumor cell attachment. J. Biol. Chem. 257: 9740-9744, 1982.

Rao, C.N., Margulies, I.M.K., Goldfarb, R.H., Woodley, D.T., Madri, J.A., and Liotta, L.A.: Differential proteolytic susceptibility of laminin alpha and beta subunits. Arch. Biochem. Biophys. 219: 65-70, 1982.

Terranova, V.P., Rao, C.N., Kalebic, T., Margulies, I.M.K., and Liotta, L.A.: Laminin receptor on human breast carcinoma cells. Proc. Natl. Acad. Sci. USA 80: 444-448, 1983.

Schiffmann, E., Geetha, V., Pencev, D., Warabi, H., Mato, J., Hirata, F., Brownstein, M., Manjunath, R., Mukherjee, A., Liotta, L.A., and Terranova, V.P.: Adherence and regulation of leukotaxis. 1st International Conference on Inflammation and Chemotaxis, Gersan, Switzerland, May, 1982. Proceedings chapter (in press)

Liotta, L.A., Rao, C.N., Thorgeirsson, U.P., Russo, R., and Terranova, V.P.: Tumor cell attachment and degradation of basement membranes. Proceedings of 36th Annual Symposium on Fundamental Cancer Research "Cancer Invasion and Metastases", book chapter (in press)

Rao, C.N., Barsky, S.H., Terranova, V.P., and Liotta, L.A.: Isolation of a tumor cell laminin receptor. Biochem. Biophys. Res. Commun. 111: 804-808, 1983.

Liotta, L.A., Rao, C.N., and Barsky, S.H.: Laminin receptors on human breast carcinoma: Role in invasion of the extracellular matrix. Book chapter (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00562-01 LP
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.) Somatic cell genetics of tumor cell invasion		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Unnur P. Thorgeirsson, Visiting Scientist, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Tumor Invasion and Metastases Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.5	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Only a small fraction of tumor cells is capable of passing through connective tissue barriers and forming metastatic foci. A quantitative <u>in vitro</u> tumor invasion assay using human amnion has made it possible to assess how external factors as well as normal cellular functions affect the invasive behavior of tumor cells. M5076 cells, derived from murine reticulum cell sarcoma, pass spontaneously through the amnion in significant numbers within 24 hours. When protein synthesis is blocked with cycloheximide, the number of invasive M5076 cells is reduced 82%. On the contrary, inhibition of DNA synthesis and cell proliferation has no effect on their invasiveness. </p> <p> The present project is focused on the genetic mechanism by which tumor cells penetrate connective tissue barriers. In an attempt to identify the genes responsible for the invasive behavior, NIH 3T3 cells and amnion epithelial cells were transfected with purified whole DNA extracted from various tumor cell lines. Tumors were formed in nude mice inoculated with 3T3 cells transfected with DNA from murine reticulum cell sarcoma (M5076) and DNA from human breast carcinoma (MCF-7). The <u>in vitro</u> amnion system is being used to select for transformed invasive cells. A second approach to examine whether the invasive behavior can be genetically transmitted is to use fused cell lines. Tumor cells of high (F10 melanoma) and low (2237 fibrosarcoma) metastatic potential were fused with benign neoplastic cells and normal cells. The present work includes comparing invasiveness and proteolytic activity of the fused lines with the parent lines. </p> <p> Besides using the amnion BM as an <u>in vitro</u> invasion model, it can be used as a growth substrate. Human and bovine endothelial cells grow well on the BM. Normal rat hepatocytes attach much better to the amnion BM than to any of the five types of collagen tested. We propose that this native BM substrate be used to study differentiation of various epithelial cells which normally reside on basement membranes. </p>		

Project Description

Other Professional Personnel: L.A. Liotta, Chief, LP, NCI; T. Turpeeniemi-Hujanen, Visiting Fellow, LP, NCI

Objectives: 1. To study which cellular functions such as protein synthesis, DNA synthesis and cell proliferation are required during tumor cell invasion.

2. To examine whether invasive behavior can be transmitted from malignant to benign cells through cell fusion and transfection.

3. To identify cellular genes which control the invasive character of tumor cells.

Methods Employed: 1. In vitro tumor invasion assay using human amnion divested of its epithelial layer.

2. Phenol extraction and DNA precipitation of DNA from tumor cell lysates.

3. Transfection of NIH 3T3 cells growing in petri dishes and on the amnion with tumor cell DNA using calcium phosphate precipitation method. Normal amnion epithelial cells in situ are also being transfected with whole DNA from tumor cell lines.

4. Type IV collagenase assay using as substrate ¹⁴C proline labeled type IV collagen. Proteolytic activity of culture supernatants and cell membranes is measured.

5. Subcutaneous inoculation of transfected 3T3 cells into nude mice.

6. Culturing of tumor cells and treatment with non-toxic concentrations of inhibitors; cycloheximide for protein synthesis, aphidicolin for DNA synthesis and sodium butyrate for cell proliferation.

Major Findings:

1. Penetration of M5076 through the basement membrane and collagenous stroma of the amnion was measured in the presence of inhibitors to protein synthesis, DNA synthesis and cell proliferation. When protein synthesis was almost completely blocked with cycloheximide, the number of M5076 cells passing through the membrane was reduced 82%. Inhibition of DNA synthesis had no effect on the invasive capacity of the tumor cells. The non-proliferating butyrate-treated cells were arrested in the G₁ phase of the cell cycle. Their invasiveness in the amnion as well as their type IV proteolytic activity was slightly increased with butyrate treatment.

2. NIH 3T3 cells transfected with whole DNA from M5076 cells and MCF-7 cells produced tumors in nude mice.

3. The amnion BM is used as a substrate for growing various normal and neoplastic cells.

Significance to Biomedical Research and the Program of the Institute: Since most cancer-related deaths are caused by metastases, it is of fundamental importance to understand the pathogenesis of tumor invasion. Although the *in vitro* amnion invasion assay does not replace the *in vivo* situation, it measures one of the most important steps in the metastatic process, i.e. penetration of tumor cells through the basement membrane. Our observations using invasion of M5076 cells in the amnion assay indicate that non-proliferating tumor cells can actively invade host connective tissues. This may be of clinical significance since most chemotherapeutic agents used today are directed against cell proliferation. The goal of this project is to understand the genetic control of tumor invasion which could lead to therapeutic strategies designed specifically to inhibit invading tumor cells.

Proposed Course: 1. To continue genetic studies on fused tumor lines. It will be tested in the amnion invasion assay whether the invasive behavior of the highly metastatic melanoma cells can be transferred to the benign fibroadenoma cells. A second parameter of aggressive behavior i.e. production of type IV collagenase will be measured in both parent and fused lines.

2. To continue transfection of 3T3 cells and normal amnion epithelial cells with DNA from different tumor lines. DNA will be extracted from tumors already produced by the MCF-7 DNA transfected 3T3 cells and human DNA sequences will be looked for by hybridization studies. If the transfected 3T3 cells contain human DNA sequences and show the invasive behavior, the goal is to sequence the human DNA and eventually clone the genes which control the invasive process.

3. To study if type IV collagenase production by tumor cells is related to cell attachment to specific substrates. Membrane-bound and secreted type IV collagenolytic enzyme will be measured in bladder carcinoma (T24) cells attached to type IV collagen, laminin and amnion BM.

Publications:

Thorgeirsson, U.P., Liotta, L.A., Margulies, I.M., Thomas, K., Rios-Candelore, M., and Russo, R.G.: Effect of natural protease inhibitors and a chemoattractant on tumor cell invasion *in vitro*. J. Natl. Cancer Inst. 69: 1049-1054, 1982.

Liotta, L.A., Thorgeirsson, U.P., and Garbisa, S.: Role of collagenases in tumor cell invasion. Cancer Metastasis Reviews 1: 277-288, 1982.

Russo, R.G., Thorgeirsson, U.P., and Liotta, L.A.: In Vitro Quantitative Assay of Invasion Using Human Amnion. In Liotta, L.A., and Hart, I.R. (Eds.): Tumor Invasion and Metastasis. The Hague, Martinus Nijhoff, 1982, pp. 173-187.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB08247-05 LP (formerly 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.) Basement membrane degradation by normal and neoplastic cells		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Lance A. Liotta, Chief, Tumor Invasion & Metastases Section, Lab. of Path., NCI		
COOPERATING UNITS (if any) Laboratory of Developmental Biology and Anomalies, NIDR, and Laboratory of Chemistry, NIAMDD		
LAB/BRANCH Laboratory of Pathology		
SECTION Tumor Invasion and Metastases Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>There are at least five genetically distinct collagen types whose degradation may be controlled independently. The initial step of collagen degradation is performed by collagenase. We were the first to find that type IV basement membrane collagen and type V collagen is not degraded by human skin collagenase suggesting that a separate collagenase may degrade types IV and V collagen. A collagenase which preferentially degrades type IV collagen has been derived from metastatic tumor cells and from mammary epithelium. This collagenase has been purified 1000-fold and its cleavage products have been partially characterized by rotary shadowing electron microscopy. We are further studying the secretion rate of this enzyme by a wide variety of cell types both normal and malignant. A collagenase which preferentially degrades type V collagen has been identified and purified from metastatic tumor cells. Membrane-associated forms of these enzymes have been discovered in migrating endothelial cells responding to a chemoattractant. Polyclonal monospecific antibodies to the type IV collagenase have been prepared. These antibodies react with human breast carcinoma cells in tissue sections. The collagenolytic susceptibility of 1α 2α 3α (a new type of cartilage collagen) has been elucidated.</p>		

Project Description

Other Professional Personnel: T. Kalebic, Visiting Fellow, LP, NCI

Objectives: The objectives of this project are to purify and characterize type IV collagen and type V collagen degrading enzymes and to study their biological significance in tumor invasion and metastases, angiogenesis, embryology and diabetes.

Methods Employed: Crude collagenase is obtained from a) serum-free cultures of highly metastatic mouse tumor, b) serum-free culture of minced involuting mammary gland, c) serum-free cultures of cultured human breast carcinoma, d) cultures of rat mammary ducts and alveoli, and e) migrating endothelial cells. The enzyme activity is precipitated with ammonium sulfate and purified by HPLC molecular sieve and collagen affinity chromatography. Collagenase activity is studied on labeled and unlabeled purified collagens I, II, III, IV, V and $\alpha 2\alpha 3\alpha$. Degradation products are studied by gel electrophoresis and electron microscopy. Polyclonal and monoclonal antibodies to the enzymes are prepared by standard methods. In order to investigate degradation of basement membrane type components by migrating normal and tumor cells, we developed a simple new quantitative assay *in vitro*. ^{14}C -labeled substrate is bound to nitrocellulose filters in the presence of cold carriers. The filter is placed into a chemotactic chamber. The test cells are placed on one side and the attractant is placed on the opposite side.

Major Findings: Metalloproteases have been extracted from the media of cultured metastatic tumor cells and purified approximately 8000 times after sequential ammonium sulfate fractionation, concanavalin A column chromatography, and molecular sieve chromatography and affinity chromatography. A type IV collagenase has an apparent molecular weight of 70-80,000, is inactive at acid pH, requires trypsin activation, and is inhibited by ethylenediamine tetracetic acid, but not phenylmethyl-sulfonyl fluoride, *n*-ethyl maleimide, or soybean trypsin inhibitor. The enzyme produces specific cleavage products for both chains of type IV collagen isolated without pepsinization and apparently cleaves at one point in a major pepsin extracted chain of placenta type IV collagen. A type V collagenase was purified 1000-fold and found to have a molecular weight of 80 Kd.

A wide variety of human and rodent cells were studied for type IV and type V collagen degrading activity. Inflammatory cells endothelial cells, epithelial cells but not normal connective tissue cells exhibited enzyme activity. Metastatic tumor cells secreted enzyme activity in proportion to their ability to produce spontaneous metastases *in vivo*. Antibodies to type IV collagenase consistently inhibited metastases from *i.v.* injected tumor cells.

Latent collagenase was present in tumor interstitial fluid. Collagenase activity was enhanced in MCF-7 breast carcinoma cultured with insulin.

Our findings suggest that increasing collagenolytic type IV activity is associated with increasing migratory activity of cells. Collagenase type IV exists in a membrane bound form in normal and tumor cells. While normal cells

do not secrete the enzyme during the growth in vitro, tumor cells secrete significant amounts of enzyme into the media.

Significance to Biomedical Research and the Program of the Institute: Breakdown of basement membranes occurs during the transition from in situ to invasive carcinoma and during penetration of vessel walls by metastasizing tumor cells. The basement membrane collagen degrading enzyme identified and purified in this project may play a role in the cancer invasion process. Detection of the enzyme may be a means of predicting the metastatic potential of a tumor. Furthermore, human breast carcinoma cells have been shown to secrete latent collagenase which degrades stromal type I collagen. Hence the specificity of different collagenase species elaborated by tumor cells may influence their pattern of invasion.

Proposed Course: a) Purification of type IV collagen degrading enzyme to homogeneity using HPLC and isoelectric focusing; b) Purifying additional antibodies against this enzyme; c) Use of the enzyme to elucidate the structure of type IV collagen; d) The effect of type IV protease on the morphologic structure of the basement will be studied by electron microscopy; e) The role of other serine, thiol and cytein proteases on the degradation of basement membranes are under study.

Publications:

Salomon, D.S., Liotta, L.A., Rennard, S., Foidart, J.M., Terranova, V.P., and Yaar, M.: Stimulation by retinoic acid of synthesis and turnover of basement membrane in mouse embryonal carcinoma-derived endoderm cells. Collagen Rel. Res. 2: 93-110, 1982.

Liotta, L.A., Kalebic, T.A., Reese, C., and Mayne, C.: Protease susceptibility of HMW, 1a, 2a, but not 3a cartilage collagens are similar to type V collagen. Biochem. Biophys. Res. Commun. 104: 500-506, 1982.

Tryggvason, K., Pihlajaniemi, T., Salo, T., Liotta, L.A., and Kivirikko, K.I.: Studies on the Biosynthesis and Degradation of Type IV Procollagen. In Kuehn, K., Schoene, H., and Timpl, R. (Eds.): New Trends in Basement Membrane Research. New York, Raven Press, 1982, pp. 187-193.

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Thorgeirsson, U.P., Liotta, L.A., Kalebic, T., Margulies, I.M.K., Thomas, K., Rios-Candelore, M., and Russo, R.G.: Effect of natural protease inhibitors and a chemoattractant on tumor cell invasion in vitro. J. Natl. Cancer Inst. 69: 1049-1054, 1982.

Siegel, 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.

Rao, C.N., Margulies, I.M.K., Goldfarb, R.H., Woodley, D.T., Madri, J.A., and Liotta, L.A.: Differential proteolytic susceptibility of laminin alpha and beta subunits. Arch. Biochem. Biophys. 219: 65-70, 1982.

Salo, T., Liotta, L.A., Keski-Oja, J., Turpeenniemi-Hujanen, T., and Tryggvason, K.: Secretion of basement membrane collagen degrading enzyme and plasminogen activator by transformed cells - role in metastasis. Int. J. Cancer 30: 669-673, 1982.

Schiffmann, E., Geetha, V., Pencev, D., Warabi, H., Mato, J., Hirata, F., Brownstein, M., Manjunath, R., Mukherjee, A., Liotta, L.A., and Terranova, V.P.: Adherence and regulation of leukotaxis. 1st International Conference on Inflammation and Chemotaxis, Gersan, Switzerland, May, 1982. Proceedings chapter (in press)

Seppa, S.T., Seppa, H.E.J., Liotta, L.A., Glaser, B.M., Martin, G.R., and Schiffmann, E.: Cultured tumor cells produce chemotactic factors specific for endothelial cells: A possible mechanism for tumor-induced angiogenesis. Tumor Invasion and Metastases (in press)

Kalebic, T., Garbisa, S., Glaser, B., and Liotta, L.A.: Migrating endothelial cells degrade basement membrane collagen. Science (in press)

Salo, T., Liotta, L.A., and Tryggvason, K.: Purification and characterization of a murine basement membrane collagen-degrading enzyme secreted by metastatic tumor cells. J. Biol. Chem. 258: 3058-3063, 1983.

Liotta, L.A., Rao, C.N., Thorgeirsson, U.P., Russo, R., and Terranova, V.P.: Tumor cell attachment and degradation of basement membranes. Proceedings of 36th Annual Symposium on Fundamental Cancer Research "Cancer Invasion and Metastases", book chapter (in press)

Salomon, D.S., Liotta, L.A., Panneerselvam, M., Terranova, V.P., Sahai, A., and Fehnel, P.: Analysis of basement membrane synthesis and turnover in mouse embryonal and human A431 epidermoid carcinoma cells in serum-free medium. Methods in Enzymology (in press)

Barsky, S.H., Togo, S., Garbisa, S., and Liotta, L.A.: Type IV collagenase immunoreactivity in invasive breast carcinoma. Lancet (I) 8319: 296-297, 1983.

Barsky, S.H., Baker, A., Siegal, G.P., Togo, S., and Liotta, L.A.: Use of anti-basement membrane antibodies to distinguish blood vessel capillaries from lymphatic capillaries. Am. J. Surg. Pathol. (in press)

Barsky, S.H., Siegal, G.P., Jannotta, F., and Liotta, L.A.: Loss of basement membrane components by invasive tumors but not by their benign counterparts. Lab. Invest. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CB08266-03 LP (formerly 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 and function of basement membrane molecules		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Lance A. Liotta, Chief, Tumor Invasion & Metastases Section, Lab. of Path., NCI		
COOPERATING UNITS (if any) NIDR, Laboratory of Developmental Biology and Anomalies		
LAB/BRANCH Laboratory of Pathology		
SECTION Tumor Invasion and Metastases Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The structure, chemical characteristics, and biologic function of purified components of the basement membrane were studied by rotary shadowing electron microscopy. Purified protease-derived fragments of laminin, type IV collagen, fibronectin, type V collagen and basement membrane proteoglycan were studied for a) biologic function, b) binding properties with other basement membrane molecules, and c) physical and chemical properties. A model was proposed for the structure of laminin and type IV collagen and which domains of these molecules mediate various biologic functions. The carbohydrate domains on the laminin molecule were mapped. The location of the type IV collagen, laminin receptor, and proteoglycan binding domains on laminin was determined. A model was proposed for the chemical and structural organization of the basement membrane.</p>		

Project Description

Other Professional Personnel: C.N. Rao, Visiting Fellow, LP, NCI;
I.M.K. Margulies, Biologist, LP, NCI

Objectives: 1. To prepare purified protease-derived fragments of basement membrane components; 2. To use these fragments to study the structure and function of basement membrane molecules.

Methods Employed:

1. Purification of laminin and laminin fragments

Laminin was purified from 0.5 M NaCl extracts of mouse EHS tumor by DEAE cellulose and agarose A 5M column chromatography and stored frozen in phosphate-buffered saline.

Protease-derived fragments of laminin were purified and examined by electron microscopy. The alpha fragment generated by digestion with α -thrombin lacks the long arm but retains the three short arms with globular end regions. The C₁ and P₁ fragments generated by chymotrypsin and pepsin, respectively, lack both the long arm and the globular end regions of the short arms. The C₁ fragment has a "T" structure with arm lengths of 32 nm. The P₁ fragment is similar in appearance but has shorter arm lengths (26 nm). Homogeneity of laminin and the three fragments was verified by electrophoresis on 5% slab gels in the presence of 0.5 M urea.

2. Iodination of laminin and laminin fragments

Laminin and the three fragments were iodinated by the lacto-peroxidase method. The reagents and the unreacted iodine were removed from the iodinated proteins by molecular sieve chromatography.

3. Binding assay

The GS I-B₄ isolectin and the GS II lectin were isolated from Griffonia simplicifolia seeds by the method of Delmotte and Goldstein. PHA lectins, concanavalin A, wheat germ agglutinin, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine were purchased from Sigma Chemical Company. Lima bean lectins I, II and III were isolated by the procedure of Roberts and Goldstein. The Dolichos biflorus lectin was the gift of Dr. Marilyn Etzler, University of California, Davis. The Datura stramonium lectin was prepared by the procedure of Crowley and Goldstein. Methyl α -D-galactopyranoside, methyl β -D-galactopyranoside, methyl α -D-mannoside, methyl α -D-glucoside and methyl β -D-glucoside were gifts from Dr. Gilbert Ashwell (NIADK, NIH). Nitrocellulose SCWP 8 μ m filters and other types of filters were purchased from the Millipore Corporation (Bedford, Massachusetts). The binding of laminin and laminin fragments to lectins or type IV collagen immobilized on nitrocellulose was carried out as follows:

Nitrocellulose filters (13 mm dia. 8 micron pore size Millipore SCWP type) were saturated with 20 μ l of the lectin in phosphate-buffered saline (+ calcium + magnesium) pH 7.4 (PBS). Following lectin binding to the nitrocellulose, the filters were immersed in a blocking solution of 3% bovine serum albumin (BSA/PBS: 2 x 90 min) and rinsed with PBS (2 x 30 min). The blocked filters were saturated with 125 I-labeled whole laminin or the laminin fragments and incubation was conducted in a humidified chamber at 25°C for 30 min. The filters were washed with PBS (2 x 45 min) and placed in a Packard Gamma Counter to measure the bound radioactivity. The radioactivity bound to NC filters blocked with 3% BSA alone was subtracted as background (8-15%) to give the final value for bound radioactivity. Carbohydrate specificity of the lectin-laminin binding was studied by two means: In the first method, the laminin-lectin binding reaction was conducted in the presence of excess haptenic sugars and in the second method, the haptenic sugars were added at the end of the binding reaction and the radioactivity was eluted from the filters over 18 hr at 25°C.

Major Findings:

I. The nature and location of carbohydrate moieties on the laminin molecule were identified by studying the binding affinity of a series of lectins for purified, protease-derived fragments of laminin. Laminin is a cross-shaped molecule containing 3 short arms (36 nm) and one long arm (76 nm). All arms contain globular end regions by electron microscopy. Purified fragments of laminin were obtained which a) lacked the long arm of the molecule but retained the intact short arms, or b) lacked both the long arm and the globular end regions of the short arms. These two types of fragments differed markedly in lectin binding capacity. Using the known sugar specificities of the lectins and hapten sugar competition for lectin binding to laminin fragments, we conclude the following:

- a) α -D-galactosyl end groups are markedly enriched in the globular end regions of the short arms compared to the rod-shaped portions of the molecule.
- b) α -D-mannopyranosyl residues are present on both the globular end regions and the rod-shaped portions of the molecule.
- c) Exposed N-acetyl-D-galactosaminy end groups are absent or present in low amounts on laminin.
- d) (NANA)-(2 \rightarrow 6)- β -D-Gal-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 2)-D-Man-terminated oligosaccharide units are enriched on the rod-shaped regions of the short arms compared to the globular end regions.

II. The location of the type IV collagen, laminin receptor, and proteoglycan binding domains on laminin was determined. Laminin is a cross-shaped molecule with three short arms and one long arm. All arms have globular end regions. The globular end regions of the short arms contain the type IV collagen binding domain. The long arm contains the proteoglycan binding domains. The intersection of the short arms contains the receptor binding domain.

Significance to Biomedical Research and the Program of the Institute: Invasive neoplasia is accompanied by profound changes in the structure and amount of basement membranes. Study of the structure of basement membrane components and their interaction with cells may lead to therapeutic approaches which involve modification of tumor cell interaction with basement membranes.

Proposed Course: 1) to chemically characterize the binding domains of laminin; 2) to characterize the binding domains on the type IV collagen molecule; 3) to study the assembly of basement membranes in vitro; and 4) to visualize (by EM) the binding of laminin, type IV collagen and proteoglycan together.

Publications:

Liotta, L.A., Garbisa, S., and Tryggvason, K.: Biochemical Mechanisms Involved in Tumor Cell Penetration of the Basement Membrane. In Liotta, L.A., and Hart, I.R. (Eds.): Tumor Invasion and Metastasis. The Hague, Martinus Nijhoff, 1982, pp. 319-333.

Yaar, M., Foidart, J.M., Rernard, S., Brown, K., Martin, G.R., and Liotta, L.A.: Goodpasture-like syndrome induced by antibodies to basement membrane components. Am. J. Pathol. 107: 79-86, 1982.

Kidwell, W.R., Salomon, D.S., Liotta, L.A., Zweibel, J.A., and Bano, M.: Effects of Growth Factors on Mammary Epithelial Cell Proliferation and Basement-membrane Synthesis. In Sato, G.H., Pardee, A.B., and Sirbasku, D.A. (Eds.): Growth of Cells in Hormonally Defined Media. Cold Spring Harbor Conferences on Cell Proliferation. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982, vol. 9, pp. 807-818.

Terranova, V.P., Liotta, L.A., Russo, R.G., and Martin, G.P.: Role of laminin in the attachment and metastasis of murine tumor cells. Cancer Res. 42: 2265-2269, 1982.

Giavazzi, R., Liotta, L.A., and Hart, I.: Laminin inhibits the adhesion of a murine tumor of macrophage origin. Exp. Cell Res. 140: 315-322, 1982.

Rao, C.N., Margulies, I.M.K., Tralka, T.S., Terranova, V.P., Madri, J.A., and Liotta, L.A.: Isolation of a subunit of laminin and its role in molecular structure and tumor cell attachment. J. Biol. Chem. 257: 9740-9744, 1982.

Shibata, S., Peters, B., Roberts, D., Goldstein, I., and Liotta, L.A.: Isolation of laminin by affinity chromatography on immobilized Griffonia simplicifolia I lectin. FEBS Lett. 142: 194-198, 1982.

Rao, C.N., Margulies, I.M.K., Goldfarb, R.H., Woodley, D.T., Madri, J.A., and Liotta, L.A.: Differential proteolytic susceptibility of laminin alpha and beta subunits. Arch. Biochem. Biophys. 219: 65-70, 1982.

Martin, G.R., Raurbach, D., Terranova, V.P., and Liotta, L.A.: Structure, function and pathology of basement membranes. Am. J. Pathol. (in press)

Foidart, J.M., Yaar, M., Figueroa, A., Wilk, A., Brown, K.S., and Liotta, L.A.: Abortion in mice induced by intravenous injections of antibodies to type IV collagen or laminin. Am. J. Pathol. 110: 346-357, 1983.

Terranova, V.P., Rao, C.N., Kalebic, T., Margulies, I.M., and Liotta, L.A.: Laminin receptor on human breast carcinoma cells. Proc. Natl. Acad. Sci. USA 80: 444-448, 1983.

Kleinman, H.K., McGarvey, M.L., Liotta, L.A., Robey, P.G., Tryggvason, K., and Martin, G.R.: Isolation and characterization of type IV procollagen, laminin, and heparin sulfate proteoglycan from the EHS sarcoma. Biochemistry 21: 6188-6192, 1982.

Salomon, D.S., Liotta, L.A., Panneerselvam, M., Terranova, V.P., Sahai, A., and Fehnel, P.: Analysis of basement membrane synthesis and turnover in mouse embryonal and human A431 epidermoid carcinoma cells in serum-free medium. Methods in Enzymology (in press)

Liotta, L.A., Rao, C.N., and Barsky, S.H.: Laminin receptors on human breast carcinoma: Role in invasion of the extracellular matrix. Book chapter (in press)

Salomon, D.S., Liotta, L.A., Foidart, J.-M., and Yaar, M.: Synthesis and Turnover of Basement Membrane Components by Mouse Embryonal Carcinoma Cells in Serum-free, Hormone-supplemented Medium. In Sato, G.H., Pardee, A.B., and Sirbasku, D.A. (Eds.): Growth of Cells in Hormonally Defined Media. Cold Spring Harbor Conferences on Cell Proliferation. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982, vol. 9, pp. 203-207.

Salomon, D.S., Liotta, L.A., Rennard, S., Foidart, J.M., Terranova, V.P., and Yaar, M.: Stimulation by retinoic acid of synthesis and turnover of basement membrane in mouse embryonal carcinoma-derived endoderm cells. Collagen Rel. Res. 2: 93-110, 1982.

Barsky, S.H., Siegal, G.P., Jannotta, F., and Liotta, L.A.: Loss of basement membrane components by invasive tumors but not by their benign counterparts. Lab. Invest. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00877-01 LP
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 desmoplasia: A study of the collagenous response to tumor invasion		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Sanford H. Barsky, Expert, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology, NCI		
SECTION Tumor Invasion and Metastases Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p style="margin-left: 40px;"> The study is designed to biochemically characterize the dense collagenous response to tumor invasion and by doing so, gain insight into the nature and purpose of this host response. Human breast cancer, because of its accessibility and because of its characteristic scirrhous or desmoplastic qualities will be the main tissue of investigation, but the study will be extended to other invasive tumors, which are, and are not associated with a desmoplastic response. </p>		

Project Description

Other Professional Personnel: T. Kalebic, Visiting Fellow, LP, NCI; S. Togo, Guest Worker, LP, NCI; C.N. Rao, Visiting Associate, LP, NCI; L.A. Liotta, Chief, Tumor Invasion and Metastases Section, LP, NCI

Objectives: 1. Characterize the collagenous types found in human breast cancer desmoplasia, determine their source and the stimulus to their formation. 2. Study the cellular composition of desmoplasia. 3. Investigate the presence of inhibition to invasion in tumor desmoplastic tissue

Methods Employed: Collagen extractions of desmoplastic (scirrhous) breast carcinoma were carried out by pepsin extraction, salt fractionation, and polyacrylamide gel electrophoresis. Desmoplastic tissue was studied morphologically by electron microscopy. Explants of desmoplastic breast tissue were cultured in RPMI media supplemented with 10% FCS. Extracts of desmoplastic tissue were studied for the presence of inhibitory activity to type IV collagenase. All extracts were studied for mitogenic effect on human fibroblasts and myofibroblasts, cells obtained from the explants of desmoplastic breast carcinoma.

Major Findings: 1. Desmoplasia of human breast cancer has a characteristic profile with marked increase in type V collagen. 2. The type V collagen is produced by myofibroblasts recruited by tumor-associated soluble factors. 3. Myofibroblasts are the predominant cell which can be cultured from explants of human breast carcinoma.

Significance to Biomedical Research and the Program of the Institute: Although tumor desmoplasia has been recognized for centuries, the mechanism of this phenomenon has been unknown. This work is the first to identify any biochemical differences in desmoplastic connective tissue and to develop a comprehensive theory explaining the mechanism of desmoplasia.

Proposed Course: a) to search for soluble factors elaborated by the invasive tumor cells which stimulate the recruitment of myofibroblasts and the elaboration of type V collagen; b) to search for natural protease inhibitors to type IV collagenase in desmoplastic tissue as a possible host defense mechanism to tumor invasion.

Publications:

Barsky, S.H., Rao, C.N., Grotendorst, G., and Liotta, L.A.: Increased type V collagen in desmoplasia of human breast carcinoma. Am. J. Pathol. 108: 276-283, 1982.

Liotta, L.A.: Tumor extracellular matrix. Lab. Invest. 47: 112-113, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00878-01 LP
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.) Human breast cancer laminin receptor: measurement and role in metastases		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Sanford H. Barsky, Expert, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Tumor Invasion and Metastases Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>We have demonstrated that certain murine tumors, eg. BL6 melanoma, contain cell surface receptors for the basement membrane glycoprotein laminin and that metastases can be increased by preincubation of BL6 cells with laminin followed by intravenous injection in C57 mice. Conversely, preincubation of these same cells with a laminin fragment (C1) containing the receptor binding site, but lacking the globular end regions necessary for binding to vascular basement membrane, inhibits metastases.</p> <p>Human breast epithelium rests on a laminin containing basement membrane, whereas invasive breast carcinoma loses its extracellular basement membrane. We have demonstrated an exposed laminin receptor in human breast carcinoma and localized it to the cell surface of the invasive tumor cells.</p>		

Project Description

Other Professional Personnel: C.N. Rao, Visiting Associate, LP, NCI;
L.A. Liotta, Chief, Tumor Invasion and Metastases Section, LP, NCI

Objectives: The laminin receptor may mediate metastasis in human breast carcinoma and therefore its presence, characterization, and measurement may have clinical usefulness. Furthermore, the concept of an exposed laminin receptor on invasive cells may serve to selectively "target" them with either the C1 fragment which blocks invasion and metastasis or a laminin molecule coupled to a chemotherapeutic agent.

Methods Employed: 1. The laminin receptor will be measured in individual samples of human breast carcinoma and correlated with available clinical history, ie. metastases in lymph nodes, pulmonary metastases, etc. 2. The receptor will be solubilized and purified further and both polyclonal and monoclonal antibodies will be raised. 3. Clones of MCF-7 cells possessing the laminin receptor and metastatic in nude mice will be studied with regards to the influence of laminin and the C1 fragment on metastases, analogous to the BL6 melanoma cells in the C57 mouse.

Major Findings: We have biochemically quantitated this receptor in pooled plasma membrane fractions of human breast carcinoma obtained at surgery. We have characterized it as a single class receptor with a kd of 10^{-8} - 10^{-9} and a Mr of 60,000. We have purified it 1000 fold on laminin affinity chromatography. We have also demonstrated the laminin receptor on MCF-7 cells, a line derived from a human breast carcinoma.

Significance to Biomedical Research and the Program of the Institute: A potential tool to influence the metastatic process is envisioned in the direction of this research.

Proposed Course: Correlation of laminin receptors with metastatic potential; purification of this receptor, and the demonstration of its role in metastases of human breast carcinoma.

Publications:

Liotta, L.A., Rao, C.N., and Barsky, S.H.: Laminin Receptors on Human Breast Carcinoma: Role in Invasion of the Extracellular Matrix. In Rich, M. (Ed.): Understanding Breast Cancer: Clinical and Laboratory Concepts. Marcel Dekker Publ., 1983 (in press)

Terranova, V.P., Rao, C.N., Kalebic, T., Margulies, I.M., and Liotta, L.A.: Laminin receptor on human breast carcinoma cells. Proc. Natl. Acad. Sci. USA 80: 444-448, 1983.

Rao, C.N., Barsky, S.H., Terranova, V.P., and Liotta, L.A.: Isolation of a tumor cell laminin receptor. Biochem. Biophys. Res. Commun. 111: 804-808, 1983.

Barsky, S.H., Siegal, G.P., Jannotta, F., and Liotta, L.A.: Loss of basement membrane components by invasive tumors but not by their benign counterparts. Lab. Invest. (in press, Aug. 1983)

Project Description

Other Professional Personnel: K. Stromberg, Senior Investigator, FCRF, NCI; T.S. Tralka, Biologist, LP, NCI; L.M. Neckers, Expert, LP, NCI

Objectives: 1. To identify factors which play a role in horizontal recruitment.
2. To isolate and characterize a HSV induced receptor for the protein A-IgG complex.

Methods Employed: Cell culture, liquid chromatography, animal tumor transplantation, cell transformation assay, fluorescence-activated cell sorter, gel electrophoresis, immunoprecipitation, transblot analysis, electron microscopy.

Major Findings: Our studies have shown that a protein A-Ig receptor is induced on herpes-infected cells. That this receptor has an apparent molecular weight of 150,000 Daltons as determined by gel electrophoresis. The protein A-IgG complex has been visualized by electron microscopy to bind specifically to HSV nucleocapsids. It can be induced by HSV-1 and HSV-2 and binds to many different animal species of IgG. This protein is the viral capsid antigen.

The human chondroblastic osteosarcoma transplanted into the nu/nu mouse continues to express biochemical and histological markers similar to the original tumor. Morphologically; the majority of the cells were of the chondroblastoid type with a minor component of fibrosarcoma. By isozyme analysis and karyology, the tumor is human with a slight contamination of mouse. However, all attempts to grow the human component of the mixed tumor in vitro have resulted in a murine cell line. A murine cell line (TC715) which was isolated caused fibrosarcomas in nu/nu mice and forms colonies in soft agar. Urine isolated from nu/nu rats injected with the chondrosarcoma tumor grown in mice, contains a growth factor of a high molecular weight (30-35,000 Daltons). This factor competes in a radioreceptor assays with epidermal growth factor and also promotes the growth of phenotypically normal rat kidney cells, consequently it is a transforming growth factor.

Significance to Biomedical Research and the Program of the Institute: In previous studies, we have shown that HSV-infected cells develop a receptor for the Fc portion of the IgG molecule. This receptor is located on the infected cell membrane as well as on the viral envelope. Our studies have now identified another protein which will bind to the complex of protein A-IgG. This protein is located on the nucleocapsids and is most likely the major capsid antigen. We propose that there exist a closely related protein similar to the protein A-IgG complex which has an affinity to both the viral membrane and the nucleocapsids, thus bringing these two proteins together during viral assembly. These studies will allow us to gain insights into the relatively poorly understood process of viral assembly.

The occurrence of horizontal recruitment has been known for some time, but the relative recent increase in the use of immunodeficient animals as recipients of human xenografts has brought the phenomenon to the attention of investigators working with grafted tissue. We have recently reported the formation of a bipartate tumor containing human chondroblastic cells and a mouse fibrosarcoma.

A transforming growth factor of high molecular weight was present in the urine of nu/nu rats bearing this tumor and was absent in the media of the recruited murine cell line which strongly suggests that TGF's of human origin could mediate the initial steps of horizontal recruitment.

Proposed Course: With Dr. Costa's departure from the NCI, these projects will be terminated.

Publications:

Yee, C.L., Costa, J.C., Hamilton, V., Klein, G., and Rabson, A.S.: Changes in the expression of Fc receptor produced by induction of Epstein-Barr virus in lymphoma cell lines. Virology 120: 376-382, 1982.

Tralka, T.S., Yee, C.L., Rabson, A.B., Stromberg, K., Rabson, A.S., and Costa, J.C.: Murine type-C retroviruses and intracisternal A-particles in human tumors serially passaged in nude mice. J. Natl. Cancer Inst. (in press)

Costa, J., Yee, C., and Rabson, A.S.: In Proceedings IV International Workshop on the Use of Immunosuppressed Animals in Research. Karger, 1982 (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00542-06 LP
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 on oncogenic primate DNA viruses		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Peter M. Howley, Chief, Viral Oncol. & Molec. Pathol. Sec., Lab. of Path., NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Viral Oncology and Molecular Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1/2	PROFESSIONAL: 1/4	OTHER: 1/4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) <p>This study involves the characterization of the primate polyomaviruses BK, JC, SV40, STMV, and SA12. In addition, zur Hausen has recently identified a lymphotropic polyomavirus in African Green monkeys and serologic evidence suggests that a human lymphotropic virus also exists. BK, JC, and SV40 have each been shown to be oncogenic in hamsters and each has been demonstrated capable of transforming certain rodent cells in tissue culture. While BK virus has not been clearly associated with any human disease, JC virus has repeatedly been isolated from diseased brains of patients with progressive multifocal leukoencephalopathy (PML). The purpose of this work is to better characterize the polyomaviruses in terms of their biologic and molecular characteristics and to characterize the biologic features of polyomavirus transformed cells. An understanding of how these viruses interact with human cells will provide a better understanding of what pathogenic roles may be involved in any human disease associated with these viruses.</p>		

Project Description

Other Professional Personnel: S.P. Banks-Schlegel, Expert, CIP, NCI

Objectives: The area of interest currently being investigated has been the study of transformation of human epidermal cells with SV40 and a temperature-sensitive mutant of SV40, tsA209. We've been interested in the biologic properties of human epidermal cells transformed by SV40 and the effect of the expression of this transformed phenotype on the differentiated properties of human epidermal cells. We are also interested in establishing whether there is a human polyomavirus which affects human lymphocytes. We would like to identify human lymphoid tissue which may contain a human polyomavirus using a cross-reactive antiserum to all polyomaviruses. We would also like to identify the viral DNA associated with such a virus and if possible, clone it.

Methods Employed: Molecular cloning, nucleic acid hybridization, restriction endonuclease analysis, tissue culture, and DNA transfection.

Major Findings: Human epidermal cells have been transformed by wild-type SV40 and by the DNA of the tsA mutant of SV40 (209). These cells are transformed as evidenced by their ability to grow under stringent conditions, the presence of SV40 tumor antigens, and by altered morphology. In addition, differentiated properties including the production of keratin proteins and the presence of a cross-linked envelope are quantitatively decreased in the transformed cells. Under the restrictive temperature (40°), however, the tsA209 transformed cells revert to a normal phenotype in terms of morphology, growth parameters and differentiated properties.

Proposed Course: 1. To further study the genetic organization in control regions of the JC viral genome. 2. To use the peroxidase anti-peroxidase techniques for studying pML brains for the evaluation of known viral antigens. 3. To further characterize the differentiated properties of SV40-transformed human epidermal cells. 4. To see whether the transformed human epidermal cells will support the complete or partial productive expression of human papillomaviruses.

Publications:

Banks-Schlegel, S.P., and Howley, P.M.: Differentiation of human epidermal cells transformed by SV40. J. Cell Biol. 96: 330-337, 1983.

Howley, P.M.: Papovaviruses - Search for evidence of possible association with human cancer. In Phillips, L.A. (Ed.): Viruses Associated with Human Cancer. Marcel Dekker, New York, 1983, pp. 253-305.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01CB00543-05 LP
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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 the papillomaviruses

PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.)
(Name, title, laboratory, and institute affiliation)
Peter M. Howley, Chief, Viral Oncol. & Molec. Pathol. Sec., Lab. of Path., NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 4 1/2	PROFESSIONAL: 3 1/2	OTHER: 1
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CHECK APPROPRIATE BOX(ES)

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

(a1) Minors

(a2) Interviews B

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

There are currently recognized to be 16 human papillomaviruses and 6 bovine papillomaviruses. Each of these viruses is associated with distinct clinical entities which in humans include common warts, condylomata accuminata, laryngeal papillomatosis, and the macular pityriasis-like lesions of epidermodysplasia verruciformis. In cattle, these lesions are associated with cutaneous fibro-papillomas and esophageal papillomatosis. To date, no tissue culture system has been developed to propagate the papillomaviruses. There is a subset of papillomaviruses which are associated with carcinomas in their natural hosts. Among the human papillomaviruses, these include the HPV-5 and HPV-6; in cattle it includes the BATV (BPV-4) in cattle which feed on bracken fern. In the laboratory we have been studying the molecular biology of the BPV-1, because it can be used to transform susceptible rodent cells in tissue culture. Transformation of rodent fibroblasts by papillomaviruses remains one of the only biologic systems available to study the biology of papillomaviruses.

Project Description

Other Professional Personnel: C.R. Schlegel, Senior Investigator, LP, NCI; M.-F. Law, Expert, LP, NCI; N. Sarver, Sr. Staff Fellow, LP, NCI; Y.C. Yang, Visiting Fellow, LP, NCI; S. Mitrani-Rosenbaum, Visiting Fellow, LP, NCI; T. Hasuma, Visiting Fellow, LP, NCI

Objectives: 1. To analyze the molecular biology of the bovine papillomaviruses and the human papillomaviruses using available in vivo and in vitro systems. 2. To localize and characterize the transforming regions of the bovine papillomavirus type 1 and to determine if corresponding regions are expressed in the human papillomaviruses which have persistently infected rodent or human cells. 3. To develop a tissue culture system for the propagation of the papillomaviruses. 4. To analyze what, if any, role the human papillomaviruses play in the etiology of human carcinomas. 5. The analysis of the viral mRNAs expressed in BPV-1 transformed and in productive fibropapillomas excised from cattle. 6. Analysis of the "early" papillomavirus products expressed in BPV-1 transformed cells. 7. An analysis of the viral specific products required for the extrachromosomal replication of papillomavirus genomes in persistently-infected cells. 8. A determination of the cis and trans functions and sequences required for the autonomous extrachromosomal replication of a papillomavirus-derived plasmid.

Methods Employed: Nucleic acid hybridization, molecular cloning of DNA genomes, restriction endonuclease analysis, tissue culture, transcriptional analysis using hybridization techniques, the S1 and exonuclease VII digestion techniques, DNA sequencing, synthetic peptides, and immunoprecipitation.

Major Findings: 1. The DNA sequence of the BPV-1 genome has been determined in a collaborative study with investigators at Genentech, Inc. The genome consists of 7,944 base pairs, although the open reading frames are located on one strand. The early region consists of 8 open reading frames and the late region contains 2 open reading frames partitioned by a single stop codon. Using a computer-assisted graphic matrix technique, the DNA sequence of the BPV-1 genome has been compared with the DNA sequence of the human papillomavirus type 1 genome as determined by Olivier Danos and Moshe Yaniv of the Pasteur Institute in Paris, France. It has been found that the organizational components of each of the genomes are similar. Regions of conservation involve the L1 and E1 open reading frames. Conserved sequences were also found in the non-coding region of the genome and their possible involvement in regulation of viral gene expression is therefore implied. The manuscripts describing the sequence of the BPV-1 genome and its comparison with the HPV-1 genome have been published in Nature and the Journal of Virology, respectively.

2. The transcriptional organization of the bovine papillomavirus genome has been determined. The viral transcripts present in bovine papillomavirus transformed cells have been mapped. A total of 5 discrete species were found in the total cellular RNA detected. The 3' ends all map at .53 map units where a polyadenylation recognition signal AATAAA is located at base 4179. The 5 transcripts are co-linear and the 5' ends of the bodies map at .41, .39, .34, .09, and .03 map units. Within the 69% transforming region, 2 "TATA" boxes are located at base 7108 and base 58. These TATA boxes are diagnostic of the eukaryotic promoter and may serve as the promoter for the early transcriptional unit. Recently,

the viral transcripts in a productively infected bovine fibropapilloma have also been mapped. In addition to the species of RNA detected in the transformed cells, a second set of RNA species with a co-terminus at 0.90 map units and 5' termini of the bodies at 0.71 and 0.42 map units was noted. These messenger RNAs have been selected and translated in vitro into the major capsid protein (53K). Preliminary studies with hybrid arrested translation map the major capsid protein of BPV-1 to the L1 open reading frame. A study describing the virus-specific transcription in BPV-transformed mouse cells has been published in Virology and a paper describing the virus-specific transcripts in productively infected bovine fibropapillomas has been submitted to the Journal of Virology.

3. Because of the unique plasmid nature of the BPV-1 genome in transformed mouse cells, we examined the effect of mouse L cell interferon on acute transformation of mouse cells by BPV-1 as well as upon established BPV-1 transformants. These studies have been done in collaboration with Dr. Douglas Lowy and Dr. Israel Dvoretzky of the Dermatology Branch, as well as with Dr. Friedman of the Uniformed Services University for Health Sciences. We found that mouse L cell interferon inhibited transformation of mouse cells by BPV approximately 20 fold. Two independent lines (ID14 and ID13) were treated for long-term culture with 200 units of interferon/ml. It was found after 10 passages (approximately 60 cell generations) that there was a 5-10 fold drop in the overall copy number of BPV-1 genomes per cell. After 10 passages, the cells were plated at low density, and it was found that the two cell lines had between 1 and 10% of the individual colonies now demonstrating a flat reverted morphology. Independent colonies were grown up and found to show morphologic similarity to the non-transformed C127 cells, to no longer be anchorage-independent, and to have a lowered saturation density. Analysis of the DNA had shown that at the level of .1 copies/cell that they had been cured of the all viral DNA sequences. These studies show that interferon affects either the plasmid replication of BPV-1 genomes in transformed cells or affects the equal partitioning of the plasmids to daughter cells. This study has been published in the Proceedings of the National Academy of Sciences in December of 1982. Subsequent studies have asked whether the hybrid interferon made in bacteria has the same effect. The human Ad beta 2 alpha hybrid interferon was chosen to be examined because it had been demonstrated previously to have effect on mouse cells. It was found that this hybrid interferon had a marked effect on inhibiting transformation of mouse cells by BPV. The magnitude of the effect was approximately that of the mouse L cell interferon. In contrast, the hybrid Ad Pvu II alpha interferon did not have this effect. The effect of the beta 2 Ad hybrid interferon on decreasing plasmid copy number and possibly inducing revertants is under investigation.

4. In a collaborative study with Paul Fisher of the Department of Microbiology at Columbia University, we have examined the ability of bovine papilloma virus to transform rat cells. Both BPV virus as well as cloned BPV viral DNA is capable of transforming cloned rat embryo fibroblasts from the Fisher rat. The BPV DNA is maintained extrachromosomally in these rat fibroblasts much the same way as it is maintained as a plasmid in mouse cells. A manuscript describing these results is in preparation.

Publications:

Howley, P.M.: The human papillomaviruses. (Special Article). Arch. Pathol. Lab. Med. 106: 429-432, 1982.

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 Merigan, T., Friedman, R.M., and Fox, C.F. (Eds.): UCLA Symposia on Molecular and Cellular Biology. Vol. XXV, Chemistry and Biology of Interferons: Relationship to Therapeutics. New York, Academic Press, 1982, pp. 181-191.

Chen, E.Y., Howley, P.M., Levinson, A., and Seeburg, P.H.: The primary structure and genetic organization of the bovine papillomavirus (BPV) type I genome. Nature 299: 529-534, 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.

Danos, O., Engel, L.W., Chen, E.Y., Yaniv, M., and Howley, P.M.: A comparative analysis of the human type 1a and bovine type 1 papillomavirus genomes. J. Virol. 46: 557-566, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00547-03 LP
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 use of papillomavirus DNAs as eukaryotic cloning vectors		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Peter M. Howley, Chief, Viral Oncol. & Molec. Pathol. Sec., Lab. of Path., NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Viral Oncology and Molecular Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3	PROFESSIONAL: 2 1/4	OTHER: 3/4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The bovine papillomaviruses are capable of transforming certain mouse fibroblast lines as well as certain rat fibroblast lines. The viral DNA in these transformed lines is maintained exclusively as extrachromosomal plasmids. The extrachromosomal nature of the viral DNA in these lines together with the selected malignant phenotypes has been utilized to develop the papillomaviruses as eukaryotic cloning vectors. We have shown that the complete genome cloned into a deletion derivative of pBR322 (pML2) is capable of serving as a shuttle vector between bacteria and eukaryotic cells. Eukaryotic and prokaryotic genes can be expressed in mammalian cells as part of BPV plasmids. We have shown that the human beta interferon gene can be inducibly regulated when maintained in a plasmid state in mouse cells. Using a stable plasmid containing the neomycin resistance gene (a phosphotransferase) from Tn5, we are testing the tissue range and host range of papillomavirus plasmid replication.		
683		

Project Description

Other Professional Personnel: N. Sarver, Staff Fellow, LP, NCI; M.-F. Law, Expert, LP, NCI; S. Mitraní-Rosenbaum, Visiting Fellow, LP, NCI; W. McAllister, IAP, LP, NCI

Objectives: 1. To develop the papillomaviruses as effective eukaryotic cloning vectors. 2. To develop an effective shuttle vector containing papillomavirus autonomous replication sequences and prokaryotic sequences containing a prokaryotic replication origin and a selectable marker. 3. To segregate the transformation function of the bovine papillomavirus from the extrachromosomal replication function of the bovine papillomavirus and assessing its use as a eukaryotic cloning vector. 4. To link a transformation-negative, replication-positive segment of a papillomavirus to a dominant selectable marker such as the *E. coli* HGPRT gene or the neomycin resistance gene from TN5 in order to assess its ability as a eukaryotic cloning vector. 5. To assess the ability of a human papillomavirus genome to replicate autonomously in mouse as well as primate in human cells when linked to a dominant selectable marker. 6. To assess the ability of this cloning system to deliver a gene to a cell in such a manner as to affect site-specific integration. 7. To introduce inducible genes into eukaryotic cells using the papillomavirus vector systems in order to define the nature of the inducible sequences. Experiments are underway utilizing the human beta interferon gene.

Methods Employed: Nucleic acid hybridization, restriction endonuclease analysis, immunologic techniques, bacterial cloning, tissue culture, RNA analysis, and DNA transfection.

Major Findings: 1. Dr. Ming-Fan Law has cloned the *E. coli* gpt gene arranged in a modified SV40 early transcriptional unit into a BPV_{69T}-pBR322 hybrid plasmid. He has used this DNA to transform susceptible mouse cells and selected transformants either for their malignant phenotype or their ability to survive in selected media containing mycophenolic acid and xanthine. Dr. Law established that cells selected for their malignant phenotype are also capable of growing in selected media. In addition, the mouse cells selected for their ability to grow in the otherwise poison media demonstrated malignant phenotype. Thus, the two phenotypic markers are closely linked. One problem became obvious in the analysis of the DNA, and that was that many of the lines contained hybrid DNA which had undergone rearrangement, acquired additional sequences, or had become integrated. Dr. Law has subsequently inserted the SV2gpt transcriptional cassette into a complete BPV/pML2 plasmid and used that DNA to select again either for malignant transformation or ability to grow in selected media. He has found again that the phenotypic markers are tightly linked. In the cells selected for malignant transformation, the DNA existed in a monomeric plasmid state. In the cells selected for mycophenolic acid, however, the DNA existed in a high molecular weight form possibly integrated. Analysis of the XGPRT levels in these cells indicate that they were the same in cells selected for malignant transformation or in cells selected for their ability to grow in selected media. In addition, cells initially selected for their malignant transformed phenotype in harboring the plasmid as a monomeric plasmid were placed under selection. These cells grew very poorly initially and few foci were able to be selected. On subsequent selection, it was found that the DNA had gone into again a high molecular weight form. It was concluded that the use of the gpt gene and

mycophenolic acid selection was not compatible with extrachromosomal plasmid replication. A manuscript has been published in a book entitled Eukaryotic Viral Vectors and a second manuscript is in preparation.

2. A second dominant selective marker has been studied by Dr. Law in the laboratory. That is the neomycin-resistance gene from the bacterial transposed on Tn5. Dr. Law has hooked the gene up into a mammalian cell transcription cassette consisting of the mouse metallothionein promoter and the SV40 small t and early region 3' end processing signals. This DNA has been cloned into a complete BPV/pML2 hybrid DNA. The mouse cells have been transfected with this plasmid and again selected for either their malignant phenotype or for their ability to grow in the presence of the selective drug G418. There was a 100% linkage of the two phenotypes. The plasmid exists as a stable plasmid as a monomeric form in cells selected either for malignant transformed phenotype or for growth in the presence of the drug G418. The DNA can be rescued out readily from the transformed cells by using the Hirt supernatant and retransformation of bacteria since this plasmid contains a bacterial plasmid origin of replication as well as the ampicillin-resistance gene which one can select for. Of interest is the fact that the initial colonies selected for neomycin resistance have a flat phenotype and it is only after propagating the cells for approximately 2-3 weeks that they develop a transformed phenotype. This says that temporally one can dissociate the G418 selection from malignant transformation. This suggests that some cascade effect must occur within mouse cells containing a BPV genome and that the presence of the BPV genome itself is not sufficient for the transformed phenotype. A reasonable hypothesis for this cascade is that there must be an amplification of the BPV genome to get the copies effectively up to a threshold level to provide a threshold level of BPV gene product to affect a transformation. A manuscript is in preparation which will be submitted to Nature.

3. Dr. Nava Sarver has completed a study in which she has shown that the complete BPV-1 genome cloned into a deletion derivative of pBR322 called pML2 is capable of transforming mouse cells at high efficiency. In the study she has shown that the pBR322 sequences are cis inhibitory to BPV transformation when they are cloned into either the 69% transforming region or into the complete BPV-1 genome. The sequences which are deleted from pBR322 degenerate the pML2d molecule are sequences which are inhibitory to the expression and replication of a covalently linked SV40 sequence in monkey cells. These same sequences appear to be therefore cis inhibitory to the replication or expression of BPV sequences in mouse cells. Dr. Sarver has shown that BPV/pML2 hybrid is present in an unmodified form in mouse cells and that this DNA can be rescued from the mouse cells using the Hirt supernatant when transformed back into susceptible E. coli. This BPV/pML2 vector therefore is sufficient for shuttling genes between animal cells and prokaryotic cells. A manuscript has been published in PNAS describing these studies.

4. While the BPV_{69T}/pML2 DNA does not transform mouse cells at a high efficiency, a number of eukaryotic DNA inserts when cloned into this molecule facilitate transformation of mouse cells. Dr. Dan DiMaio in Dr. Maniatis' lab at Harvard first showed this with the human beta hemaglobin gene. Drs. Sarver and Rosenbaum in this laboratory have shown that a 5.3 kb genomic segment containing the rat preproinsulin gene, a 2.6 kb genome containing the

human growth hormone, and a 4.5 kb segment of the rat genomic DNA between the alpha and gamma rat fibrinogen genes, are each capable of providing this facilitative function. The mechanism of this facilitating function is still unknown. These studies have been published in a book entitled Gene Transfer in Cancer by Raven Press.

5. Dr. Stella Rosenbaum has studied the question of whether an inducibly regulated gene introduced into a BPV plasmid vector can be properly regulated when maintained extrachromosomally. This study was done in collaboration with Michele Revel from the Weissmann Institute for Science in Rehovot, Israel. She inserted the 1.6 kb genomic DNA segment of the human interferon beta 1 gene into a recombinant plasmid BPV_{69T}. The DNA was maintained extrachromosomally and she demonstrated that the interferon gene could be induced with either poly IC or with Newcastle disease virus. She has subsequently inserted the same genomic DNA into a complete BPV/pML2 hybrid gene and shown that that also is inducibly regulated as an extrachromosomal element. The authentic use of the 5' ends of the human beta 1 interferon gene is only detected after induction.

Publications:

Law, M.-F., Howard, B., Sarver, N., and Howley, P.M.: Expression of Selective Traits in Mouse Cells Transformed with a BPV DNA Derived Hybrid Molecule Containing E. coli gpt. In Gluzman, Y. (Ed.): Eukaryotic Viral Vectors. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory, 1982, pp. 79-86.

Sarver, N., Byrne, J.C., and Howley, P.M.: Transformation and replication in mouse cells of a bovine papillomavirus/pML2 plasmid vector that can be rescued in bacteria. Proc. Natl. Acad. Sci. USA 79: 7147-7151, 1982.

Mitrani-Rosenbaum, S., Maroteaux, L., Mory, Y., Revel, M., and Howley, P.M.: Inducible expression of the human interferon beta 1 gene linked to a bovine papillomavirus DNA vector and maintained extrachromosomally in mouse cells. Molec. Cell. Biol. 3: 233-240, 1983.

Howley, P.M., Sarver, N., and Law, M.-F.: Eukaryotic Cloning Vectors Derived from Bovine Papillomavirus DNA. In Wu, R., Grossman, L., and Moldave, K. (Eds.): Methods in Enzymology. New York, Academic Press, Inc., 1983, vol. 101, pp. 387-402.

Sarver, N., Mitrani-Rosenbaum, S., Law, M.-F., McAllister, W.T., Byrne, J.C., and Howley, P.M.: Bovine Papillomavirus Shuttle Vectors. In Setlow, J.K., and Hollaender, A. (Eds.): Genetic Engineering. Vol. 5, New York, Plenum Press (in press)

Sarver, N., Mitrani-Rosenbaum, S., Law, M.-F., Byrne, J.C., and Howley, P.M.: A Bovine Papillomavirus/pML2 Hybrid Vector - A Dual Host Replicon. In Sternberg, N.L., and Pearson, M.L. (Eds.): Gene Transfer and Cancer. New York, Raven Press (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00564-01 LP
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.) Early events in VSV: host cell interaction		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) C. Richard Schlegel, Senior Investigator, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Viral Oncology and Molecular Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.) <p>VSV infects a wide variety of animal cells and has been used as a prototype for studying the mechanism of replication and assembly of enveloped viruses. Recently, there has been renewed interest in the study of virus uptake into host cells. Such studies have not only elucidated basic characteristics of cell function but have also given focus to new antiviral therapies. Our laboratory has been investigating the internalization of VSV into host cells in an attempt to (1) define the plasma membrane binding site for VSV, (2) determine the specificity characteristics of this binding, (3) dissect the mechanism by which VSV fuses with cell membranes, and (4) explore possible mechanisms for inhibiting or perturbing the early steps of infection.</p> <p>We will use multiple approaches to study the internalization of VSV. Binding assays with purified S³⁵-VSV will permit the detection of specific VSV binding. In addition, IF and EM techniques will be used to monitor the morphologic pathway of VSV entry. To analyze the fusion of virus and cell membranes, we will utilize liposomes containing VSV G protein (virosomes). These virosomes will be studied for their specificity of interaction with host cells and then used to study their interaction with other liposomes. Energy transfer fluorescence, fluorescence quenching, and EM will be used to quantitate and monitor the fusion process mediated by G protein. In addition, attempts will be made with circular dichroism and infra-red spectroscopy to follow possible conformational changes in G protein which occur during the fusion event. Neutralizing antibodies will be used to confirm relevant changes in protein conformation.</p>		

Project Description

Other Professional Personnel: R. Blumenthal, Chief, Membrane Structure and Function Section, LMMB, NCI

Objectives: 1. Identify the cell binding site for VSV. 2. Study the mechanism of VSV endocytosis and virus/cell fusion. 3. Analyze the mechanism of virus neutralization by anti-G protein antibodies.

Methods Employed: 1. S^{35} -VSV binding assays. 2. Electron microscopy (EM). 3. Immunofluorescence microscopy (IF). 4. Liposome and cell fusion assays monitored by energy transfer fluorescence and fluorescence quenching. 5. Detection of G-protein conformation changes by circular dichroism and infra-red spectroscopy. 6. Density gradient centrifugation.

Major Findings: 1. There is a saturable cell surface receptor for VSV. 2. The VSV receptor may be phosphatidylserine.

Significance to Biomedical Research and the Program of the Institute: 1. The delineation of the mechanism of virus internalization will be crucial to designing inhibitors of virus infection. 2. Knowledge of the mechanism of protein-mediated membrane fusion is important for understanding viral infection as well as the many cellular processes involved in endocytosis and exocytosis.

Proposed Course: 1. Use purified S^{35} -VSV and S^{35} -virosomes to study their interaction with the cell surface. These biochemical studies will be complemented by IF and EM studies. 2. Use circular dichroism, infra-red spectroscopy, energy fluorescence transfer, and fluorescence quenching to characterize the fusion of VSV with cells, virosomes with cells, and virosomes with liposomes.

Publications:

Schlegel, R., Willingham, M.C., and Pastan, I.: Saturable binding sites for vesicular stomatitis virus on the surface of Vero cells. J. Virol. 43: 871-875, 1982.

Schlegel, R., Tralka, T.S., Willingham, M.C., and Pastan, I.: Inhibition of VSV binding and infectivity by phosphatidylserine: is phosphatidylserine a VSV-binding site? Cell 32: 639-646, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00565-01 LP
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 immortalization and transformation by papovaviruses		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) C. Richard Schlegel, Senior Investigator, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Viral Oncology and Molecular Pathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.4	PROFESSIONAL: 0.9	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The development of the tumorigenic state appears to be a complex, multi-step process. Studies with chemical carcinogens, for example, have indicated that there are probably at least two discernible steps, initiation and promotion, which are involved in carcinogenesis. Although this conceptual construction may be too simple, it does provide a framework for evaluating the distinct, but sometimes overlapping, effects of mutagens and promoters. Most studies with viral tumorigenesis have focused on <u>in vitro</u> transformation and, because of the nature of the assay, have characterized the transformation as a one-step phenomena which is usually ascribed to the presence and activity of a specific viral transforming protein. The goal of this project is to use a different assay system to define the role of two papovaviruses (polyomavirus and bovine papillomavirus) in the multi-step process of cell transformation. While established rodent fibroblast lines (e.g. NIH-3T3 and Fisher rat) can be used to illustrate the ability of virus to effect one-step transformation, the use of primary rodent cultures allows the analysis of at least two distinct steps necessary for tumorigenesis: cell immortalization and cell transformation. Current information indicates that cell immortalization is necessary but not sufficient for ultimate tumorigenesis. Preliminary information suggests that polyoma but not bovine papillomavirus can transform primary rodent cells and that bovine papillomavirus may be deficient in a necessary tumorigenic function. Our approach will be to 1) determine whether polyoma and BPV truly differ in their ability to transform primary fibroblasts and primary epithelial cells, 2) determine which of the three early polyoma gene functions are involved in cell immortalization and cell transformation, 3) complement the deficient BPV-transforming functions with the appropriate polyoma early proteins, 4) determine whether known promoters (for example TPA) can complement any deficient transforming functions of BPV, and 5) delineate the cellular changes associated with the acquisition of the viral-induced immortalized state, including growth factor requirements, growth factor production, changes in ploidy, and alterations in specific cell differentiation functions.</p>		

Project Description

Other Professional Personnel: P.M. Howley, Chief, Viral Oncology and Molecular Pathology Section, LP, NCI; S. Banks-Schlegel, Expert, CIP, NCI

Objectives: 1. Define the immortalization and transformation functions of polyoma and BPV. 2. Analyze the cellular alterations during the course of in vitro transformation. 3. Attempt complementation studies between polyoma and BPV for in vitro transformation.

Methods Employed: 1. Bacterial plasmid preparation, purification and transfection. 2. Keratinocyte culture and transfection. 3. Immunofluorescence of viral antigens. 4. SDS-PAGE analysis and immunoprecipitation of keratinocytes for the detection of keratin involucrum and envelope protein. 5. FACS analysis of cellular DNA content.

Major Findings: Study not yet initiated.

Significance to Biomedical Research and the Program of the Institute: 1. To clarify the role of virus infection in tumorigenesis. 2. To define the interaction between chemical and viral agents in transformation.

Proposed Course: See above.

Publications:

Study not initiated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00548-03 LP
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.) Monoaminergic receptors on lymphocytes visualized by FACS analysis		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Leonard M. Neckers, Expert, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Hematopathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0	PROFESSIONAL: 0	OTHER: 0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>In the last year we have determined that this project, while potentially fruitful, was of less importance than the others in which we have been engaged, and so have suspended any further work in this area. No further progress was made during the current fiscal year.</p>		

Project Description

Objectives: To examine the feasibility of using anti-receptor antibodies to study lymphocyte aminergic receptors by FACS analysis.

Methods Employed: Fluorescent derivatization of biogenic amines followed by FACS analysis; making anti-receptor antibodies to be used with FACS analysis.

Major Findings: This approach can be used to visualize beta receptors on lymphocytes but not receptors with low surface densities.

Significance to Biomedical Research and the Program of the Institute: Although various lymphocyte receptors for biogenic amines have been found, in vivo measurement of these receptors has not been possible. Visualization and subtyping of these receptors will add to our understanding of their function in both normal and abnormal immunological processes. If our current approach proves useful, then we will have a new technique to use anti-receptor antibodies for study of lymphocyte aminergic receptors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00550-03 LP
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.) Immunologic characterization of malignant lymphomas		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Elaine S. Jaffe, Chief, Hematopathology Section, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Hematopathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">3.0</div>	PROFESSIONAL: <div style="text-align: center;">1.5</div>	OTHER: <div style="text-align: center;">1.5</div>
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
A		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>In order to assess the clinical and pathologic significance of the immunologic characterization of human malignant lymphomas, fresh biopsy tissues are obtained from patients referred to the Clinical Center for treatment. Biopsies are obtained with patient permission prior to therapy and processed in the Hematopathology Section. The neoplastic cells are characterized as to their origin from T cells, B cells, or histiocytes, and can in addition be identified as belonging to specific developmental and functional subpopulations. This data is then correlated with clinical and pathologic data.</p>		

Project Description

Other Professional Personnel: J. Cossman, Sr. Assistant Surgeon, LP, NCI;
R.I. Fisher, Senior Investigator, M, NCI

Objectives: To determine the clinical importance of immunologic phenotype, and determine if it correlates with clinical presentation, stage, response to therapy or survival. To determine the correlation of immunologic phenotype with conventional morphology.

Methods Employed: Analysis of a wide variety of immunologic, cytochemical and biochemical markers including the identification of the following: complement receptors (CR1 and CR2), receptors for the Fc fragment of IgG and IgM, sheep erythrocyte receptors, surface immunoglobulins for individual heavy and light chains, terminal deoxynucleotidyl transferase, and alpha-naphthyl butyrate esterase. Additionally, a large battery of monoclonal antibodies reactive with normal and neoplastic lymphoreticular cells are employed. This data is correlated with clinical and cytogenetic data provided by the Medicine Branch, NCI.

Major Findings: Diffuse aggressive non-Hodgkin's lymphomas are immunologically heterogeneous. Surface immunotype cannot be predicted by conventional morphology. Lymphomas of peripheral T-lymphocytic origin have some unique clinical and pathologic features, in part due to the production of lymphokines by the neoplastic cells. Immunotype alone is probably not a major factor in influencing response to therapy.

Significance to Biomedical Research and the Program of the Institute: This information will affect future development of clinical protocols, as tumors of differing immunotypes may require different therapies. This has already been shown to be true for lymphoblastic lymphoma. Neoplastic expansions often permit the identification of normal cellular phenotypes not previously recognized, and lead to increased understanding of the immune system.

Publications:

Gelmann, E., Anderson, T., Jaffe, E.S., and Broder, S.: Chemotherapy for lymphoma in a patient with common variable immunodeficiency. Case report, literature review, and recommendations for chemotherapy in immunodeficient patients. Arch. Intern. Med. 142: 90-92, 1982.

Fisher, R.I., Silver, B.A., Vanhaelen, C.P., Jaffe, E.S., and Cossman, J.: Objective regressions of T- and B-cell lymphomas in patients following treatment with anti-thymocyte globulin. Cancer Res. 42: 2465-2469, 1982.

Jaffe, E.S.: Hypothesis: Follicular Lymphomas - Are They Benign Tumors of the Lymphoid System? In Vitetta, E., and Fox, C.F. (Eds.): B and T Cell Tumors: Biological and Clinical Aspects. UCLA Symposia on Molecular and Cellular Biology, Vol. XXIV, New York, Academic Press, 1982, pp. 91-95.

Jaffe, E.S.: Follicular lymphomas: Possibility that they are benign tumors of the lymphoid system. (Guest Editorial) J. Natl. Cancer Inst. 70: 401-403, 1983.

Zeller, N., Cossman, J., Jaffe, E.S., and Tsichlis, P.: Expression of c-myc Sequences in Human Lymphomas. In Magrath, I., Ramot, B., and O'Connor, C. (Eds.): Monograph - title unknown at present. Raven Press (in press)

Cossman, J., and Jaffe, E.S.: Analysis of Immunologic Phenotype of Non-Hodgkin's Lymphomas at the National Cancer Institute. In Magrath, I., Ramot, B., and O'Connor, G. (Eds.) Monograph - title unknown at present. Raven Press (in press)

Cossman, J., Chused, T.M., Fisher, R.I., Magrath, I., Bollum, F., and Jaffe, E.S.: Diversity of immunologic phenotypes of lymphoblastic lymphoma. Cancer Res. (in press)

Braziel, R.M., Keneklis, T., Donlon, J.A., Hsu, S.-M., Cossman, J., Bollum, F.J., and Jaffe, E.S.: Terminal deoxynucleotidyl transferase in non-Hodgkin's lymphoma. Am. J. Clin. Pathol. (in press)

Greene, W.C., Waldmann, T.A., Cossman, J., Hsu, S.-M., Neckers, L.M., Marshall, S.L., Jensen, J.P., Bakhshi, A., Leonard, W.J., Depper, J.M., Jaffe, E.S., and Korsmeyer, S.J.: Hairy Cell Leukemia: A Malignant Expansion of B Cells which Express TAC Antigen. In Marks, P., and Golde, D.W. (Eds.): Normal and Neoplastic Hematopoiesis. New York, Alan R. Liss, Inc. (in press)

Korsmeyer, S.J., Greene, W.C., Cossman, J., Hsu, S.-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 (in press)

Project Description

Other Professional Personnel: C.R. Simrell, Sr. Assistant Surgeon, LP, NCI; L.M. Neckers, Expert, LP, NCI; G. Crabtree, Medical Officer, LP, NCI

Objectives: To determine whether certain human malignant lymphomas (especially those of peripheral T-cell origin) produce a factor (or factors) which can stimulate the phagocytic ability of human macrophages.

Methods Employed: Neoplastic cells are placed in tissue culture and 24 hour supernatants tested for their ability to induce an increase in the number of Fc receptors and to enhance the phagocytic activity of the human promyelocytic cell line HL60, the macrophage-like cell line U937, and normal peripheral blood monocytes. Fc receptors are assayed by measuring the specific Fc receptor dependent binding of ^{125}I -IgG. Phagocytosis is assayed using IgG coated OX-RBC or 1.5 μ fluorescent beads.

Major Findings: Preliminary results suggest that occasional peripheral T-cell lymphomas may secrete a factor which stimulates the phagocytosis of antibody-coated RBC but not beads. This effect is independent of an increase in the number of Fc receptors on the macrophage cell surface. Normal peripheral blood lymphocytes stimulated in an allogeneic mixed leucocyte reaction or by CON-A elaborate a factor which induces phagocytosis and with CON-A stimulated lymphocytes, this effect is not associated with an increase in Fc receptors. However, Fc receptors appear to be required. Phagocytosis of IgG-coated particles but not IgM coated particles is induced. Furthermore, free IgG will inhibit phagocytosis but not free IgM.

Further attempts to define the clinicopathologic characteristics of the patients which elaborate this factor have revealed that it is associated almost exclusively with angiocentric immunoproliferative lesions, i.e. lymphomatoid granulomatosis in both its benign and malignant phases. Other mature T-cell malignancies including HTLV-positive and HTLV-negative cases have been negative.

Preliminary attempts to characterize the factor (phagocytosis inducing factor or PIF) have indicated that it is temperature sensitive unlike MAF.

Further studies were undertaken to investigate other effects of CON-A and tumor cell supernatants on U937 cells. There is induction of lysosomal enzymes as well as certain normal differentiation antigens seen on mononuclear phagocytes.

Significance to Biomedical Research and the Program of the Institute: Some patients with peripheral T-cell lymphomas develop a syndrome resembling malignant histiocytosis characterized by fever, hepatosplenomegaly, and pancytopenia associated with histiocytosis and marked erythrophagocytosis within the reticulo-endothelial system. The demonstration that neoplastic T cells from such a patient can secrete a factor capable of stimulating macrophage in vitro gives some insight into the pathogenesis of this syndrome, and also contributes to a greater understanding of the nature of normal lymphocyte/macrophage interaction.

Proposed Course: Further studies are underway to define the patient population. Other vasculitides including Wegener's granulomatosis are being investigated. Further studies to define PIF and distinguish it from other known lymphokines are underway.

Publications:

Simrell, C.R., Crabtree, G.R., Cossman, J., Fauci, A.S., and Jaffe, E.S.: Stimulation of Phagocytosis by a T-cell Lymphoma-derived Lymphokine. In Vitetta, E., and Fox, C.F. (Eds.): B- and T-cell Tumors: Biological and Clinical Aspects. UCLA Symposia on Molecular and Cellular Biology, vol. XXIV, New York, Academic Press, 1982, pp. 247-252.

Jaffe, E.S., Costa, J.C., Fauci, A.S., Cossman, J., and Tsokos, M.: Malignant lymphoma and erythrophagocytosis simulating malignant histiocytosis. Am. J. Med. (in press)

Project Description

Other Professional Personnel: L.M. Neckers, Expert, LP, NCI; E.S. Jaffe, Chief, Hematopathology Section, LP, NCI

Objectives: To identify and characterize human malignant lymphomas.

Methods Employed: Immunofluorescence FACS and computer analysis; tissue culture; hybridoma development.

Major Findings: 1. Intra-tumor phenotype heterogeneity suggestive of potential selective problems during therapy. 2. A large class of peripheral T-cell lymphomas previously considered rare. 3. Documentation of reported specificity of monoclonal antibodies. 4. Unique immunologic phenotype of many low-grade B-cell lymphomas. 5. Regulation of cell growth via modulation of the transferrin receptor. 6. Inducibility of differentiation of common acute lymphoblastic leukemia cells to successive pre-B and early B-cell stages. 7. Capability of FACS-monoclonal antibody analysis to diagnose lymphoma from body fluids and fine needle aspirations. 8. Expression of so-called "common acute lymphoblastic leukemia antigen" (CALLA) in B-cell lymphomas, T-cell lymphoblastic lymphomas, and human neutrophils.

Significance to Biomedical Research and the Program of the Institute: Monoclonal antibodies have great potential for both diagnosis and treatment of malignant lymphomas. We have the best characterized series of monoclonal antibody phenotyping of lymphoma in existence. This information is invaluable to our understanding of the biology of neoplastic lymphocytes, for diagnosis, for conventional therapy and for future monoclonal antibody therapy.

Proposed Course: 1. Determine phenotypes of malignant lymphomas using monoclonal antibodies. 2. Determine correlation between immunologic phenotype and clinical course. 3. Develop monoclonal antibodies and flow cytometry as methods for the diagnosis of malignant lymphoma in leukemia.

Publications:

Cossman, J., Neckers, L.M., Arnold, A., and Korsmeyer, S.J.: Induction of differentiation in a case of common acute lymphoblastic leukemia. N. Engl. J. Med. 307: 1251-1254, 1982.

Cossman, J., Neckers, L.M., Leonard, W.J., and Greene, W.C.: Polymorphonuclear neutrophils express the common acute lymphoblastic leukemia antigen. J. Exp. Med. 157: 1064-1069, 1983.

Cossman, J., Neckers, L.M., Braziel, R., Bakhshi, A., Arnold, A., and Korsmeyer, S.: Induction of Differentiation in B-cell Leukemias. In Bernard, A., Boumsell, L., Dausset, J., Milstein, C., and Schlossman, S.F. (Eds.): Human Leucocyte Markers Detected by Monoclonal Antibodies. Springer-Verlag, 1983.

- Cossman, J., Chused, T.M., Fisher, R.I., Magrath, I., Bollum, F., and Jaffe, E.S.: Diversity of immunologic phenotypes of lymphoblastic lymphomas. Cancer Res. (in press)
- Cossman, J., and Jaffe, E.S.: Analysis of Immunologic Phenotype of Non-Hodgkin's Lymphomas at the National Cancer Institute. In Magrath, I., Ramot, B., and O'Connor, G. (Eds.): Title of book unknown at present. Raven Press (in press)
- Neckers, L.M., and Cossman, J.: Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin-2 (TCGF). Proc. Natl. Acad. Sci. USA (in press)
- Blayney, D.W., Jaffe, E.S., Blattner, W.A., Cossman, J., Robert-Guroff, M., Longo, D.L., Bunn, P.A., Jr., and Gallo, R.C.: The human T-cell leukemia/lymphoma virus (HTLV) associated with American adult T-cell leukemia/lymphoma (ATL). Blood (in press)
- Braziel, R.M., Keneklis, T., Donlon, J.A., Hsu, S.-M., Cossman, J., Bollum, F.J., and Jaffe, E.S.: Terminal deoxynucleotidyl transferase in non-Hodgkin's lymphoma. Am. J. Clin. Pathol. (in press)
- Greene, W.C., Waldmann, T.A., Cossman, J., Hsu, S.-M., Neckers, L.M., Marshall, S.L., Jensen, J.P., Bakhshi, A., Leonard, W.J., Depper, J.M., Jaffe, E.S., and Korsmeyer, S.J.: Hairy Cell Leukemia: A Malignant Expansion of B Cells which Express TAC Antigen. In Marks, P., and Golde, D.W. (Eds.): Normal and Neoplastic Hematopoiesis. New York, Alan R. Liss, Inc. (in press)
- Korsmeyer, S.J., Greene, W.C., Cossman, J., Hsu, S.-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 (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00553-03 LP
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 fibrinogen gene expression		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Gerald R. Crabtree, Medical Officer (Path.), Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Hematopathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>We are studying the regulation and structure of the genes which code for fibrinogen, the major blood coagulation protein. We have found that fibrinogen mRNA levels are controlled through a complex feedback-like regulation involving the plasmin degradation products of fibrinogen and interleukin I. This same mechanism also appears to account for the induction of the acute phase reaction in response to injury or inflammation. This regulatory influence somehow coordinates the levels of each of the three fibrinogen mRNAs so that the genes are activated at the same time and to the same extent. We have begun studying the mechanisms underlying this coordinate regulation and have obtained cDNA and genomic clones for each of the three fibrinogen chains in the rat and human. Thus far, we have found that the three fibrinogen genes are linked on human chromosome four, that the activation of the three genes occurs by increasing the rate of transcription of mRNA from each of the three genes, and that homologous sequences exist at the 5' ends of the genes which might account for this regulation.</p> <p>We have also begun studying the hereditary human afibrinogenemias as models of defective fibrinogen production. Patients with these diseases do not make circulating fibrinogen. We have found that by examining the DNA using Southern blotting from patients with afibrinogenemias, that at least two genotypes underlie these diseases.</p> <p>The goals of our research are to understand the factors controlling and coordinating the expression of families of genes during differentiation and development.</p>		

Project Description

Other Professional Personnel: J.A. Kant, Expert, LP, NCI; A.J. Fornace, Jr., Expert, LP, NCI; C.M. Comeau, Biologist, LP, NCI

Objectives: 1) To develop a model system to study the factors controlling and coordinating the expression of genes during differentiation and development. 2) To understand the molecular genetics of the human dysfibrinogenemias and afibrinogenemias.

Methods Employed: cDNA was prepared from purified mRNA and cloned in pBR322. Fibrinogen clones were identified by hybrid-selection and translation. Human and rat genomic libraries were screened, and the clones mapped by Southern blotting, R-looping and heteroduplex mapping. DNA was purified from patients with afibrinogenemias and examined by Southern blotting.

Major Findings: We have found that mRNA levels for fibrinogen increase 10-38 fold following defibrination. This response is mediated by a feedback mechanism involving fibrin split products and interleukin I. We have mapped the genes for α , β , and γ chains of fibrinogen and find they are tightly linked on human chromosome 4 and that the γ chain gene produces two mRNAs by alternate splice patterns.

Significance to Biomedical Research and the Program of the Institute: Recent evidence indicates that malignancy may be related to inappropriate expression of normal cellular genes. If this proves correct, it will be essential to understand the factors controlling gene expression to permit specific therapeutic intervention. We hope that the system we have developed will be useful in understanding the general mechanisms controlling gene expression. Secondly, understanding the control of fibrinogen synthesis may allow more effective treatment of thrombotic disease, and also hereditary coagulation disorders related to the deficient production of fibrinogen.

Proposed Course: To map the transcriptionally active regions in or about the fibrinogen genes, develop *in vitro* systems to study their expression, and explore the mutations which result in the human afibrinogenemias.

Publications:

Crabtree, G.R., and Kant, J.K.: Coordinate accumulation of the mRNAs for the α , β , and γ chains of rat fibrinogen following defibrination. J. Biol. Chem. 257: 7277-7279, 1982.

Crabtree, G.R., Kant, J.A., Fornace, A.J., Jr., Rauch, C.A., and Fowlkes, D.M.: Regulation and structure of the mRNAs for the α , β , and γ chains of rat fibrinogen. Ann. N.Y. Acad. Sci. (in press)

Crabtree, G.R., and Kant, J.A.: Organization of the rat γ fibrinogen gene: Alternative mRNA splice patterns produce the γ_A and γ_B (γ') chains of fibrinogen. Cell 31: 159-166, 1982.

Kant, J.A., and Crabtree, G.R.: Linkage of the genes for the α and γ chains of fibrinogen. J. Biol. Chem. (in press)

Kant, J.A., Lord, S., and Crabtree, G.R.: Partial sequences for the mRNAs of the α , β , and γ chains of fibrinogen. Proc. Natl. Acad. Sci. USA (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00574-01 LP
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.) Anti-idiotypic in the investigation and therapy of B-cell lymphoma and Leukemia		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Jeffrey Cossman, Senior Assistant Surgeon, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Hematopathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1	PROFESSIONAL: 3/4	OTHER: 1/4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>We have developed an <u>in vitro</u> system to induce immunoglobulin secretion by malignant B cells (lymphomas and leukemias). The immunoglobulin secreted by these cells (IgM) is purified on an affinity column developed in our laboratory. This highly purified monoclonal immunoglobulin will be used for the immunization of mice and subsequent development of monoclonal (hybridoma) antibodies specific to idiotypic determinants associated with the malignant cells. This antibody will have use both in our basic investigations of immunoglobulin production and cell differentiation as well as for passive immunotherapy. Preliminary evidence in another institution has shown that such antibodies may be efficacious in certain types of low-grade B-cell lymphomas.</p>		

Project Description

Other Professional Personnel: L.M. Neckers, Expert, LP, NCI; J. Trepel, Biologist, LP, NCI; R. Braziel, Medical Staff Fellow, LP, NCI

Objectives: To induce the secretion of immunoglobulin by malignant B cells, to purify this immunoglobulin, and to prepare anti-idiotypic antibodies.

Methods Employed: Cell culture, ELISA quantitative assay system, affinity chromatography, hybridoma preparation, and solid phase ELISA screening assay.

Major Findings: We have found that cells from all cases of chronic lymphocytic leukemia/well-differentiated lymphocytic lymphoma can be induced to secrete immunoglobulin in vitro when exposed to the phorbol ester TPA. In addition, greater than 80% of cases of follicular (nodular) lymphoma will also secrete immunoglobulin either spontaneously or upon exposure to a combination of TPA and allogeneic T lymphocytes. We have developed an IgM affinity column which purifies and concentrates the IgM secreted by these malignant cells with 95-100% recovery of the IgM.

Significance to Biomedical Research and the Program of the Institute: These studies demonstrate that human B-cell neoplasms are monoclonal proliferations that retain the capacity to differentiate under appropriate inducing conditions in vitro. The mechanisms that suppress such differentiation in vivo are of great interest and may relate to the cause and maintenance of the malignant situation. In addition, the production of anti-idiotypic antibodies will allow further dissection of the regulatory system of B-cell differentiation in vitro. Finally, these anti-idiotypic antibodies, which have been shown to have profound therapeutic value in follicular lymphoma, will be provided to Dr. Dan Longo of the Medicine Branch for use as passive immunotherapy for these otherwise incurable lymphomas.

Proposed Course: We will provide purified IgM to Hazelton Laboratories to inject mice and produce monoclonal (hybridoma) anti-idiotypic antibodies under our direct supervision. In addition, we will immunize sheep with the purified IgM to produce heterologous anti-idiotypic antisera for in vitro studies. These studies will include the regulation of immunoglobulin secretion in vitro and the surface signals which affect this secretion.

Publications:

Cossman, J., Neckers, L.M., Arnold, A., and Korsmeyer, S.J.: Induction of differentiation in a case of common acute lymphoblastic leukemia. N. Engl. J. Med. 307: 1251-1254, 1982.

Cossman, J., Neckers, L.M., Braziel, R., Bakhshi, A., Arnold, A., and Korsmeyer, S.: Induction of Differentiation in B-cell Leukemias. In Bernard, A., Boumsell, L., Dausset, J., Milstein, C., and Schlossman, S.F. (Eds.): Human Leucocyte Markers Detected by Monoclonal Antibodies. Springer-Verlag, 1983.

Project Description

Other Professional Personnel: L.M. Neckers, Expert, LP, NCI; J. Trepel, Biologist, LP, NCI; R.M. Brazziel, Medical Staff Fellow, LP, NCI; A. Bakhshi, Research Associate, M, NCI; S.J. Korsmeyer, Senior Investigator, M, NCI

Objectives: To determine the capacity of B-cell neoplasms to further differentiate in vitro.

Methods Employed: Cell culture, ELISA assay, fluorescence-activated cell sorter analysis, cytoplasmic immunoglobulin staining by ABC technique, specific RNA analysis by hybridization techniques including extracted RNA blots, cytoplasmic blots and Northern blots.

Major Findings: 1. We have found that all cases of chronic lymphocytic leukemia/well differentiated lymphocytic lymphoma and the majority of cases of follicular lymphoma can be induced to secrete immunoglobulin in vitro upon exposure to TPA, TPA with T cells, or spontaneously. 2. Immunoglobulin secretion is associated with a conversion to a plasmacytoid or immunoblastic morphology and accumulation of intracytoplasmic immunoglobulin. 3. Of all cell surface markers analyzed, only surface IgD changed and it had a marked decrease. 4. Addition of antibody to the transferrin receptor, OKT9, inhibited immunoglobulin secretion. 5. Immunoglobulin secretion was associated with accumulation of mRNA specific for μ chain of IgM and was predominantly in the form encoding for secretory IgM (2.4 kb) in contrast to the membrane form (2.7 kb) which did not show a relative increase. 6. In a case of precursor B-cell acute lymphoblastic leukemia (common ALL), we have shown that although the cells did not make immunoglobulin in vivo, they could be induced in vitro to make both cytoplasmic and surface immunoglobulin of the isotype (IgM kappa) that was predicted by the immunoglobulin gene rearrangements present in the cells in vivo. 7. The conclusions are that B-cell malignancies appear to be arrested at certain stages of differentiation in vivo but retain the capacity to differentiate further upon manipulation in vitro. The mechanisms governing the differentiation of these cells in vivo remain unknown.

Significance to Biomedical Research and the Program of the Institute: B-cell leukemias and lymphomas are the most frequent type of non-Hodgkin's lymphoma and lymphocytic leukemias in man. Recent advances in the understanding of both the immune system and the molecular biology of the immunoglobulin gene have provided us with tools to dissect the differentiation status of these malignancies. We have found that malignant B cells from a variety of stages of differentiation in vivo retain the capacity to differentiate further. This suggests that in vivo mechanisms either suppress or are ineffective in allowing the differentiation events to occur. Failure of leukemia and lymphoma cells to differentiate further are likely related to the development and maintenance of the malignancy.

Proposed Course: 1. Identify the receptor, if other than the transferrin receptor, that is responsible for inhibition of immunoglobulin secretion when engaged by antibody OKT9. 2. Determine whether accumulation of μ chain mRNA represents increased transcriptional rates or stabilization of the message. 3. Further analyze the effects of allogeneic T cells in their role in inducing immunoglobulin secretion in B-cell neoplasms.

Publications:

Cossman, J., Neckers, L.M., Arnold, A., and Korsmeyer, S.J.: Induction of differentiation in a case of common acute lymphoblastic leukemia. N. Engl. J. Med. 307: 1251-1254, 1982.

Cossman, J., Neckers, L.M., Braziel, R., Bakhshi, A., Arnold, A., and Korsmeyer, S.: Induction of Differentiation in B-cell Leukemias. In Bernard, A., Boumsell, L., Dausset, J., Milstein, C., and Schlossman, S.F. (Eds.): Human Leucocyte Markers Detected by Monoclonal Antibodies. Springer-Verlag, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00851-01 LP
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 TPA-induced immunoglobulin secretion by CLL cells		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Leonard M. Neckers, Expert, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Hematopathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2	PROFESSIONAL: 1	OTHER: 1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p style="margin-left: 40px;"> We have demonstrated that the phorbol ester TPA is capable of causing induction of immunoglobulin synthesis in chronic lymphocytic leukemia cells (CLL). This induction involves increased levels of mRNA coding for the secretory form of IgM. Our goal in this study is to discern the mechanism(s) whereby TPA exerts its effects on these CLL cells. </p>		

Project Description

Other Professional Personnel: J. Cossman, Sr. Asst. Surgeon, LP, NCI; J. Trepel, Biologist, LP, NCI; R. Brazier, Med. Staff Fellow, LP, NCI

Objectives: To uncover and study the way in which TPA induces CLL cells to secrete immunoglobulin.

Methods Employed: Cells from blood or lymph nodes of CLL patients are cultured in the presence of TPA and various enzyme assays are determined. Immunoglobulin levels are measured by ELISA assay and total RNA, DNA and protein synthesis are determined by radioactive tracer methods.

Major Findings: Our findings to date suggest that TPA induces immunoglobulin secretion in CLL cells by increasing calcium flux across the cell membrane. Cells are maintained in G₁ phase of the cell cycle, even though TPA induces an increase in ³H-thymidine incorporation by these cells. This increased uptake of thymidine may be serving a repair function.

Significance to Biomedical Research and the Program of the Institute: B-cell lymphomas and leukemias are thought to be models for normal B cells arrested at different stages of development. The plasma cell stage of B-cell differentiation, when B cells secrete immunoglobulin, is thought to be the end stage for these cells. If we can determine how CLL cells, B cells arrested at an earlier stage of development, can be made to progress to end stage differentiation by TPA, we will have a better understanding of the regulatory processes of normal B-cell differentiation as well as what goes wrong in CLL cells to prohibit them from fully differentiating.

Project Description

Other Professional Personnel: W.A. Blattner, EEB, NCI; D.W. Blayney, EEB, NCI; P. Bunn, DCT, NCI; R.C. Gallo, LTCB, NCI

Objectives: To determine if HTLV, the human retrovirus, is associated with a distinct clinicopathologic entity and to determine if pathologic criteria can be developed to recognize this entity.

Methods Employed: Screening of sera for antibodies to the viral core proteins p19 and p24. Isolation of virus from tumor cells. Pathologic review of all positively identified samples.

Major Findings: HTLV is associated with a distinct clinicopathologic syndrome. Although there is some overlap with other mature T-cell malignancies, some unique features have been identified.

Significance to Biomedical Research and the Program of the Institute: The recognition of defined clinicopathologic criteria for the identification of HTLV-associated malignancies should aid in diagnosis and further definition of the epidemiology of this disease.

Publications:

Blayney, D.W., Jaffe, E.S., Fisher, R.I., Schechter, G.P., Cossman, J., Robert-Guroff, M., Kalyanaraman, V.S., Blattner, W.A., and Gallo, R.C.: The human T-cell leukemia/lymphoma virus, lymphoma, lytic bone lesions, and hypercalcemia. Ann. Intern. Med. 98: 144-151, 1983.

Blattner, W.A., Blayney, D.W., Robert-Guroff, M., Sarngadharan, M.G., Kalyanaraman, V.S., Sarin, P.S., Jaffe, E.S., and Gallo, R.C.: Epidemiology of human T-cell leukemia/lymphoma virus. J. Infect. Dis. 147: 406-416, 1983.

Blattner, W.A., Robert-Guroff, M., Kalyanaraman, V.S., Sarin, P., Jaffe, E.S., Blayney, D.W., Zener, K.A., and Gallo, R.C.: Preliminary Epidemiological Observations on a Virus-associated with T-cell Neoplasm in Man. In Magrath, I., and O'Connor, G. (Eds.): The Influence of the Environment in Leukemia and Lymphoma Subtypes. New York, Springer-Verlag (in press)

Blattner, W.A., Blayney, D.W., Jaffe, E.S., Robert-Guroff, M., Kalyanaraman, V.S., and Gallo, R.C.: Epidemiology of HTLV-associated Leukemia. In Modern Trends in Human Leukemia V, New York, Springer-Verlag (in press)

Bunn, Jr., P.A., Schechter, G.P., Jaffe, E.S., Blayney, D.W., Young, R.C., Matthews, M.J., Blattner, W.A., Broder, S., Robert-Guroff, M., and Gallo, R.C.: Retrovirus-associated adult T-cell lymphoma in the United States: Staging evaluation and management. N. Engl. J. Med. (in press)

Blayney, D.W., Jaffe, E.S., Blattner, W.A., Cossman, J., Robert-Guroff, M., Longo, D.L., Bunn, P.A., Jr., and Gallo, R.C.: The human T-cell leukemia/lymphoma virus (HTLV) associated with American adult T-cell leukemia/lymphoma (ATL). Blood (in press)

Project Description

Other Professional Personnel: N. Holbrook, Guest Worker (American Leukemia Society Fellow)

Objectives: To understand the mechanism of action of mitogens at the genetic level and to understand how glucocorticoids negatively influence the transcription of the TCGF gene. To investigate if and how this gene is involved in certain human T cell lymphoma and leukemias.

Methods Employed: mRNA was isolated from a cloned human cell line (Jurkat 6.8) selected for high levels of TCGF production. This mRNA was used to prepare a cDNA library of over one million clones and the clones for TCGF were selected from the library using chemically synthesized oligonucleotide probes. The gene for this clone will be isolated by screening genomic libraries prepared from normal and malignant lymphocytes. Its structure will be studied using R looping, restriction analysis and S₁ nuclease mapping in normal and malignant cells.

Major Findings: We have obtained a sequence for the TCGF protein from the cloned cDNA which matches the partial sequence obtained by Dr. Kendall Smith of Dartmouth Medical School. We have found that the gene for this growth factor is not present or highly rearranged in normal cells and we are presently trying to isolate this gene from libraries of malignant cells.

Significance to Biomedical Research and the Program of the Institute: Cloning the TCGF gene will allow us to determine if and how it is involved in the pathogenesis of human leukemias. The regulation of this gene will provide a useful system to study the way in which mitogens and glucocorticoids control the expression of genes.

Proposed Course: We will obtain genomic clones for TCGF from normal and malignant cells and define the precise structure of the gene in these cells. We will attempt to relate the enhanced expression of the TCGF gene in certain human malignancies to the uncontrolled growth of these cells. Finally, we will study the control of the cloned TCGF gene in eukaryotic vectors by mitogen and glucocorticoid and attempt to locate sequences in or near the gene which might be responsible for regulating TCGF expression.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00873-01 LP
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.) Recombination of parent and daughter strand DNA after UV in mammalian cells		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Albert J. Fornace, Jr., Expert, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Hematopathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The mechanism, by which DNA is replicated past a non-coding lesion in parent strand DNA in mammalian cells, is poorly understood. Unlike bacteria, general recombination-like events have not been convincingly seen in higher eukaryotes after DNA damage by an agent such as UV-radiation (UV). In this study, I have demonstrated a low level of recombination events as measured by the exchange of pyrimidine dimers from parent to daughter strand DNA. In order to demonstrate clearly such recombination events, cells were irradiated in the G₀ or G₁ phases of the cell cycle and then allowed to enter DNA synthesis. Pyrimidine dimers were measured by a very sensitive enzymatic assay using alkaline elution which I recently developed. Human peripheral blood lymphocytes (PBL), normal human (NF) and Group A xeroderma pigmentosum (XP) fibroblasts, and 3T3 cells were irradiated and then stimulated to enter DNA synthesis by addition of phytohemagglutinin or release from contact inhibition. [¹⁴C]-tdr was added to cells 20h after UV and the cells were analyzed 35h after UV. The frequency of dimers in daughter strand DNA increased with UV dose in all cells and was 1-3% of the dimers estimated to be present in the parent DNA. After 4 Jm⁻², 0.2, 1.2, or 1.3 dimers/10⁹d of DNA were detected in the newly synthesized DNA of NF, 3T3 cells, or PBL respectively, and 1.0 dimer/10⁹d after 1 Jm⁻² in XP. Analysis by fluorescence activated cell sorter analysis, incorporation studies, and autoradiography indicated that the cells were irradiated prior to S phase. Appreciable incorporation into parent strand DNA by repair synthesis was also excluded.</p>		

Project Description

Other Professional Personnel: L.M. Neckers, Expert, LP, NCI (FACS analysis)

Objectives: To determine if general recombination-like events occur in mammalian cells after UV-radiation.

Methods Employed: Cell culture, alkaline elution in combination with prior digestion of the cell DNA by a preparation from M. luteus containing a pyrimidine dimer specific endonuclease, autoradiography, fluorescence-activated cell sorter analysis.

Major Findings: 1. Pyrimidine dimers were detected in the daughter strand DNA from normal human fibroblasts, peripheral blood lymphocytes, xeroderma pigmentosum fibroblasts, and mouse 3T3 cells. 2. The frequency of exchange events was approximately 1-3% of the total dimers in the parent strand DNA. 3. Incorporation of radioactive label into daughter strand prior to UV, appreciable incorporation of label into parent strand DNA by repair synthesis, and anomalous elution of daughter strand DNA were excluded. 4. These results indicate that recombination events between parent and daughter strand DNA do occur in mammalian cells after UV. A reasonable interpretation is that a small length of parent and daughter strand DNA is exchanged, and occasionally parental DNA containing the dimer remains in daughter DNA.

Significance to Biomedical Research and the Program of the Institute: Recombination-like events have been observed in viral and plasmid DNA in mammalian cells. On a cytogenetic level, sister chromatid exchanges (SCE) occur and increase in frequency after treatment of the cells with mutagens or carcinogens. My approach offers a biochemical method to detect recombination events. This may offer an approach to study the mechanisms of SCE. It should prove useful in the study of recombination events in mammalian cells induced by various mutagens and carcinogens. The biologic consequences of cells deficient in this activity may be of particular interest.

Proposed Course: Undetermined.

Publications:

Fornace, A.J., Jr.: Recombination of parent and daughter strand DNA after UV-irradiation in mammalian cells. Nature (in press)

Project Description

Other Professional Personnel: J. Cossman, Sr. Assistant Surgeon, LP, NCI; W. Funkhouser, Clinical Associate, Surg., NCI; E. Grimm, Expert, Surg., NCI; S. James, Clinical Associate, I, NCI; G. Yenokida, Clinical Associate, I, NCI

Objectives: To discern the regulation of transferrin receptor appearance in mitogen-stimulated peripheral blood lymphocytes as well as transformed cell lines, and to study the role of these receptors in proliferation and growth.

Methods Employed: Quantitation of transferrin receptors is made by use of the cell sorter. DNA, RNA and protein synthesis are measured by incorporation by cell of radioactive substrates.

Major Findings: A. Cell lines: We have shown that transferrin receptor expression is directly correlated with cellular metabolic rate as well as growth rate. Antibodies to the receptor stop cell proliferation with cells accumulating in S phase of the cell cycle. Receptor expression in normal cells is not cell cycle stage dependent. Enhancement of receptor expression has been observed in cells treated with a DNA polymerase inhibitor. The mechanism by which this occurs appears to be via inhibition of internalization of the receptor. Transferrin receptor density on the cell surface may be closely linked to DNA polymerase activity. Modulation of receptor density may internally regulate DNA synthesis via polymerase inhibition. B. Mitogen-stimulated lymphocytes: We have shown that transferrin receptor expression is dependent on the presence of monocytes. The phorbol ester TPA can replace monocytes in this regard. Without the presence of the transferrin receptor, mitogen-stimulated lymphocytes will not enter S phase. This has been shown by removing monocytes from the culture or by using an anti-receptor antibody. The role of TPA or monocytes is related to the stimulation of IL-2 production in the stimulated T cells. IL-2 receptor induction and IL-2 synthesis are required for transferrin receptor induction on T cells. Once the transferrin receptor is induced, entry of the cell into S phase can only be prevented by blocking transferrin binding. In B cells, transferrin receptors are induced by mitogen treatment and are required for B-cell proliferation (clonal expansion) but not for immunoglobulin secretion. Thus, B cells do not have to pass through a clonal expansion step before they can differentiate to plasma cells.

Significance to Biomedical Research and the Program of the Institute: What triggers a cell to divide is currently under intense investigation. Learning in a normal cell what this trigger(s) is(are), would be a monumental step in the understanding of cancer. Since the transferrin receptor is present on every type of dividing cell studied to date, and since its removal prevents cell division, we feel that studying the regulation of this receptor in normal and transformed lymphocytes will add to the understanding of what regulates cell division.

Proposed Course: We plan to continue our studies in two basic areas: 1) the role of the transferrin receptor in cell growth (using transformed cell lines) and 2) the role of the receptor in activation, proliferation and differentiation of normal lymphoid cells.

Publications:

Neckers, L.M., and Cossman, J.: Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin-2 (TCGF). Proc. Natl. Acad. Sci. USA (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00882-02 LP
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.) Use of the cell sorter to clone hybridoma cells secreting idiotype		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Leonard M. Neckers, Expert, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any) Transplantation Biology Section, Immunology Branch, NCI; Laboratory of Molecular Biology, NCI		
LAB/BRANCH Laboratory of Pathology		
SECTION Hematopathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.2	OTHER: 0.3
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The ability to select hybridoma cells secreting an antibody of choice without resorting to cumbersome screening techniques would greatly decrease the time involved in obtaining those cells. We have perfected a method which uses fluoresceinated anti-idiotypic antibody to identify those hybridoma cells making antibody of a specified idiotype. These cells are identified and cloned by the cell sorter in a one step procedure, greatly reducing the effort needed to obtain hybridomas of choice.</p>		

Project Description

Other Professional Personnel: J.B. Trepel, Biologist, LP, NCI; I. Pastan, Chief, LMBGY, NCI; G. Merlino, LMBGY, NCI

Objectives: To be able to rapidly identify and clone idiotype-secreting hybridomas from a mixed population of hybridoma cells, as well as transfectants bearing unique surface antigens.

Methods Employed: Cells are tagged with fluoresceinated anti-idiotypic antibody and tagged cells are visualized and cloned by the cell sorter in a one-step procedure.

Major Findings: To date, we have observed a direct correlation between the presence of surface immunoglobulin and secretion of immunoglobulin. Likewise, there is a direct correlation between the presence of surface idiotype and idiotype secretion. Thus, by cloning surface idiotype-positive hybridoma cells using the cell sorter, we obtain idiotype-secreting clones. We have now expanded this approach to other systems and have found it to be a consistently successful technique for hybridoma screening. We are currently compiling the data for submission to a professional journal.

Significance to Biomedical Research and the Program of the Institute: Using such a rapid procedure, isolating idiotype-secreting hybridomas is much quicker. This has some clinical significance in that it was recently reported that anti-idiotypic antibody killed (*in vivo*) a patient's idiotype-bearing B-cell lymphoma (Miller et al., *N. Engl. J. Med.* 306: 517, 1982). Using our technique, we could easily isolate hybridomas making anti-idiotypic. Production of relevant antibody would thus be greatly speeded up.

Proposed Course: We now are using the technique for screening and cloning mouse cells transfected with human DNA, to find those cells expressing human transferrin receptors and EGF receptors. Positive cells will be FACS-cloned, expanded and used to obtain cDNA for these receptors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00883-02 LP
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.) Malignant lymphomas: analysis with monoclonal antibodies on tissue sections		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Su-Ming Hsu, Medical Staff Fellow, Laboratory of Pathology, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Hematopathology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.2	PROFESSIONAL: 1.2	OTHER: 1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>A variety of monoclonal antibodies (hybridomas) have been recently developed that distinguish among classes of normal human lymphocytes and identify discrete stages of differentiation. We are using a battery of these antibodies to determine the phenotypes of human malignant lymphomas using an immunohistochemistry technique. The phenotypic expression of these neoplastic lymphocytes is then related to normal lymphocytes and is useful in diagnosis and monitoring of patients' tumors during therapy.</p>		
723		

Project Description

Other Professional Personnel: E.S. Jaffe, Chief, Hematopathology Section, LP, NCI; J. Cossman, Sr. Assistant Surgeon, LP, NCI

Objectives: To identify and characterize human malignant lymphomas.

Methods Employed: Immunohistochemistry; tissue culture; hybridoma development.

Major Findings: 1. Documentation of reported specificity of monoclonal antibodies. 2. Screening and determination of specificities of antibodies to Hodgkin's tumor cells. 3. Minor diversity of phenotypic expression of hairy cell leukemias and Hodgkin's diseases. The neoplastic cells bear similarity in their marker expression. 4. Determination of marker expression associated with normal differentiation. 5. A great majority of cutaneous T cell lymphomas express leu 1⁺/3A1⁻ phenotype. 6. Documentation of tumor cell uptake of monoclonal antibody after serotherapy.

Proposed Course: 1. Determine phenotypes of malignant lymphomas using monoclonal antibodies. 2. Correlation of marker expression with morphology and clinical course. 3. Determination of selective markers that can be used for differential diagnosis and staging.

Publications:

Hsu, S.M., Cossman, J., and Jaffe, E.S.: Lymphocyte subsets in normal human lymphoid tissues. Am. J. Clin. Pathol. (in press)

Hsu, S.-M., Yang, K., and Jaffe, E.S.: Hairy cell leukemia: A B-cell neoplasm with a unique antigenic phenotype. Am. J. Clin. Pathol. (in press)

Hsu, S.-M., Zhang, H.-Z., and Jaffe, E.S.: Utility of monoclonal antibodies directed against B and T lymphocytes and monocytes in paraffin-embedded sections. Am. J. Clin. Pathol. (in press)

Hsu, S.-M., Cossman, J., and Jaffe, E.S.: A comparison of ABC, unlabeled antibody and conjugated immunohistochemical methods with monoclonal and polyclonal antibodies--an examination of germinal center of tonsils. Am. J. Clin. Pathol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00517-42 LP
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.) Report from the Pathological Technology Section		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Barbara J. Coolidge, Chief, Pathological Technology Sect., Lab. of Pathol., NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathology		
SECTION Pathological Technology Section		
INSTITUTE AND LOCATION NCI, FCRF, Frederick, Maryland 21701		
TOTAL MANYEARS: 5	PROFESSIONAL: 0	OTHER: 5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Stained tissue sections are the fundamental basis of all clinical and experimental studies of cancer. The Section prepares histological sections for the investigators of the National Cancer Institute. It makes available all the established routine and special stains and in addition develops and provides the current experimental methods of tissue preparation such as enzyme stains and specific histological stains.</p>		

October 1, 1982 - September 30, 1983

Number of investigators.....	64
Number of pieces of tissue.....	22,175
Number of bottles of tissue.....	7,963
Number of blocks cut.....	11,054
Number of blocks cut serially.....	1,971
Number of frozen blocks cut.....	349
Number of blocks recut.....	908
Number of slides stained H & E.....	49,469
Number of special stained slides.....	2,463
Number of unstained slides.....	10,311
Total number of slides - H & E, special, unstained.....	62,243

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00885-02 LP
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.) Automated 2D gel analysis using computerized data base methods		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Peter F. Lemkin, Computer Scientist, Image Processing Sec., Lab. of Path., NCI		
COOPERATING UNITS (if any) NIAID, Lab. Parasitic Disease; University of Chicago, Department of Medicine (E.P. Lester, Asst. Prof. Med.); MNS, LMB, NINDS, NICHD		
LAB/BRANCH Laboratory of Pathology		
SECTION Image Processing Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.5	PROFESSIONAL: 2.5	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Employing GELLAB, a system of state of the art computer/data base techniques, we automatically detect and relate qualitative and quantitative differences among protein moieties produced by differing experimental conditions in sequences of 2 dimensional electrophoretic polyacrylamide gels prepared by our collaborators. A series of differences have been found in preliminary analysis of a small leukemic data base between AML, ALL, CLL, and HCL leukemias. Additional gels were added to the leukemia data base to bring it up to 114 gels. Additional gels were added to the whole cell HL60 data base. Additional search is now underway on both of these data bases. Differences in red cell surface proteins correlating with partial immune response to Plasmodium knowlesi has been further investigated. The quantitative aspects of the silver stain on mouse embryo 48 hr cultures have shown it to be linear for low concentrations of protein (within a particular protein curve). Several spot differences were also found in the mouse embryo cultures when treated with several anticonvulsants. Results of a feasibility study in measuring protein synthesis of presynaptic axonal proteins during synapse formation of chicken embryonic dorsal root ganglia in TC have shown a small number of changes. Final results of protein changes in mRNAs in HL60 cell line and changes in Friend virus infected murine ethroleukemia cells under chemical induced differentiation were submitted for publication. Additional refinement of the component algorithms continues to make GELLAB an even more effective a tool for cell biological and clinical investigation. Recent work has allowed us to measure very light noisy spots previously seen by the system as fragmented. Extensive set operations have been added which aids in manipulating sets of spots found in the merged gel data base under various statistical search conditions.		

Project Description

Other Professional Personnel: L.E. Lipkin, Chief, Image Processing Section, LP, NCI; M.L. Schultz, Senior Engineer, Image Processing Section, LP, NCI; E.M. Smith, Expert, Image Processing Section, LP, NCI; R.P. Howard, Expert, Malaria Section, LPI, NIAID; S. Aley, Research Fellow, Malaria Section, LPI, NIAID

All gels and the radioautographs related to them are produced by our collaborators and are entered as images via our image processing system. Detection of quantitative differences among similar spots in 2D gels is complicated by local inhomogeneties in the polyacrylamide base. Biochemical changes and variations in temperature and preparative technique also make the gel density and x-y coordinate correspondences quite imprecise. This plus the obvious need to deal with a thousand or more spots in each of as many as a hundred gels required the construction of a system of GELLAB's complexity. The system is essentially a 3 stage one in which the first is concerned with segmentation (i.e. pulling out the spots from noise background) and the establishing some local integral correspondences (landmarks). The next stage deals with a group of derived parameters, i.e. constructs developed from images taken two at a time based on regional correspondences established by the landmarks. The last stage brings to bear a powerful array of interactive, statistical and display procedures which allow the user to vary the constraints under which he operates while searching the spot data base for various types of changes in spot patterns. The keystone of this analysis is the R-gel, the representative gel image. It is the reference around which the total spot data base is organized. The primary data base consists of the totality of the lists of corresponding spots and their associated properties and interrelations. The multiple gel data base system is very general in that gel segmentation and spot pairing algorithms other than those now used in GELLAB may be substituted in the early phases of data base construction. The final phase offers the user the ability to interactively and/or automatically employ data base construction techniques, partition data bases according to external or developed information, search the data base under various constraints and/or restrictions. He is allowed to extrapolate into the data base missing spots and can format the data base almost at will.

Publications:

Lemkin, P.F., Lipkin, L.E., and Lester, E.P.: Some extensions to the GELLAB two-dimensional electrophoretic gel analysis system. Clin. Chem. 28: 840-849, 1982.

Lemkin, P.F., and Lipkin, L.E.: 2D electrophoresis gel data base analysis: Aspects of data structures and search strategies in GELLAB. Electrophoresis 4: 71-81, 1983.

Lemkin, P.F., and Lipkin, L.E.: Data Base Techniques for Two-Dimensional Electrophoretic Gel Analysis. In Geison, M.J., and Barrett, A. (Eds.): Computing in Biological Science. Amsterdam, Elsevier/North Holland Biomed. Press, 1983, pp. 181-231.

Lester, E.P., Lemkin, P.F., and Lipkin, L.E.: A two-dimensional gel analysis of autologous T and B lymphoblastoid cell lines. Clin. Chem. 28: 828-839, 1982.

Lester, E.P., Lemkin, P.F., Lowery, J.F., and Lipkin, L.E.: Human leukemias: A preliminary 2D electrophoretic analysis. Electrophoresis 3: 364-375, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB00886-02 LP
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.) Analysis and synthesis of nucleic acid secondary structure		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Bruce A. Shapiro, Computer Scientist, Image Processing Sec., Lab. of Path., NCI		
COOPERATING UNITS (if any) NICHD, Laboratory of Molecular Genetics; NCI, Laboratory of DNA Tumor Viruses		
LAB/BRANCH Laboratory of Pathology		
SECTION Image Processing Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.6	PROFESSIONAL: 1.6	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p style="margin-left: 40px;"> This research may be viewed as two sides of the same coin, i.e. 1) the analysis of nucleic acid structure as revealed by electron micrographs and 2) the computer synthesis of the secondary structure of nucleic acid molecules, given the sequence of bases in their known order, the synthesis being based on thermodynamic (minimum free energy) considerations. Novel methods of representing structure are being developed as well as interactive programs which allow clarification of these structures. Exploration of algorithms which will measure similarity is continuing. Both aspects would benefit from information provided by the other and one of the eventual goals is a combined analysis-synthesis procedure, which will reduce the huge combinatoric overload of secondary structure computation, which will eliminate the ambiguities of electron-micrographic images of partially denatured nucleic acid molecules. </p>		

Project Description

Other Professional Personnel: L.E. Lipkin, Chief, Image Processing Section, LP, NCI; M.L. Schultz, Senior Engineer, Image Processing Section, LP, NCI; E.M. Smith, Expert, Image Processing Section, LP, NCI; J.V. Maizel, Chief, Molecular Structure Section, LMG, NICHD, R. Dhar, Visiting Scientist, LDNATV, NCI; K. Currey, LMG, NICHD

The two drawing algorithms that were previously reported have been enhanced. The polygonal algorithm now allows the searching for specific base patterns and allows the superposition of these base patterns onto the secondary structure to determine in what types of structures these patterns reside. The ability to label base positions has also been added. Over 150 molecules have been drawn using this algorithm. The size molecule that the algorithm can accommodate has also been increased to permit sizes up to 1500 bases.

The canonical spider-like drawing algorithm has also been enhanced to accommodate similarity comparisons amongst several folded molecules. The facility now exists to extend a sequence from its 3' end to allow molecules that are of different lengths to be mapped to the same length. The canonical drawing may then be rotated to facilitate comparison. Currently, similarity experiments using this technique are underway with adenovirus molecules, various viroids and Harvey sarcoma virus.

Another similarity measuring technique has been enhanced and expanded. This involves the use of circle graphs. Chords drawn from one part of the circumference of a circle to another represent base pairings. Morphologic entities of secondary structure are discernible by the different patterns the chords make within the circle. Similarity determinations are done by extending sequences from their 3' end to map them to the same size and then rotating the circle graphs to obtain the proper orientation. Similarity comparisons are now being done using this technique on the same class of molecules that are mentioned above.

The circle graph technique is also being used in a novel way for the generation of movies to examine how the secondary structure changes as a molecule is being synthesized from the 5' end. by using the energy matrix generated by the paging folding algorithm (an extension by our section of the original folding algorithm by Michael Zuker), one can generate a circle graph of the molecule at each stage of its development and thereby determine how its secondary structure is changing. We have found, for example, that certain 5srRNA's remain as linear structures for most of their development until they form their final "Y" configuration.

The paging folding algorithm has also been enhanced to increase its speed. Since paging by its very nature is slow because the algorithm has to go to a slow storage media, i.e. disk, an examination was made of the access pattern in the various matrices that are used in the folding algorithm. A program that used a graphical display was developed that clearly showed a particular access pattern. Using this information two line buffers were added to ensure fewer disk accesses. This increased the running speed of the paging folding algorithm by about a factor of 2.

In order to analyze secondary structures and their significance, we have compared the predicted structure(s) of natural sequences with the same sequence which had been randomly shuffled. In sequences known to have high secondary structure such as viroids and 5srna, we found the natural sequence to have a more stable predicted structure and agree better with the chemical data. This approach is being pursued on selected adenovirus sequences. We tentatively observed that the natural sequence not only has regions of greater stability than the shuffled one but also that some regions have lower stability, suggesting that the potential both for forming and avoiding secondary structures has evolved in genomes. Further analysis of the natural and shuffled sequence stability data led to the observation that stability (or energy of the structure) is linear with G+C content. Thence, from these data it is possible to predict a stability for a given region or molecule and assess the likelihood of the presence or absence of significant secondary structure for that sequence. In addition, artificially created, shuffled sequences of varying length and specific base composition were examined and the energy (stability) was found to be directly proportional to length. These properties are currently being analyzed in finer detail with regard to how varying the base composition influences the stability (energy) of single strand RNAs produced by genomes.

Publications:

Dhar, R., Ellis, R., Shih, T.Y., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D., and Scolnick, E.: Nucleotide sequence of the p21 transforming protein of Harvey murine sarcoma virus. Science 217: 934-936, 1982.

Shapiro, B.A., Lipkin, L.E., and Maizel, J.: An interactive technique for the display of nucleic acid secondary structure. Nucl. Acids Res. 10: 7041-7052, 1982.

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SUMMARY REPORT
IMMUNOLOGY BRANCH
October 1982 - September 1983

The Immunology Branch carries out laboratory investigations in basic immunobiology with particular emphasis in the following areas: 1) Regulation and control of immune responses; 2) Structure and function of cell surface molecules; 3) Transplantation biology; 4) Molecular Biology and 5) Tumor immunology including clinical studies in immunotherapy. In addition, the Immunology Branch maintains a fluorescence activated cell sorter facility which is involved integrally in many of the studies carried out in the Branch in each of the above areas, and is also used in a large number of collaborative investigations with other laboratories at NIH. This report summarizes research efforts in each of these areas during the past year. More detailed information on specific accomplishments can be found in the individual annual reports.

A. REGULATION AND CONTROL OF IMMUNE RESPONSES

Work in Dr. Hodes' laboratory 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. Two pathways have been characterized for the regulation of cytotoxic T lymphocyte responses to alloantigens and modified self antigens. One pathway is antigen non-specific and is mediated by Lyt 1⁺2⁻ T cells, while the other is antigen-specific and is mediated by Lyt 1⁺2⁺ T cells. These regulatory mechanisms provide models for the study of allograft tolerance and regulation of T cell mediated immune response. (5055) In addition, T cell dependent antibody responses were shown during the past year to be regulated by two independent T_G cell pathways. The mechanism of this suppression involves a previously undescribed form of MHC restriction in which antigen-specific and MHC-restricted T_G cells act to suppress only these responses generated by T_H cells expressing the same antigen specificity and MHC restriction specificity. Cloned T_G cells with these characteristics have been generated and are now being characterized. (5108)

Dr. Hodes' laboratory has also studied the cellular mechanism of Ir gene expression for a series of immune responses controlled by genes mapping to I-A, I-B or I-E. The effect of Ir genes on the interaction of T cells with B cells and/or accessory cells has been shown to depend upon the pathway of B cell activation functioning under given response conditions. Thus, the ability to circumvent Ir gene restrictions may be dependent upon the ability to select specific pathways for immune response activation. (5086)

Studies in the laboratory of Dr. Sachs have utilized idiotypic determinants on anti-Staphylococcal nuclease (nuclease) antibodies from different mouse strains as markers for investigations of genetic control of the immune response to nuclease. Several hybridomas reactive with nuclease and/or anti-idiotype have been produced. In a collaborative effort between the laboratories of Drs. Sachs and Hodes, an investigation of idiotype expression on cells involved in the in vitro immune response to nuclease has been pursued. T helper cells in the in vitro anti TNP-nuclease response have been found to bear idiotype on their surface. T cell clones reactive with nuclease have

been produced and are being examined for idiotypic reactivities.

Collaborative studies of Drs. A. Singer and R. Hodes have been directed toward identifying the cell surface elements which regulate cellular collaborations. The recent observation that the activation of B cells can involve a genetically regulated interaction with macrophages (5064) suggests the existence of a new mechanism by which MHC genes regulate the immune response. Recent studies in Dr. Singer's laboratory on the activation requirements of distinct B cell subsets have revealed that the Lyb 5⁻ B cell subset is functionally heterogeneous as determined by responsiveness to lyopolysaccharide (5090). This observation bears on our understanding of B cell development and activation. Elucidation of the roles performed by macrophages in the activation of allospecific CTL (5111) has demonstrated that there exists two distinct T cell activation pathways mediated by macrophage. There exists an Ia dependent activation pathway which involves self-recognition of macrophage Ia determinants, and a distinct Ia independent activation pathway which involves recognition by macrophage K/D determinants. These observations provide important insights into potential mechanisms of modulating alloreaction and of prolonging allografts.

Dr. Shearer's laboratory is investigating the immunosuppressive potential of chronic graft-versus-host (GVH) reactions in mice. These studies indicate that: (a) allogeneic class II major histocompatibility antigens are all that needs to be recognized in order to initiate profound immunosuppression *in vivo*; and (b) such suppression can be prevented by treating donor or recipient mice with anti-H-2 monoclonal antibodies. These observations may be relevant for elucidating various aspects of GVH disease and could be important in the etiology of acquired immune deficiency syndrome (AIDS). (5088) Other studies in Dr. Shearer's laboratory have shown that under certain conditions cyclophosphamide (Cy) selectively abrogates suppressor cell effects *in vivo*. Cy has been used to prevent the induction of immunosuppression induced by graft-versus-host reactions and to "rescue" mice suppressed by graft-versus-host. These studies could be of potential value in treating AIDS patients if graft-versus-host-induced suppression is shown to be a factor in the etiology of this syndrome. (5109) Synergistic effects of cytomegalovirus and graft-versus-host-induced immunosuppression have been observed, depending on the strain of mouse used and the sequence of exposure to virus and lymphocytes. These studies could likewise be of relevance in graft-versus-host disease and in the etiology of AIDS. (5099)

Dr. Shearer's laboratory is also following a number of immune response profiles of healthy human donors (homosexual men) who are at high risk for acquired immune deficiency syndrome (AIDS) in a prospective study. Peripheral blood leukocytes from approximately 20% of donors repeatedly exhibit reduced cytotoxic T cell and interferon responses to influenza virus *in vitro*, although they do not exhibit reversals in helper:suppressor T cell ratios (considered to be characteristic and predictive for AIDS). These findings may be important in predicting a very early stage in AIDS and/or in identifying individuals who are immune-compromised and susceptible to AIDS. (5110)

Work in Dr. Segal's laboratory has shown that treatment of ADCC effector cells with antibody which has been chemically cross-linked with an anti-FcγR monoclonal antibody, renders them potent effectors against cells bearing the appropriate antigenic determinants. Unlike normal ADCC effectors these "franked" effectors are not inhibited by monomeric IgG or

Since all IgG mediated effectors are strongly inhibited by serum IgG, this technique may provide one way to artificially override the normal control exerted by serum IgG on IgG-dependent effectors, in vivo, thus producing enhanced responses against various types of targets including tumor cells and pathogenic organisms. (5050)

Work in the laboratory of Dr. Howard Dickler 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: (a) illuminate a mechanism for perpetuation of humoral immunity; and (b) provide a theoretical basis for obtaining immunity in the absence of antigen. (5058) Studies by Dr. Stephen Shaw (in collaboration with Dr. W. Biddison, NINCDS) have explored the functional role of T cell differentiation markers in human T cell recognition and activation. Evidence from studies of blocking of function of HLA-SB-specific T cells indicate that the OKT4 molecule functions in activation of many MHC class II specific T cell clones. (5067)

B. STRUCTURE AND FUNCTION OF CELL SURFACE MOLECULES

Work in the laboratory of Dr. Pierre Henkart directed at the mechanism of lymphocyte mediated cytotoxicity has established that cytoplasmic granules are the source of cytolytic material during NK cell cytotoxicity. Purified granules from NK tumor cells have been shown to be highly cytolytic to a wide variety of cells in a rapid and calcium dependent process. (5103, 5018)

Studies in the laboratory of Dr. Howard Dickler have been directed toward mechanisms involved in the triggering and regulation of immunocompetent cells and the role that cell surface molecules play in this triggering. Current studies in this area are focused on extending the finding that monoclonal anti-Fc γ receptor antibodies trigger B cells to secrete antibody. This work may have important implications for understanding the mechanisms of B cell triggering (5035).

The laboratories of Drs. Segal and Shearer have collaborated in studies which show that the modification of K^b spleen cells with TNP causes them to bind several monoclonal antibodies specific for K^k. This appearance of neo H-2 like antigens resulting from chemical modification of the cells parallels previous observation on the cytotoxicity of CTL against hapten modified targets, and may be important in the functioning of MHC class I molecules in general. (5038, 5050) In addition, Dr. Segal's laboratory has studied the distributions of Fc γ R on subpopulations of B and T cells in the mouse using a dual parameter flow cytometric technique. This information is essential in understanding the multiple ways in which Fc γ R function in the immune system. (5050)

C. TRANSPLANTATION BIOLOGY

Studies in Dr. David Sachs' laboratory have been directed toward understanding of the structure and function of products of the major histocompatibility complex, and manipulations of the immune response to these products.. A large number of hybridoma cell lines producing antibodies to H-2 and Ia antigens have been produced and characterized. (5021) 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. (5021, 5112) Mechanisms of tolerance in bone marrow chimeric animals have also been investigated. Evidence for non-specific suppressor cell generation in bone marrow chimeras was obtained at times up to six weeks following grafting. At later dates no evidence for suppressor cells of either specific or non-specific nature was obtained, supporting the hypothesis of clonal deletion rather than active suppression. (5021) Reconstitution of lethally irradiated mice with mixtures of syngeneic and either allogeneic or xenogeneic bone marrow has been performed. These mice are being evaluated for specific allo-immune hyporeactivity.

Studies by Dr. Jeffrey A. Bluestone have been directed toward dissecting the recognition structures of alloreactive T cells. The recent findings that anti-idiotypic antibodies modify in vivo alloantibody responses but do not appear to affect T cell-mediated alloreactivity, suggest that either the recognition structures of T cells and B cells are substantially different or the allodeterminants recognized by these cells are not the same. (5112)

Studies of transplantation biology in the miniature swine model have been continued. Milligram quantities of histocompatibility antigens have been prepared from individual pig spleens, and N-terminal amino acid sequences have now been obtained for the SIA antigens of all three partially inbred lines of miniature swine. (5023) Two new recombinants within the MHC have been detected within the miniature swine herd, both separating the MLC stimulatory locus (SIA-D) from the serologic loci (SIA-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. (5023)

The alloreactive repertoire has been examined by Dr. Hodes and colleagues for responses to cell surface antigens including K^b mutant-encoded class I antigens and the non-MHC-linked mls antigens. Findings indicate that the T cell repertoire for recognizing K^b mutant products resembles the conventional "self + X" repertoire in its dependence upon the interaction of responder genotype and T cell maturation environment. These results have theoretical implications for studies of the T cell repertoire as well as potentially practical implications for immune competence in clinical bone marrow transplantation. (5106)

Work in the laboratory of Dr. Alfred Singer has focused on the induction of T cell tolerance to self components. Recent studies 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 (5093). These findings have important implications for our understanding of the T cell repertoire and which elements induce T cell tolerance to MHC and non-MHC encoded self antigens. Other studies have shown that the induction of T cell tolerance to self antigens involves recognition of self H-2 determinants (5107). Thus, T cells are not tolerant to self antigens per se but are only tolerant to the antigenic complex of self antigens + self H-2. These findings are among the first demonstration that MHC genes not only regulate T cell activation but also regulate the induction of self-tolerance.

The laboratory of Dr. Stephen Shaw has continued to characterize the new HLA gene HLA-SB. This work has led to recognition of Dr. Shaw's laboratory as the world reference for studies relating to the HLA-SB locus, originally described in this lab. Rapid progress has continued in defining the genetics, structure and function of this locus and its products through local and collaborative projects. This locus is proving to be important in regulation of human immune responses and association with disease states. (5101, 5100) Studies have been also initiated in Dr. Shaw's laboratory to systematically analyze cellular reactivity to human minor histocompatibility antigens. New approaches to generate specific cloned cells promise to provide powerful tools to explore such responses which are important in clinical tissue transplantation and as a model, relevant to tumor immunology, of responses to weak immunogens. (5101)

D. MOLECULAR BIOLOGY

Work in the laboratory of Dr. Dinah Singer has been directed towards the elucidation of the structure, genomic organization and regulation of expression of porcine MHC genes. It has been established that the structure of 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 about the evolution of this multigene family. The over-all organization of the swine MHC is being determined by analysis of over-lapping cosmid clones containing class I and class II genes. The regulation of expression of class I genes is being investigated in a model system in which the expression of a swine class I gene transferred into mouse L cells has been shown to be actively regulated. The role of MHC-linked sequences in regulating gene expression is being studied by analyzing the ability of deletion mutants to express MHC antigens following transfer to mouse L cells. The mechanisms leading to differential expression of MHC genes in vivo are also under investigation. (5083)

E. TUMOR IMMUNOLOGY

Dr. John Wunderlich's laboratory has been studying mechanisms in mice which generate cell-mediated cytotoxic reactions against chemically-induced, syngeneic tumor cells. One mechanism, which is mediated by T lymphocytes, is generated by a response which differs from previously described cytotoxic T cell responses; it may offer new opportunities for manipulating host anti tumor activity. (5003)

Ultrastructural studies, by Dr. M. Henkart's laboratory, 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. These observations suggest that the granule contents might contain the product(s) that mediate cytotoxic function. (5102) These observations were the basis for to study of the mechanism of NK killing by isolating granules from NK cells. The isolated granules have been found to be highly cytotoxic by a strictly calcium dependent mechanism. (5018) Recent studies in the laboratory of Dr. Howard Dickler have shown that monoclonal anti-Fc γ receptor antibodies will stimulate natural killer (NK) cell activity against tumors. This work has implications for the mechanisms of NK cell triggering. (5035)

The S-49 T cell lymphoma line of BALB/c origin should express H-2K^d, 2-2D^d and H-2L^d antigens. Studies in Dr. Shearer's laboratory have shown that five sublines of S-49 do express all of these H-2 antigens, and four different patterns of H-2^d expression have been observed. This model may be useful for investigating regulation of expression of MHC antigens and raises the possibility of a high rate of modulation of these antigens among tumor cell lines of the same origin. (5104)

A controlled randomized clinical trial has been conducted comparing MeCCNU chemotherapy to immunotherapy with BCG or BCG plus allogeneic tumor cell vaccine for the adjuvant treatment of human malignant melanoma. A total of 181 patients was entered and treated, and preliminary evaluation reveals no significant differences between treatment groups in either recurrence or survival. (5033)

F. FLUORESCENCE ACTIVATED CELL SORTER

The Immunology Branch has continued to maintain an active Fluorescence Activated Cell Sorter Facility operated by Ms. Susan Sharrow and under the guidance of Dr. J. Wunderlich. The Fluorescence Activated Cell Sorter (FACS) has been used both to analyze and to separate lymphoid populations by rapid flow microfluorometry. Studies performed in this facility have been an integral part of many of the investigations described in the above sections, as well as of numerous collaborative studies with other investigators at NIH and elsewhere. Among the studies supported by flow microfluorometry are: 1) Mutation-induced changes in I-region genes have been correlated with functional changes in MHC-restricted, antigen-presenting cells by FMF phenotyping of these cells and isolation of viable, low-frequency mutants for subsequent functional testing (5062). 2) Cell phenotyping and purification by FMF has been used in the study of immunoglobulin gene rearrangement which occurs early in the course of human B cell maturation (5062). Several projects have been supported by the ability of FMF to analyze three independent parameters of individual cells at rates of several thousand cells a second: a) correlating human lymphocyte phenotypes with function (5062), b) examining biologic processes which are initiated by cell-cell conjugation (5062, 5050), c) measuring the effects of lipid packing density and the cell cycle on the expression of class I MHC determinants (5003), and d) analyzing determinants detected serologically which are shared by the products of different genes (5062).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05003-18 I
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-Mediated Cytotoxicity		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) John R. Wunderlich, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.0	PROFESSIONAL: 2.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Mouse effector cells mediating broadly reactive anti-tumor cytotoxic activity, induced under syngeneic conditions in vitro with polyinosinic acid and phorbol-induced lymphocyte growth factors, can be generated not only by unprimed spleen, thymus, and fetal liver cells, but also (unlike classic cytotoxic T lymphocytes) at augmented levels by spleen cells depleted of Thy 1-bearing cells and thymocytes depleted of Lyt 2-bearing cells. Higher levels of H-2 expression on mitogen-induced mouse lymphoblasts in the G2 than G0 or G1 portions of the cell cycle have been demonstrated by flow microfluorometry. This difference cannot be attributed to differences in cell size determined by low angle light scatter. The cholesterol probe, filipin, has been adapted to flow microfluorometry for quantitative detection of cholesterol at the single cell level.		

Project Description

Other Professional Personnel Engaged on the Project:

N. A. Dorfman, Guest Worker, Immunology Branch, NCI
C. Muller, Visiting Fellow, Immunology Branch, NCI

Objectives: This project has been directed at understanding and manipulating mechanisms of cell-mediated immune cytotoxicity in both humans and mice. Long-range goals are: (1) to identify and characterize factors which influence induction, maturation and expression of cytotoxic cellular immune responses; (2) to define changes in cytotoxic cellular immunity which occur in tumor bearing hosts; and (3) to find means of manipulating the cytotoxic cellular immune response for therapeutic purposes.

Methods Employed: Cell-mediated cytotoxicity has been measured in vitro by release of ^{51}Cr from labelled target cells incubated with lymphoid cells for 2-6 hours. Immune cells have been collected from spleen, marrow, lymph node, thymus and peripheral blood of sensitized hosts. Cells have been fractionated by 1 g velocity sedimentation, density gradient separation in Percoll, removal of cells which ingest iron particles, removal of cells which adhere to anti-Ig coated petri dishes and lysis of cells by monoclonal antibodies and complement. Hybridoma cell lines producing monoclonal antibodies have been obtained from the Salk Cell Distribution Center, L. Herzenberg (Stanford) and D. Sachs (Immunology Branch). In addition effector cells have been produced by in vitro sensitization of lymphoid cells using previously established Mishell and Dutton tissue culture conditions. Broadly reactive effector cells have been generated primarily by culturing cells from normal mouse spleen, thymus or fetal liver with polyinosinic acid and/or growth factors in medium supplemented with syngeneic plasma. Growth factors have been generated by stimulating EL4 (C57BL leukemia) cells with a phorbol ester. Target cell lines have been adapted to growth in medium supplemented with mouse plasma. This medium also has been used to support cytotoxicity assays. Target cells have been provided by freshly explanted cells (including primary MCA induced tumor cells dispersed with highly purified collagenase and DNase), tissue culture lines and established tumor cell lines passed in vivo or in vitro.

Sarcomas have been induced with methylcholanthrene (MCA) in mice which are routinely screened for potentially immunosuppressive pathogens before admission to the colony at the Frederick Cancer Research Center. Tumor cell lines established from these sarcomas are also screened for potentially immunosuppressive pathogens and contaminating virus-related antigens: mycoplasma (culture and serology) and a variety of viruses (serology) including MVM, Sendai, LDH and LCM. The tumor cell lines used in the study have been induced by a relatively low dose of MCA (100ug) and do not stimulate generation of anti-tumor cytotoxic cells under standard in vitro conditions for generating cytotoxic cells against allogeneic transplantation antigens.

The following two techniques have been adapted to dual laser, multiparameter flow microfluorometry (FMF) in order to study mechanisms which may regulate expression of cell-surface molecules, particularly those on the surfaces of tumor cells. 1) In a previous study we demonstrated that treatments of mouse

spleen cells with agents which change cholesterol levels also change the expression of H-2 determinants. However, we were not able to measure cholesterol levels in individual cells. In order to measure cholesterol content at the single cell level, we have adapted the cholesterol probe, filipin, to use with FMF. Two million cells, fixed with 1-4% p-formaldehyde for 30 min at room temperature, are stained for 2-4 hours with 100 mcg/ml filipin; equilibrium is reached within about 1/2 hr. Treated cells are excited at 350.7/356.7 nm in a FACS instrument with a krypton ion laser. Fluorescence emission above 510 nm. is measured. 2) In order to test the role of the cell cycle in a relatively simple model, mitogen-stimulated mouse spleen cells have been indirectly fluoresceinated with monoclonal antibodies (green emitting) against cell-surface determinants, then ethanol fixed and treated with RNase, and finally treated with propidium iodide (red emitting) to label DNA. Cells in G2 have been distinguished from those in G1 or G0 by DNA content; cell size has been approximated by forward light scatter; and expression of cell-surface H-2 determinants has been measured by the level of binding of fluoresceinated monoclonal antibody.

Major Findings: Previous work in this laboratory has demonstrated that normal mouse spleen cells cultured for 5 days in medium supplemented with syngeneic plasma and polyinosinic acid (Poly I) generate Thy 1⁺ cytotoxic cells, whose broad pattern of target cell reactivity in ⁵¹Cr-release assays includes freshly dispersed syngeneic primary tumor cells induced by MCA but not freshly dispersed or mitogen-stimulated lymphocytes. A close relationship of these effector cells to natural killer cells is apparent from a) their development from unprimed lymphoid cells in the absence of known antigen, b) the broad pattern of target cell reactivity which is primarily monomorphic as defined by cold target cell inhibition tests, c) the phenotype of effector cells generated from splenocytes (Thy 1⁺, NK 1.2⁺, Lyt 1⁻, 2⁻ - albeit asialo GM1⁻, and d) the strain distribution pattern of high vs low responses. The response is controlled by multiple dominant or co-dominant genes, and there is evidence for gene dosage effects (F₁ responses intermediate between high and low parental responses and a unimodal distribution of backcross responses intermediate between the F₁ and low parent responses). In the course of last year's work, we found that unprimed thymocytes will also generate this type of cytotoxic activity in vitro; however, an exogenous source of T-cell growth factor(s) is necessary. This effort has been extended by the following findings.

We postulated that variability of the cytotoxic cell response results in part from shifting levels of ancillary cell control. Thus, if helper-suppressor ancillary cells could be removed and replaced by a constant source of T-cell growth factor(s), complexity of the cytotoxic cell response might be reduced. To this end, we have collected supernatants from EL4 cells stimulated with phorbol esters as a source of mitogen-free, T-cell growth factor(s). Stimulated EL4 cells are cultured in medium containing syngeneic (C57B1/6) plasma rather than FCS in order to minimize the presence of antigens in the system.

Growth factor(s) alone produced by phorbol-stimulated EL4 cell stimulates generation of cytotoxic activity by unprimed B6 thymocytes against syngeneic, MCA-induced tumor cells. Poly I acts synergistically with growth factor(s).

Both agents affect generation of cytotoxicity insofar as pulsing experiments fail to show an effect on the cytotoxicity assay.

Five important observations have been made of cytotoxic activity generated by responding cells cultured 5 days in syngeneic medium with Poly I and growth factor(s). 1) B6 thymocytes treated with monoclonal anti Lyt 2 and complement (90% depletion of thymocytes) and B6 spleen cells treated with monoclonal anti Thy 1.2 and complement (40% depletion of spleen cells) generate high levels of cytotoxicity against syngeneic tumor cells. 2) compared to their untreated counterparts, thymocytes depleted of Lyt 2⁺ cells and spleen cells depleted of Thy 1⁺ cells generate higher levels of cytotoxic activity against syngeneic tumor cells but lower levels against allogeneic cells. The allogeneic responses are generated in the presence of irradiated allogeneic stimulator cells and growth factor(s) and the absence of Poly I. 3) Thy 1-depleted spleen cells from mouse strains normally classified as low responders (e.g., DBA/2J, H-2^d; and SJL/J, H-2^S) generate high levels of cytotoxic activity when cultured with growth factor(s) and Poly I and tested against two independent tumor lines (B6MCl, H-2^b; and YAC, H-2^a). 4) Fifteen-day old B6 fetal liver cells generate cytotoxic activity. 5) Phenotyping by depletion of cytotoxic activity with monoclonal antibody and complement shows that the effector cells generated by anti Thy 1 treated spleen cells are, in fact, Thy 1⁺. All cells in the cultured thymocyte populations are Lyt 1⁺ by flow cytometry analysis, even though cytotoxic activity is not depleted by treatment with anti Lyt 1 and complement.

In a related effort, procedures have been adapted to flow microfluorometry for analysis of factors which affect the quantitative expression of cell-surface determinants. The cholesterol probe, filipin, has been adapted to flow microfluorometry with the following results. 1) Filipin treatment of mouse spleen cells alters the forward light scatter profile, a parameter which is useful in the unmodified state for identifying various subpopulations of cells. The original light scatter profile can be preserved, however, if cells are fixed with formaldehyde before filipin treatment. 2) The fluorescence intensity of filipin-treated cells excited at 350.7/356.7 nm. is clearly increased above autofluorescence levels. 3) Average filipin fluorescence levels from a variety of types of mouse cells correlate well with an independent chemical assay for cholesterol when 100 mcg/ml filipin is used for treatment and cholesterol content is less than 2nmoles per million cells. Higher cholesterol levels will require more filipin in order to reach saturation.

Flow microfluorometry has also been used to correlate the expression of cell-surface H-2 determinants with different segments of the cell cycle. Con A-induced mouse lymphoblasts have been treated with both indirectly fluoresced antibodies against H-2 determinants and propidium iodide, the latter a DNA label in RNase-treated cells. Because cells in the G2 portion of the cell cycle are physically larger than those in G0 or G1, cell-size corrections have been made on the basis of forward low-angle light scatter, which approximates cell size. Thus, three parameters have been independently measured for each cell in a typical analysis of 50,000 cells: 1) light scatter (cell size), 2) green fluorescence (H-2 expression), and 3) red fluorescence (DNA content). Analysis of the expression of H-2K^d and H-2D^d

determinants shows that levels of both are highest in cells which are in the G2 portion of the cell cycle compared with cells in G0 or G1. The difference in H-2 expression cannot be explained by a difference in cell size, at least as measured by light scatter.

Significance to Biomedical Research and the Program of the Institute: The Poly I-induced cytotoxic cell response being characterized in this project is important because of 1) the high level of effector cell activity against primary, syngeneic tumor cells which are induced by low levels of chemical carcinogen and 2) the association of such effector cells with previously described natural killer (NK) cell activity, which has attracted interest as a possible major host anti-tumor surveillance mechanism.

The ability of thymocytes to generate anti tumor cell activity in the presence of Poly I and growth factor(s) aligns the response at least in part with the T cell lineage. The finding that 15-day fetal liver cells and thymocytes depleted of Lyt 2 bearing cells both generate anti tumor cell activity suggests that the response is a primitive one which first appears in relatively immature cells. The response differs from that which generates classic cytotoxic T lymphocytes against alloantigens in each of the following characteristics: a) augmentation of the spleen cell response following removal of Thy 1⁺ cells, and b) augmentation of the thymus cell response following removal of Lyt 2⁺ cells. Thus, although the cytotoxic anti tumor response under study here is related to T cells, it reacts positively to forms of manipulation not pursued with classic cytotoxic T cell responses because of ablative effects in those cases.

Earlier studies demonstrated that mouse strains can be classified as high or low responders depending on the level of broadly reactive cytotoxic cell activity generated in vitro by spleen cells in the presence of Poly I. Now it is clear that low responses can be raised to the level of high responses by culturing spleen cells depleted of Thy 1⁺ cells in the presence of both Poly I and growth factor(s).

Cell-surface determinants permit tumor cell recognition by host defense mechanisms. Techniques for analyzing factors which affect the quantitative expression of these determinants are being adapted to flow microfluorometry, which will permit rapid analysis of tens of thousands of cells. Flow microfluorometry has been used to demonstrate effects of the cell cycle on expression of H-2 determinants and to measure cholesterol content at the single cell level, which we previously demonstrated affect H-2 expression by lymphocytes. These techniques can now be adapted to use with tumor cells.

Proposed Course of Project: To test in vivo the anti tumor effects of various populations of cytotoxic cells generated with Poly I and growth factor(s), we will use Winn assays with tumor cells induced by low concentrations of MCA. All aspects of the work, including tissue culture growth of tumor and lymphoid cells, will be done under syngeneic conditions.

Genetic control of anti tumor cytotoxicity induced by Poly I and growth factor(s) will be pursued using techniques which enhance the difference between high and low responding mouse strains and minimize the role of non

responding cells which modulate the response.

Flow microfluorometry analysis of factors which affect expression of cell-surface determinants on tumor cells will be delayed during the coming year while technical modifications are made on the instrument to facilitate the use of tumor cells. We will then examine the effect of cellular cholesterol, lipid packing density, and cell cycle on H-2 expression of MCA-induced tumors.

Publications:

Muller, C. P., Stephany, D. A., Shinitzky, M., and Wunderlich, J. R.: Changes in cell-surface expression of MHC and Thy 1.2 determinants following treatment with lipid modulating agents. J. Immunol. In press.

Dorfman, N. A., Winkler, D., and Wunderlich, J. R.: Supplement-induced cytotoxic cells (SICC) generated from mouse thymus or spleen cells cultured in the presence of interleukin-2 and/or polyinosinic acid. Cell. Immunol. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05018-13 I
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 Damage by Immune Mechanisms		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Pierre A. Henkart, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.6	PROFESSIONAL: 1.2	OTHER: 0.4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The results of a careful electron microscope study of NK and ADCC lysis of target cells had suggested that the contents of cytoplasmic granules were secreted in the vicinity of target cells during the cytotoxic process. These findings are strongly supported by the recent experiments described here in which we have demonstrated the presence of a very potent lytic agent in cytoplasmic granules purified from rat NK tumors. The activity of this lytic agent is controlled by calcium which is absolutely required ($<10^{-4}M$) for its expression. Magnesium is totally inactive. The lytic activity is most easily demonstrated in red cell targets (human or sheep), where less than 1 $\mu g/ml$ granule protein suffices to lyse 100% of the target cells in less than 15 minutes. This is less than one equivalent NK cell per target cell. When red cell ghosts are treated under these conditions they can be seen to remain intact, and fluorescence microscopy reveals that very large molecules ($<500,000$ MW) remain inside while small molecules are released. This is evidence for aqueous pores in the ghost membranes, similar to the membrane damage inflicted by intact effector cells. Granule preparations from non-cytotoxic cells do not show cytolytic activity.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

M. Henkart, Expert, Immunology Branch, NCI

P. Millard, Immunology Branch, NCI

Collaborator: Craig Reynolds, NCI

Objectives: Our overall objective is to define the mechanisms by which lymphocytes destroy foreign cells. To this end we have dissected the killing process into discrete steps which can be studied independently. In this project we have concentrated on the membrane lesion induced by lymphocytes in the target cell. Over the past few years, we have produced evidence that killer lymphocytes secrete molecules which become implanted in the target cell membrane, compromising its permeability properties. Our current objectives are to understand this process at the molecular level and to understand the various levels of control of the secretory process process, in NK/ADCC killer lymphocytes.

Methods Employed: In vivo passaged rat NK tumor cells were harvested from the spleens of leukemic rats, and purified by Ficoll Hypaque. These contained $1-4 \times 10^9$ cells, of which 80-90% are large granular lymphocytes. Cells at 10^8 /ml were lysed by nitrogen decompression, the nuclei removed by Nucleopore filtration and 5 ml of the homogenate layered on 20 ml of 48% Percoll. After centrifugation at 60,000 xg, the gradient was pumped out and the fractions analyzed by biochemical and enzymatic analysis. The band at the bottom of the gradient displayed a peak of β glucuronidase, acid phosphatase and other lysosomal enzymes but was free of mitochondrial, plasma membrane, and cytosol marker enzymes. The resulting material was seen to be intact granules plus remaining Percoll by electron microscopy. This fraction was analyzed by SDS gels and other biochemical procedures. Granule cytolytic activity was measured by ^{51}Cr release using various target cells. Granules were diluted in calcium free PBS and mixed 1:1 with ^{51}Cr labeled target cells in BSS containing 1 mg/ml BSA to start the assay. After 0.5 hr at 37° supernatants were harvested and counted.

Major Findings: The results of a careful electron microscope study of NK and ADCC lysis of target cells had suggested that the contents of cytoplasmic granules were secreted in the vicinity of target cells during the cytotoxic process. These findings are strongly supported by the recent experiments described here in which we have demonstrated the presence of a very potent lytic agent in cytoplasmic granules purified from rat NK tumors. The activity of this lytic agent is controlled by calcium, which is absolutely required ($<10^{-4}\text{M}$) for its expression. Magnesium is totally inactive. The lytic activity is most easily demonstrated in red cell targets (human or sheep), where less than 1 $\mu\text{g}/\text{ml}$ granule protein suffices to lyse 100% of the target cells in less than 15 minutes. This is less than one equivalent NK cell per target cell. When red cell ghosts are treated under these conditions they can be seen to remain intact, and fluorescence microscopy reveals that very large molecules ($<500,000$ MW) remain inside while small molecules are released. This is evidence for aqueous pores in the ghost membranes, similar to the membrane damage inflicted by intact effector cells. Granule preparations from non-cytotoxic cells do not show cytolytic activity.

Significance to Biomedical Research and the Program of the Institute: The process of lymphocyte destruction of foreign cells may be one of the most important mechanisms for the immunological rejection of allografts and tumors in vivo. Studies such as ours, directed at the cellular and molecular nature of this process, allow a more complete understanding of the basic knowledge of the body's immunological defense system against foreign cells, including malignant cells.

Proposed Course of Project: Purification of the granule component(s) responsible for the lytic activity is being attempted, but is not simple because the active material is not soluble (apparently it is membrane bound, as suggested by the EM). Antibodies against the granules are being prepared and characterized. Further studies to define the pore induced in red cell ghosts are being carried out. A variety of conditions known to inhibit cell mediated cytotoxicity are being tested to see which will inhibit granule mediated killing.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05021-12 I
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.) Antigens Determined by the Murine Major Histocompatibility Locus		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) David H. Sachs, Chief, Transplantation Biology Section, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION Transplantation Biology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 5.0	PROFESSIONAL: 3.5	OTHER: 1.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Studies are being directed toward understanding the major histocompatibility complex, the structure and function of the products of this complex, and manipulations of immune responses to these products. Current studies include: 1) Characterization of major histocompatibility antigens: Congenic resistant strains of mice are developed, maintained, and used in serologic and immunochemical analyses of the MHC products of the mouse; 2) Studies of monoclonal antibodies to H-2 and Ia antigens: Hybridoma cell lines are produced by fusion of immune mouse spleen cells with mouse myeloma cells. The monoclonal anti-H-2 and anti-Ia antibodies produced by these hybridomas are analyzed by serologic and immunochemical means and are used to further characterize the fine structure of the MHC; 3) Characterization of receptor sites for histocompatibility antigens: Anti-idiotypic antisera are being produced against anti-H-2 and anti-Ia hybridoma antibodies, and the effects of these antisera on in vitro and in vivo parameters of histocompatibility are being assessed; and 4) Mechanism of tolerance to H-2 and Ia antigens: The humoral and cellular responses of radiation bone marrow chimeras are being examined, and the mechanism for maintenance of tolerance in these animals is being studied.		

Project Description

Other Professional Personnel Engaged on the Project:

Jeffrey A. Bluestone, Senior Staff Fellow, Transplantation Biology Section, I, NCI
Suzanne L. Epstein, Senior Staff Fellow, Transplantation Biology Section, I, NCI
Saswati Chatterjee-Hasrouni, Visiting Fellow, Transplantation Biology Section, NCI
Suzanne T. Ildstad, Medical Staff Fellow, Transplantation Biology Section, I, NCI
Ruth Rabinovitz, Visiting Fellow, Transplantation Biology Section, I, NCI

Objectives: 1) To produce antibodies against the products of defined regions of the major histocompatibility complex. 2) To characterize the reactions of these antibodies with the cell surface of lymphocytes, and to determine the nature of the cells bearing individual antigens. 3) To characterize the products with which these antibodies react by immunochemical means. 4) To attempt to produce anti-idiotypic antibodies against the receptors on these antibodies which detect cell surface histocompatibility antigens.

Methods Employed: 1) Congenic resistant strains of mice differing only at their major histocompatibility loci have been reciprocally immunized in order to produce antibodies of known, restricted specificity. The antibodies have been characterized by assays of complement-mediated lymphocytotoxicity and by fluorescence microscopy using a fluoresceinated rabbit antimouse immunoglobulin as a developing agent.

2) Hybridoma cell lines are obtained by fusion of immune mouse splenic lymphocytes with mouse myeloma cells (SP2/0, P3U1, and NS1). The cells are mixed and exposed to polyethylene glycol, 30% for 8 minutes, and fused cells are then cultured in the presence of a selective medium (HAT) for 2 weeks in microtiter wells. Hybridoma cells secreting anti-MHC antibodies are detected by a complement-mediated cytotoxicity assay on individual microwell supernatants. Positive cultures are then sequentially cloned in vitro and in some cases passed in vivo to produce large amounts of ascites hybridoma antibodies.

3) Cell surface antigens reactive with anti-H-2 and anti-Ia antibodies and monoclonal antibodies are isolated and studied immunochemically. Cells are labeled in vitro with ^3H -leucine, solubilized in a nonionic detergent, purified by lentil lectin chromatography, and then mixed with the antibodies being analyzed. Complexes are precipitated with Staphylococcus aureus Cowan I strain which contains protein A on its surface, and the precipitated complexes are then dissociated in SDS and mercaptoethanol and analyzed by polyacrylamide gel electrophoresis.

4) Hybridoma antibodies against H-2 and Ia antigens are purified by affinity chromatography on protein A Sepharose columns and are then used to immunize heterologous animals. Pig, rabbit, and goat antihybridoma reagents have been produced. These reagents are absorbed exhaustively on normal immunoglobulins or myeloma proteins in order to remove anti-isotype and antiallotype antibodies, and the putative anti-idiotypic antibodies are then absorbed and eluted from the relevant hybridoma antibody. These anti-idiotypes are then analyzed by hemagglutination and hemagglutination inhibition assays using cells to which the immunizing hybridoma, different hybridomas, or normal immunoglobulins have been coupled.

5) Cell-mediated cytotoxicity assays are carried out using mouse splenic lymphocytes as both responders and stimulators. The effects of antibodies to cell surface antigens at both the target and killer cell level are analyzed in the 4 hour cytotoxicity assay.

6) Radiation bone marrow chimeras are prepared by lethal irradiation of recipients and reconstitution with bone marrow from allogeneic donors exsanguinated before harvest of long bones. As these animals mature, in vitro assays are performed in an attempt to determine the mechanisms for the tolerance observed.

7) Mice are treated in vivo with purified anti-idiotypic antibodies. The effect of such treatment on idiotype levels and anti-H-2 antibody activity in the serum of these animals is examined. In addition, spleens from these animals are examined for in vitro MLC and CML reactivity. Treated animals are also examined for in vivo reactivity to appropriate skin grafts, and humoral and cellular immunity following grafting are examined by complement-mediated cytotoxicity assays and by CML assays.

Major Findings: 1) Fusion of spleen cells from mice hyperimmunized against H-2 antigens and boosted 2-3 days before fusion has been found to give satisfactory results in the production of anti-H-2 hybridomas. About 60 stable hybridomas have so far been produced, most of which detect H-2 or Ia antigens of a variety of haplotypes. Panel testing has indicated that most of these antibodies react with public specificities of the H-2 and Ia antigens, while a few appear to detect private specificities. Numerous crossreactions have been detected using these monoclonal antibodies, which define a variety of new public H-2 and Ia specificities.

2) Analysis of anti-H-2 antisera and monoclonal antibodies by gel electrophoresis of labeled cell surface antigens has demonstrated two new H-2 products determined by loci within the D region, named H-2L and H-2R. Three hybridoma antibodies reactive with H-2L and/or H-2R antigens have been produced. These antibodies are being used for structural studies of the H-2L molecule.

3) Antibodies directed against the killer cell have been shown to block cell-mediated cytotoxicity against the H-2 products. The specificity of these antisera has been shown to be directed to a product of a gene closely linked or identical to the Ly-2 locus. Additional antibodies have been produced by immunizing rats and mice with cloned cytotoxic T cell lines. Attempts are in progress to produce hybridomas from such immunized mice in order to determine other cell surface antigens of importance to the T cell cytotoxic phenomenon.

4) Evidence for nonspecific suppressor cell generation in bone marrow chimeras has been obtained at times up to 6 weeks following grafting. At later dates no evidence for suppressor cells of either specific or nonspecific nature was obtained, supporting the hypothesis of clonal deletion rather than active suppression. Mixed allogeneic and xenogeneic chimeras have been produced by grafting irradiated mice with graded mixtures of T cell depleted bone marrow from appropriate sources. Such mice are being evaluated for tolerance by skin graft challenge and by in vitro techniques.

5) Anti-idiotypic antibodies reactive with six of the hybridoma anti-H-2 antibodies and with four of the hybridoma anti-Ia antibodies have been produced and have been shown to be specific by a variety of assays. Assessment of hybridoma antibodies and immune sera for the presence of these idiotypic specificities has indicated that at least one of the anti-H-2 and one of the anti-Ia idiotypes are prevalent in the corresponding normal immune responses.

6) The effect of monoclonal anti-H-2 antibodies on the effector phase of CML reactions has been examined. All anti-H-2K^k monoclonal antibodies tested were found to block CML effectively, confirming that serologic specificities are on the same molecules which are detected by CML reactions.

7) Several monoclonal antibodies detecting non-H-2 antigens have been produced along with our anti-H-2 and anti-Ia hybridomas, and several of these have been characterized. One antibody appears to detect a T cell antigenic determinant, probably part of the Thy-1 molecule but distinct from other Thy-1 determinants. Several other antibodies detect a family of Ly-6 antigenic determinants.

8) Treatment of animals with anti-idiotypic antibodies has been found to induce appearance of idio type in the serum of these animals. This has been true for all of the anti-H-2 and anti-Ia anti-idiotypes so far examined. In addition, a percentage of the induced idio type has been shown to bear the same anti-H-2 or anti-Ia specificity as the original monoclonal antibody. These findings therefore represent the induction of anti-H-2 and anti-Ia antibody responses in the absence of exposure to the actual antigens.

9) Adoptive transfer experiments utilizing fractionated lymphoid cells from spleens of anti-idio type treated mice have been carried out. These studies indicate that T cells are involved in the effect of anti-idiotypic treatment on repertoire expression. Mice receiving such T cells and then challenged with a skin graft were found to produce a higher percentage of anti-H-2 antibodies bearing the idio type than did control animals treated with normal T cells.

Significance to Biomedical Research and the Program of the Institute: 1) The H-2 and Ia antigens are cell surface determinants which appear to be involved in physiologic cell-cell interactions in the immune response. Therefore, antisera against these antigens provide tools for dissecting the mechanism of these cell interactions and possibly for modifying responses.

2) The specificity of the receptor for histocompatibility antigens should reside in the variable portion of the heavy and light chains of the relevant antibody molecules. Thus, anti-idiotypic antibodies against such receptors might be expected to distinguish those cells capable of reacting against individual histocompatibility antigens. Such antibodies thus provide an approach to modification of the immune response to cell surface antigens. Our findings on induction of idio type by in vivo treatment with anti-idio type indeed indicate that such modifications are possible.

Proposed Course of Project: 1) In order to maintain isogenicity of the background of our congenic lines, backcrosses of these lines to the reference

congenic partner will be performed at least once every ten generations. Back-crosses of congenic lines will be examined for further recombination events within the H-2 and I regions. Recombinants will then be examined for fine structure analysis of the MHC and for the production of antisera against new H-2 and Ia specificities.

2) Anti-idiotypic antibodies directed against anti-H-2 and anti-Ia receptors will continue to be produced and studied. These reagents will be examined for reactions with antibodies produced in conventional immunization schemes in order to determine the prevalence of the individual combining sites.

3) The effects of anti-H-2 and anti-Ia anti-idiotypic reagents on MLC and CML reactions will be examined, in order to detect possible sharing of idiotypes between T and B cell receptors.

4) Further fusions of immunized cells from a variety of different strain combinations will be performed in order to produce additional monoclonal hybridoma antibodies to a variety of H-2 and Ia specificities. The library thus obtained will be screened by serologic and immunochemical means in order to further characterize the products of the MHC. Attempts will be made to identify products of new MHC genes, the presence of which has been suggested by DNA hybridization techniques.

5) The effect of in vivo treatment with anti-idiotypic antibodies will continue to be examined. The effects of such treatment on skin graft rejection and on humoral antibody production and CML reactivity will be studied. The possible sharing of idiotypes between alloantigen receptors and modified self receptors will also be examined.

6) Bone marrow transplantation studies will be continued, assessing the effect of mixed allogeneic and xenogeneic donor inocula on long-term tolerance to allografts and xenografts. Mechanism of such tolerance will be examined by techniques similar to those utilized for complete allogeneic chimeras.

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Auchincloss, H., Jr., and Sachs, D. H.: Mechanisms of tolerance in murine radiation bone marrow chimeras. I. Non-specific suppression of alloreactivity by spleen cells from early but not late chimeras. Transplantation, in press.

Auchincloss, H., Jr., and Sachs, D. H.: Mechanisms of tolerance in murine radiation bone marrow chimeras. II. Absence of non-specific suppression in mature chimeras. Transplantation, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05023-12 I
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.) Transplantation Antigens of Swine		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) David H. Sachs Chief, Transplantation Biology Section, Immunology Branch, NCI		
COOPERATING UNITS (if any) NIH Animal Center, Poolesville, Maryland		
LAB/BRANCH Immunology Branch		
SECTION Transplantation Biology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.5	PROFESSIONAL: 2.5	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) A breeding program has been carried out starting with two miniature pigs from different sources and selecting offspring according to tissue typing procedures aimed at defining the major histocompatibility complex of this species. By this procedure three herds of miniature swine, each homozygous for a different set of histocompatibility antigens at the MHC have been developed. Current projects include: 1) Assessment of survival of organs and tissue transplants among and between members of these herds as a model for tissue typing and transplantation; 2) Purification and characterization of the major histocompatibility antigens of this species, and isolation and characterization of peptides from these antigens for sequence analyses and for assessment of immunologic reactivity; 3) Assessment of the immunologic parameters involved in tolerance to allografts in this species; and 4) Detection and characterization of intra-MHC recombinants. Two intra-MHC recombinants have been obtained and bred to homozygosity. Kidney transplants utilizing these new recombinants have shown that selective matching for Class II antigens frequently permits long-term kidney graft survival across a Class I difference. The mechanism of this apparent tolerance is under further study.		

Project Description

Other Professional Personnel Engaged on the Project:

Joan K. Lunney, Senior Staff Fellow, Transplantation Biology Section, I, NCI
Steven A. Rosenberg, Chief, Surgery Branch, NCI
Stuart Rudikoff, Microbiologist, Laboratory of Cell Biology, NCI
Mark D. Pescovitz, Medical Staff Fellow, Transplantation Biology Section, I, NCI
Dinah S. Singer, Senior Investigator, Immunology Branch, NCI

Objectives: To develop and maintain three strains of miniature swine, each homozygous for a different set of histocompatibility antigens at the major histocompatibility locus (MSLA). The animals are used for in vivo experiments in organ and tissue transplantation and as a source of large numbers of cells from which cell surface antigens are isolated, purified, and characterized chemically.

Methods Employed: Unlike classical inbreeding schemes which require about twenty generations to produce homozygosity, the approach used with these swine involved selective breeding on the basis of histocompatibility typing of parents and offspring.

Four males and four females were purchased from commercial sources, and mating pairs were selected to be as varied as possible in order to assure the selection of different histocompatibility genes. Skin grafts were exchanged between the members of each pair and sera were obtained from the animals two weeks after the rejection of the grafts. The sera were tested for cytotoxic antibodies by lymphocytotoxic typing, and pairs which produced strong cytotoxic antibodies were bred.

Offspring from each breeding were tested serologically to determine which histocompatibility antigens had been inherited from the parents, and offspring which could be shown to possess the same antigen combinations were selected for further breeding. This process has now been repeated for eight consecutive generations.

Transplantation of tissues in these animals has been performed in the large animal facility of the Surgery Branch in Building 14. Methods have been developed for the transplantation of split thickness skin grafts from the ear to the dorsal thorax. Allografts are always placed side by side with autografts as a control. The use of ear skin permits very accurate assessment of viability of the grafts and determination of rejection times. Surgical techniques for vascularized grafts (kidney and liver) have been developed.

Chemical purification studies on swine transplantation antigens are being performed using both detergent and papain solubilized cell surface preparations from spleen and lymph node cells. Initial studies involved radiolabelling of the surface antigens with tritiated leucine by published methods, and the assessment of molecular weight of those components which react with alloantisera by the use of SDS polyacrylamide gels.

In order to produce large quantities of histocompatibility antigens without the use of radiolabeled amino acids, membranes have been prepared from lymphoid organs and solubilized with detergents or by limited papain digestion. The extracts were then purified by lentil lectin chromatography and by affinity chromatography on anti- $\beta 2$ microglobulin columns, or, in the case of papain solubilized antigen, by DEAE ion exchange chromatography and by gel filtration. Sera produced between outbred swine which react with public specificities shared in the inbred minipig population are being used to separate products of different histocompatibility loci. Amino acid sequencing of heavy and light chains from purified SLA antigens is being performed. Preparation, isolation, and characterization of peptides from these isolated antigens have been initiated.

All progeny of heterozygous animals have been screened by serology and by MLC reactivity to detect intra-MHC recombinants.

Monoclonal antibodies against swine cell surface antigens have been prepared by immunizing mice with thymocytes from one of the partially inbred herds (SLA^{dd}), and then fusing the mouse spleen with SP2/0 to produce hybridoma cell lines. The cell lines have been screened for activity by cytotoxicity and fluorescence activated cell sorter analysis.

In collaboration with Dr. Dinah Singer, genomic clones encoding SLA antigens are isolated and characterized. Characterization includes detection of SLA products on transfected cell lines by antisera and cellular assays.

Major Findings: 1) Breeding of further generations has continued to be successful. We therefore now have three different herds of swine, each homozygous for a different SLA antigen, as well as two herds of recombinant haplotypes.

2) Skin graft survival has been found to be prolonged within each of the homozygous herds. The mean survival time for grafts within homozygous herds was $11.8 \pm .89$ days, while the mean survival time of skin grafts between animals of the three different herds was $7.0 \pm .36$ days.

3) Renal allografts within the DD homozygous herd appeared to survive indefinitely despite minor histocompatibility antigen differences. Rejection occurred following allografts within the other two herds, with variable kidney survival times.

4) Skin grafts to DD recipients maintaining a DD allografted kidney showed marked prolongation of survival. DD kidneys transplanted after skin graft rejection by DD recipients were rejected in hyperacute fashion, but no antibody was detectable.

5) Alloantisera between the three herds have been analyzed by gel electrophoresis using detergent solubilized cell surface antigen preparations. Peaks were obtained at 45,000 molecular weight corresponding to the mouse H-2 antigen analog. Peaks at 35,000 and 28,000 molecular weights corresponding to mouse Ia antigens were also observed. Both by size criteria and by genetic

criteria these antigens thus appear to be the precise homologs of H-2 and Ia antigens of the mouse.

6) Milligram quantities of unlabeled histocompatibility antigens have been prepared from individual pig spleens by lentil lectin chromatography and anti- β 2 microglobulin affinity chromatography. The material eluted from these columns has been shown to consist predominantly of 42,000 and 11,000 dalton molecules and has been assessed immunologically by its ability to inhibit complement-mediated lysis of pig cells by anti-MHC antisera.

7) N-terminal amino acid sequences have been obtained for SLA antigens isolated from all three of our partially inbred lines. Comparisons of these sequences with each other and with sequences of MHC antigens from other species reveal high levels of homology, as well as possible allotypic differences.

8) Two recombinants within the MHC were detected by screening of the progeny of MHC heterozygous animals using MLC reactivity and the cytotoxic assay as markers. Both new recombinants involved separation of the MLC stimulatory locus (SLA-D) from the serologic loci (SLD-A,B,C). SDS-PAGE analyses of cell surface antigens from these animals have indicated that the Ia antigens segregated with the MLC stimulating determinants in both recombination events, confirming the identity or close linkage of the genes responsible for both of these products in this species. These recombinants have also been used to produce large amounts of antisera specific for Ia or SD antigens of this species, which were not previously available.

9) Transplants of kidneys between recombinant and nonrecombinant haplotypes have permitted evaluation of the effects of selective Class I or Class II matching on renal allograft survival. In approximately 50% of animals tested, selective matching for Class II antigens permitted long-term survival. Skin grafts in the same combinations were rejected promptly, but following a kidney transplant a subsequent skin graft from the same donor showed prolonged survival, indicating that systemic tolerance had apparently been induced by the kidney graft.

10) A series of monoclonal antibodies reactive with a variety of swine lymphocyte surface antigens have been prepared. One of these antibodies recognizes an SLA antigen of the SLA^{dd} haplotype, at least two others react with selective T cell subpopulations, and at least one other antibody is reactive with macrophages. Further characterization of these antibody reactivities is in progress.

11) A genomic clone encoding SLA^{dd} antigen has been isolated and shown to cause expression of SLA^{dd} determinants on L cells following transfection.

Significance to Biomedical Research and the Program of the Institute: One of the major problems in the study of cell surface antigens of human beings is lack of control of genetic constitution. The use of mice and rats as experimental models avoids this problem, but creates two new ones: 1) physiologically and anatomically these animals are often so different from human beings as to make comparisons and applications of findings difficult. 2) The

size of these rodents makes it extremely difficult to obtain sufficient cells and tissues to permit quantitative chemical characterization of relevant cell surface antigens.

For both of these reasons it is desirable to have animals of size comparable to human beings, whose genetic constitution with respect to histocompatibility can be controlled. The miniature pig, which attains an adult weight of about 200 lbs, is ideal for this purpose, and to date the experimental breeding pattern outlined above appears to be working well.

The availability of large quantities of MHC antigens make it possible to determine the effects of soluble antigen and possibly of peptides from these antigens on tissue transplantation. This will be assessed both by in vitro assays (MLC and CML), as well as in vivo in the transplantation models which have now been developed in these swine. In addition, large amounts of soluble SLA antigens should permit both primary and secondary structural studies to be performed.

Proposed Course of Project: The breeding plan and typing will be continued. Attempts to identify new recombinants by serologic and MLC screening of progeny from heterozygous breeders will be continued. Recombinants thus obtained will be bred to homogeneity as has been accomplished for the first two recombinants.

Experiments in collaboration with the Surgery Branch will be continued in order to: 1) characterize the immune response to transplantation of skin, kidney, and liver in pigs across defined histocompatibility differences, and 2) to determine the basis of allograft tolerance which has previously been reported in pigs.

Studies of the MSLA antigens at the biochemical level will be continued. Using the isolated unlabeled histocompatibility antigens, we will begin to prepare peptide fragments of these antigens and to analyze these fragments determinants involved in serologic assays (by inhibition of complement-mediated lysis), as well as in cellular assays (CML and MLC). The antigens will also be subjected to further sequence analysis in an attempt to determine for immunologic reactivities. An attempt will be made to localize those the structural basis for antigenicity in this system. An attempt to crystallize the SLA antigens for x-ray crystallographic studies will be made in collaboration with Drs. Gary Gilliland (Laboratory of Immunogenetics, NIAID) and David Davies (Laboratory of Molecular Biology, NIAMDD).

Investigations of the mechanism of tolerance induced by a vascular graft across a Class I only difference will be carried out. These will include assays both of cellular immunity (e.g., suppressor T cells) and of humoral immunity (IgM-IgG switch, soluble factors, etc.).

Publications

Shinohara, N. and Sachs, D. H.: Interspecies cross-reactions of murine anti-Ia antibodies. In Ferrone, S. and David, C. S. (Eds.): Ia Antigens Vol. 1. Mice. Boca Raton, FL, CRC Press, Inc., 1982, pp. 219-240.

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- Almaraz, R., Ballinger, W., Sachs, D. H., and Rosenberg, S. A.: The effect of peripheral lymphoid cells on the incidence of lethal graft versus host disease following allogeneic mouse bone marrow transplantation. J. Surg. Res. 34: 133-144, 1983.
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- Lunney, J. K., Osborne, B. A., Devaux, C., Pierres, M., and Sachs, D. H.: Interspecies cross reactivity of monoclonal antibodies directed against polymorphic mouse Ia determinants. Transplant. Proc. 15: 139-141, 1983.
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- Lunney, J. K., Osborne, B. A., Devaux, C., Pierres, M., and Sachs, D. H.: Interspecies cross reactivity of monoclonal anti-I-E antibodies specific for polymorphic Ia determinants. In Ir Genes: Past, Present and Future, Clifton, NJ, The Humana Press Inc., in press.
- Lunney, J. K., Osborne, B. A., Sharrow, S. O., Devaux, C., Pierres, M., and Sachs, D. H.: Sharing of Ia antigens between species. IV. Interspecies cross reactivity of monoclonal antibodies directed against polymorphic mouse Ia determinants. J. Immunol., in press.
- 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.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 201CB05033-12 I
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.) Immunotherapy of Human Cancer		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Richard J. Hodes Chief, Immunotherapy Section, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.2	OTHER: 0.1
CHECK APPROPRIATE BOX(ES)		
<input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) A controlled, randomized trial comparing immunotherapy to chemotherapy in stage I and stage II malignant melanoma has been initiated. A total of 181 patients have entered the trial, which is closed to further accrual of patients. Preliminary evaluation of data has demonstrated no significant effect of adjuvant therapies on clinical course.		

Project Description

Other Professional Personnel Engaged on the Project:

Steven A. Rosenberg, Chief, Surgery Branch, NCI
 Richard I. Fisher, Senior Investigator, Medicine Branch, NCI

Objectives: This project is designed to study various approaches to the immunotherapy of human malignancies.

Methods Employed: A new protocol (designed IB-2) was initiated in August 1975. This protocol will evaluate the effect of two types of immunotherapy or one type of chemotherapy on the remission duration and survival of patients with stage I (level 4 or 5) and stage II malignant melanoma. Patients are stratified by stage, site of primary, and for stage II patients, presence or absence of clinically palpable lymph nodes and the number of histologically positive lymph nodes. Following stratification, they are randomized to receive no further therapy (this is standard treatment for these patients) or chemotherapy with methyl-CCNU or immunotherapy with BCG, or immunotherapy with BCG plus a vaccine that consists of three tissue culture-grown allogeneic melanoma cell lines. These cell lines were a gift from Dr. Donald Morton, UCLA, and are grown in PPL0-free conditions at Litton Bionetics, Inc., under the supervision of Dr. Edwin Matthews. Cells are treated with the enzyme neuraminidase to remove sialic acid (and thus render them more immunogenic) and frozen until ready for use.

All patients are worked up by the Immunology Branch or the Surgery Branch. Following randomization, they are assigned to the Medicine Branch for chemotherapy, the Immunology Branch for immunotherapy, and the Surgery Branch for follow up if there is no further treatment.

Bloods are drawn during pre-randomization work-up and throughout the treatment cycle for the purpose of serum banking.

Major Findings: As of Dec. 1, 1982, 181 patients have been randomized into this protocol. The state of the trial is summarized in the following table:

	Control	MeCCNU	BCG	BCG & Vaccine
Total Patients Entered	43	46	47	45
Recurrences	32	25	38	25
Deaths	24	23	27	21

In vitro assays have only been carried to the point of indicating that patients are being effectively immunized with the vaccine (see Project No. Z01-CB-05016-10 I). In addition, PPD tests indicate that all patients receiving BCG have converted to a positive skin test.

Significance to Biomedical Research and the Program of the Institute:

Immunotherapy studies will explore the clinical effectiveness of manipulating the immune system in patients with cancer, and will also provide new information about the biology of the tumor-host relationship.

Proposed Course of Project: No further patient accrual will occur. For those patients already on study, treatment and follow-up as described by the IB-2 protocol will be continued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05035-11 I
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 the Lymphocyte Receptor for IgG (FcγR)		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Howard B. Dickler, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any) Dr. F. D. Finkelman, Dept. Medicine, USUHS, Bethesda, MD		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.50	PROFESSIONAL: 2.50	OTHER: 1.00
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The goals of this project are to characterize lymphocyte Fcγ receptors genetically and functionally. Previous findings indicate that the Fcγ receptors of B lymphocytes interact with: a) the lymphocyte cytoskeleton, b) Ia antigens and Lym antigens, c) surface IgM, and d) surface IgD. Each of these interactions is distinct, specific, and non-random. Recently monoclonal antibodies specific for FcγR have been shown to induce B lymphocytes to both proliferate and secrete antibody (function). This response does not require T lymphocytes or accessory cells and is specific by a variety of criteria. This is the only antibody specific for a B lymphocyte receptor which triggers function. The monoclonal anti-FcγR antibody stimulates primarily a subset of B lymphocytes characterized by increased size and the presence of the marker LyB5. It also triggers B lymphocytes to antibody secretion in vivo. These findings strongly suggest that B lymphocyte Fcγ receptors are central to B lymphocyte activation and immunoregulation. Finally, preliminary studies indicate that the monoclonal anti-FcγR antibodies stimulate natural killer cell activity in vitro.		

Project Description

Other Professional Personnel Engaged on the Project:

M. C. Lamers, Postdoctoral Fellow, Immunology Branch, NCI
S. Heckford, Postdoctoral Fellow, Immunology Branch, NCI

Objectives: 1) To evaluate the genotypic and phenotypic expression of the Fc γ receptor; 2) to evaluate the role of this receptor in the immune response.

Methods Employed: Heat-aggregated immunoglobulin and/or antigen-antibody complexes are fluorochrome or radioactive isotope labelled, and then allowed to interact with isolated lymphocytes or macrophages from animals. Binding is assayed by U.V. and phase microscopy, the Fluorescence Activated Cell Sorter (FACS), or by isotope counting. The interaction is studied by manipulation of the conditions of interaction, and by immunologic and chemical modifications of both lymphocytes and complexes. Genetic studies are carried out utilizing inbred, congenic, and/or recombinant mouse strains. Functionally, the effect of monomeric Ig, antigen-antibody complexes, ligands specific for molecules which interact with Fc receptors, and monoclonal antibodies specific for Fc γ R are evaluated using the B lymphocyte responses of proliferation as measured by tritiated-thymidine incorporation, and maturation (antibody secretion) as measured by plaque forming cell assays. The techniques of complement and antibody mediated cytolysis and immunoprecipitation are also employed. Finally, assays for natural killer (NK) activity are employed.

Major Findings: A series of cell surface molecules (Ia antigens) is encoded by genes within the I region of the murine H-2 complex. These are either identical to or closely linked to genes (immune response genes) which regulate immune responsiveness. Anti-Ia antibodies bound to Ia antigens inhibit binding of immune complexes (heat-aggregated IgG or antigen-antibody complexes) to specific cell surface receptors (Fc γ receptors). The inhibition is specific: (a) the Fc portion of the anti-Ia antibody is not required; (b) binding of ligands to other cell surface molecules (including H-2K, H-2D, IgM, IgD, and lectin receptors) does not produce inhibition; (c) anti-Ia antibodies bound to Ia antigens do not inhibit detection of other surface molecules; and (d) inhibition is observed with some cell types (B lymphocytes and a subpopulation of T lymphocytes) but not others (macrophages and null lymphocytes).

The nature of the Ia antigen-Fc γ receptor interaction has been examined: (a) these molecules are not identical since binding of antibodies to only a portion of Ia antigens produces maximal inhibition of Fc γ receptors and the latter can be redistributed without affecting distribution of Ia antigens; (b) ligand-bound Ia antigens do not appear to bind directly to the same site on Fc γ receptors as immune complexes since occupancy of these receptors by Ig complexes but not ligand-Ia antigens results in an interaction with surface IgM (see below); (c) the interaction does not appear to be mediated by the cytoskeleton since disruption of cytoskeletal function by drugs does not affect the interaction; and (d) the interaction may be steric since monoclonal antibodies against single antigenic determinants on Ia antigens produce only partial inhibition of Fc γ receptors whereas mixtures of the same monoclonal antibodies

produce maximal inhibition; however, immune complexes bound to Fc γ receptors do not sterically inhibit detection of Ia antigens. Thus, while the nature of the Ia antigen-Fc γ receptor interaction has not been fully elucidated, the simplest interpretation is that the two molecules lie in close proximity on the cell surface. A functional role for the interaction is suggested by the non-random and specific nature of the association.

Binding of ligand (F[ab']₂ anti-Mu) to surface IgM of B lymphocytes or ligand-induced redistribution of surface IgM has no effect on Fc γ receptors. However, if the latter receptor is occupied by antigen-antibody complexes which themselves provide insufficient cross-linking to cause redistribution or monomeric IgG at physiologic concentrations then ligand-induced redistribution of IgM results in redistribution of the Fc γ receptors. The interaction is specific and unidirectional: (a) redistribution of Fc γ receptors by further cross-linking does not result in redistribution of monomeric ligand occupied IgM; (b) ligand-mediated redistribution of IgM does not result in redistribution of ligand occupied Ia antigens or monomeric ligand occupied IgD; (c) cross reactions between ligands was excluded. A similar interaction has been demonstrated for surface IgD and Fc γ receptors except that the interaction only occurs on a subpopulation of B lymphocytes, and only with complexed IgG but not monomeric IgG. These results suggest that whenever IgM is involved in a B lymphocyte response, then the Fc γ receptor is also involved, and the differences between the sIgM-Fc γ receptor and sIgD-Fc γ receptor interactions provide a mechanism whereby the two antigen receptors could provide different signals to the B lymphocyte.

Normal spleen B lymphocytes in the presence of purified monoclonal antibody specific for Fc γ R both proliferate and differentiate to a functional stage (antibody secretion). These responses were shown to be specific and possible technical artifacts were excluded. B lymphocyte responses to monoclonal anti-Fc γ R do not require T lymphocytes or their products and do not require accessory cells. The responses are polyclonal, and mainly of the IgM class. Responses are obtained primarily from larger B cells which bear the LyB5 alloantigen. Responses are also obtained in vivo after injection of the antibody i.v. This is the only known example of an antibody specific for a B lymphocyte receptor which by itself triggers function.

Preliminary studies indicate that incubation of normal spleen cells with the monoclonal anti-Fc γ R antibody for 18 hr induces NK activity. This is specific in that control antibodies do not stimulate similar function.

Significance to Biomedical Research and the Program of the Institute: The B lymphocyte Fc γ receptor appears to play a central role in B cell activation and immunoregulation. Interactions between membrane molecules may be a general mechanism employed by cells to respond to stimuli. There is a probability that various manipulations of monoclonal anti-Fc γ R antibodies will allow functional triggering (antibody secretion) or inactivation (tolerance) of B lymphocytes specific for any antigen. The preliminary studies which suggest triggering of NK activity by the same monoclonal antibodies suggest that Fc γ R may play a central role in the function of all cells which bear this receptor. These approaches may provide significant new therapeutic modalities for the treatment of cancer and other diseases.

Proposed Course of Project: 1) Characterization of the molecular requirements of monoclonal anti-FcγR for B lymphocyte triggering such as valency and affinity. 2) Development of modifications of this antibody which will inactivate B lymphocytes as well as activate. 3) Analysis of the in vivo effects for specificity, requirement for various cell types, and mechanism. 4) Analysis of NK activity generated by anti-FcγR antibodies for specificity, cell types involved, and mechanisms.

Publications:

Dickler, H. B.: Interactions between receptors for antigen and receptors for antibody - a review. *Molec. Immunol.* 19: 1301-1306, 1982.

Lamers, M. C., Heckford, S. E. and Dickler, H. B.: Monoclonal anti-Fc IgG receptor antibodies trigger B lymphocyte function. *Nature* 298: 178-180, 1982.

Dickler, H. B.: Interaction of B lymphocyte Fc IgG receptors with other B lymphocyte surface membrane molecules. In: *Structure and Function of Fc Receptors* (Ed. A. Froese and F. Paraskevas) Marcel Decker Pub., New York, 1983, pp. 111-129.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05036-11 I
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 Control of the Immune Response to Staphylococcal Nuclease		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and Institute affiliation) David H. Sachs Chief, Transplantation Biology Section, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION Transplantation Biology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Antibodies directed against idiotypic determinants on anti-Staphylococcal nuclease antibodies from different mouse strains have been produced in rats and in pigs. The idiotypes are detected by ELISA assays and by the inhibition of antibody-mediated inactivation of nuclease. By screening a variety of strains and offspring from appropriate matings between strains for the presence of idiotypes and other markers, it has been shown that idiomorph expression is linked to the heavy chain allotype markers. By means of an in vitro anti-TNP nuclease plaque-forming cell response, idiotypic markers have been demonstrated on T helper cells. Administration of anti-idiotypic antibodies to mice has been found to induce idiomorph expression in the serum of these animals. This effect appears to involve T cells, since it is not observed in nude mice, and since idiomorph-bearing T helper cells for in vitro anti-TNP nuclease response have been found in spleens from such treated animals. Several hybridomas reactive with nuclease and/or anti-idiomorph have been produced. Syngeneic anti-idiotypes and one hybridoma anti-idiomorph have also been produced and are presently being characterized in both antibody and T cell systems.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

Richard J. Hodes, Chief, Immunotherapy Section, Immunology Branch, NCI
Alison Finnegan, Guest Worker, Immunotherapy Section, Immunology Branch, NCI
Christian Devaux, Visiting Fellow, Transplantation Biology Section, I, NCI

Objectives: Staphylococcal nuclease (nuclease) is a protein enzyme, the immune response to which we have found to be under genetic control by H-2-linked Ir genes in the mouse. Because of the wealth of available data on structural and immunochemical properties of nuclease, it is an ideal model antigen for use in dissecting the mechanism of genetic control of immune responses. Such mechanistic studies at both the T cell and B cell levels are the objective of this project.

Methods Employed: 1) Antibodies to NASE have been prepared in groups of mice of a variety of strains (both high and low responders) differing in H-2 type or allotype or both. These antibodies have been purified by affinity chromatography. Inbred Lewis rats and miniature swine have been immunized with the purified anti-NASE antibodies obtained from immune ascites from A/J mice, SJL mice, B10.A(2R) mice, and BALB/c mice after immunization with NASE. Immuno-absorptions with normal immunoglobulins from the same strains have been used in order to determine whether or not antibodies reactive with the variable region (i.e., anti-idiotypic antibodies) have been produced.

2) Anti-idiotypic reactions have been quantified by hemagglutination, by ELISA, and by the inhibition of antibody-mediated enzyme inactivation. Such reactivities have been screened against anti-NASE antibody populations from the other strains of mice in order to determine the possible genetic linkage of idio type to allotype and/or H-2 type.

3) Backcross animals have been screened for antibody levels and for allotype and idio type expression in order to determine linkage of idio type to other genetic markers.

4) Antibodies to nuclease have been separated on affinity columns into subpopulations directed against different determinants of nuclease. The reactions of anti-idiotypic antisera with these subpopulations have been characterized in order to determine new idiotypic markers.

5) Animals have been injected with purified anti-idio type antibodies and the effect of this treatment on their subsequent expression of idio type and anti-nuclease activity has been examined by means of spectrophotometric, ELISA and hemagglutination assays.

6) Anti-nuclease antisera and purified antinuclease antibodies have been examined by isoelectric focusing, using either protein stains or autoradiography employing ¹²⁵I-labeled nuclease or purified anti-idio type.

7) An assay for secondary immune responses to TNP-nuclease in vitro has been developed. This assay has been used to examine the genetics of secondary responses to nuclease, as well as to assess the effects of anti-idiotypic on the in vitro response and the cell level of expression of idiotype in this response.

8) Spleen cells from mice immunized with nuclease or treated with xenogeneic anti-idiotypic have been fused to SP2/0 myeloma variant cells. Screening has been performed by an ELISA assay for binding either to nuclease or to anti-idiotypic.

9) Syngeneic anti-idiotypic have been produced in a collaboration with Dr. Pierre-André Cazenave, of the Institut Pasteur, Paris, France. Such anti-idiotypic are produced by immunizing mice with KLH conjugates of purified anti-nuclease antibodies. Mice producing such anti-idiotypic are selected for fusions to produce hybridoma cell lines secreting anti-idiotypic antibodies.

Major Findings: By two separate criteria, anti-idiotypic antibodies have been obtained. 1) After exhaustive absorption with whole normal A/J ascites or repeated passages over affinity chromatography columns to which normal A/J ascites was bound, antibodies remained which reacted with A/J anti-NASE but not with B10.A anti-NASE antibody. Prior to absorption these rat antibodies contained precipitating antibodies to either type (A/J or C57BL/10) of antibody but no precipitating antibodies remained following absorption. 2) The rat anti-A/J anti-NASE antibodies prior to and following absorption reacted with the combining site of anti-NASE antibodies as evidenced by their ability to inhibit the anti-NASE antibody-mediated inactivation of NASE in an enzymatic assay.

Among (B10.Ax(A/J)xB10.A backcross animals the A/J anti-NASE idiotype was found to be linked ($p < .005$) to heavy chain allotype. However, a large recombination frequency was found (7-10 percent). The use of a very sensitive assay for allotype indicated that this recombination frequency was not the result of faulty allotypic typing. Also, progeny testing of the putative recombinant animals showed approximately 50 percent of the offspring to also have recombinant phenotypes, further indicating that these were true recombinant animals.

Injection of pig anti-idiotypic antibodies into virgin mice has led to an increase in the level of idiotype in the mouse serum. That the reactive immunoglobulin molecules induced represented true idiotype and not anti-anti-idiotypic was indicated by the fact that this induced idiotype was detectable using anti-idiotypic reagents produced in a variety of species. Similar treatment of nude mice did not lead to idiotype expression in the serum, suggesting that the effect may involve T cells. The idiotype detected in serum from the anti-idiotypic treated mice was predominantly found on immunoglobulin molecules not detectably specific for nuclease. Treatment of such animals with nuclease led to an even greater increase in idiotype expression, and in this case there was also an increase in idiotype expression on anti-nuclease antibody molecules.

Spleen cells from anti-idiotypic primed animals were found to provide T cell help in an in vitro anti-nuclease TNP plaque-forming cell response. This T

cell help was equivalent to that obtained from nuclease-primed spleens. Anti-idiotypic was found to inhibit this T cell help and in fact could eliminate the help if complement was added.

Isoelectrofocusing studies on anti-nuclease antibodies from a variety of strains have indicated that each strain has a characteristic spectrotypic. Development of the isoelectrofocusing gels with ^{125}I -labeled anti-idiotypic antibodies has shown a striking similarity in the clonotypes detected in all mice of a given strain, and there appears to be marked correlation with the expression of crossreactive idiotypes as detected by serologic means.

Several hybridomas producing anti-nuclease antibodies have been prepared from immunized BALB/c and SJL mice immunized with nuclease. Also, several hybridomas producing idiotype without detectable antigen binding activity (ID') have been prepared from BALB/c mice treated with anti-idiotypic.

Syngeneic anti-idiotypes have been produced in BALB/c mice by immunizing with KLH conjugates of BALB/c anti-nuclease antibodies. In one case a hybridoma cell line has been established from such a mouse, providing a monoclonal anti-idiotypic reagent for further in vitro and in vivo studies.

Significance to Biomedical Research and the Program of the Institute: Many of the antigens being studied in this laboratory are difficult to obtain in pure form (such as transplantation antigens and tumor antigens), and it is therefore important to have a well-characterized model protein on which initial experiments can be performed. In choosing models both for mechanistic studies and for studies in manipulation of the immune response, Staphylococcal nuclease is a particularly attractive protein. The immune response to this antigen has previously been shown in this laboratory to be under genetic control by an H-2-linked Ir gene.

The development of anti-idiotypic reagents directed toward antibodies under the control of an H-2-linked Ir gene provides another handle to study the mechanism of Ir gene function. These antibodies may help to determine whether the specificity of T cell recognition of antigens depends on similar variable region gene products to those responsible for B cell or antibody specificity. They may also lead to possible methods of controlling Ir gene expression. Finally, the detection of idiotypic determinants on T helper cells may provide a means of studying the elusive T cell receptor. Cloned lines of T helper cells bearing idiotype should provide a source for chemical characterization of the putative receptor.

Proposed Course of Project: The mechanism by which in vivo treatment with anti-idiotypic leads to increased idiotype production will be pursued. Transfers of purified populations of T cells and B cells from immunized mice and anti-idiotypic primed mice into irradiated recipients will be carried out. The effect of administration of inappropriate anti-idiotypic antibodies to mice of various strains will be assessed. An examination of the control of idiotype expression at the T cell level will be attempted using the in vitro anti-TNP nuclease plaque-forming cell response.

The isoelectrofocusing patterns on serum from animals treated with anti-idiotypic will be performed. These patterns will be compared to those we have previously found following antigen immunization. Attempts will be made to determine the nature of the idiotype expression in anti-idiotypic primed animals which do not demonstrate measurable anti-nuclease activity. Such antibodies will be purified by affinity chromatography on anti-idiotypic columns and will be examined for spectrotypic as well as for their ability to induce anti-idiotypic reactive with anti-nuclease antibodies. These studies may help to shed light on the network normally involved in the immune response to nuclease and may permit intervention into that network in a specific manner.

Attempts to produce hybridomas secreting monoclonal anti-nuclease antibodies will be continued. These antibodies will be examined for expression of individual anti-nuclease idiotopes and will be used to produce monospecific anti-idiotypic reagents for further characterization in in vitro and in vivo assays. In addition, attempts will be made to produce cloned T cell lines capable of providing T cell help for the in vitro anti-nuclease-TNP response.

Such lines will be examined both for anti-nuclease reactivity and reactivity with our anti-idiotypic reagents. Should such lines be established, attempts will be made to isolate and characterize the relevant T cell receptors.

Anti-idiotypic antibodies will be fractionated on immunoabsorbants bearing monoclonal anti-nuclease antibodies. These fractions, as well as syngeneic anti-idiotypic reagents, will be examined for effects on humoral and cellular immunity to nuclease.

Publications

Glimcher, L. H., Hamano, T., Asofsky, R., Sachs D. H., Pierres, M., Samelson, L. E., Sharrow, S. O., and Paul, W. E.: Ia mutant functional antigen presenting cell lines. J. Immunol. 130: 2287-2294, 1983.

Sacks, D. L., Kelsoe, G. H., and Sachs, D. H.: Induction of immune responses with anti-idiotypic antibodies: Implications for the induction of protective immunity. Springer Seminars in Immunopathol., in press.

Sachs, D. H.: Staphylococcal nuclease as a model antigen. In Schechter, A. N. and Goldberger, R. F. (Eds.): Contributions of Chemical Biology to the Biomedical Sciences. New York, Academic Press, Inc., in press.

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

PROJECT NUMBER

Z01CB05038-11 I

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-Mediated Immunity to Hapten Modified Syngeneic Lymphocytes in Mice

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

(Name, title, laboratory, and institute affiliation)

Gene M. Shearer, Senior Investigator, Immunology Branch, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

4.5

PROFESSIONAL:

3.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

B

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

Cytotoxic T lymphocytes (CTL) were generated in vitro against syngeneic spleen cells conjugated with a number of different haptens: trinitrophenyl (TNP-self), two different isomers of fluorescein isothiocyanate and I-AEDANS (AEDANS self). Genetic control of CTL responses to these haptens was compared. H-2^{k,a} and H-2^{b,d} mouse strains were the respective genetic high and low responders to haptens which conjugate to -NH₂ groups. The reverse genetic pattern was observed for CTL responses to hapten-self which conjugate to -SH groups. These differential response patterns raise some interesting possibilities concerning genetic control of immune responses and the self determinants recognized. The role of helper T cells in Ir gene control and hapten specificity was also studied for the above haptens. Ir gene defects were detected at the helper cell level and in CTL precursors or accessory cells. Activation of CTL helpers was found to be specific, but once activated, these helper effects were non-specific.

Project Description

Other Professional Personnel Engaged on the Project:

R. B. Levy, Senior Staff Fellow, Immunology Branch, NCI
T. Tsuchida, Visiting Fellow, Immunology Branch, NCI
J. Costa, Senior Investigator, P, NCI
D. Segal, Senior Investigator, Immunology Branch, NCI

Objectives: The primary objectives of this laboratory are to investigate the function of T lymphocytes, the role of self recognition, and the effects of major histocompatibility genes on the murine and human immune systems. These studies are being pursued using mouse and human leukocytes which are sensitized to autologous cells either modified with chemical agents (e.g. the trinitrophenyl group) or infected with viruses (e.g. influenza, cytomegalovirus). The murine TNP-self cytotoxic system serves as a basic model for testing many immunogenetic questions concerning the role of the MHC in immune regulation. The specific objective of this project was to continue in the investigation of immunogenetic parameters associated with the in vitro generation of T-cell mediated immunity of murine cells to syngeneic cells modified with the TNP-group. More explicitly, the project was designed to: (a) analyze the dominance of K^k TNP-self responses over D end TNP-self responses; (b) investigate the possibilities of generating helper T cells in vivo for secondary in vitro cytotoxic responses to TNP-self, and to study the effects of helpers on restriction, Ir gene control, cross-reactivity, CTL specificity, etc.; (c) to compare the in vivo priming effects on different types of T-cell mediated immunity, (e.g., cytotoxicity, delayed hypersensitivity, suppression); (d) to compare the CTL results observed using TNP-self with those obtained using the FITC-self and AEDANS-self haptens; and (e) to determine whether modification of H-2 antigens with TNBS can result in neo-determinants that resemble other H-2 antigens.

Methods Employed: For in vitro generation of CML activity mouse spleen cells were sensitized in vitro to autologous cells conjugated with trinitrobenzene sulfonic acid (TNP-self), N-iodoacetyl-N-(5-sulfonic-naphthyl) ethylene diamine (AEDANS-self) or various isomers of the fluorescein hapten, one of which was NH_2 -reactive (FITC) and others of which were SH-reactive (5AAF and 6AAF). The effector cells generated were assayed on the appropriate hapten conjugated or untreated ^{51}Cr -labelled target cells.

Helper cells for CML responses were generated by injection of hapten-conjugated syngeneic cells or by skin painting with the hapten. Helper cell activity was determined by co-culture of irradiated spleen cells from immunized mice with those from normal spleen cells.

Major Findings: Helper cell activity for TNP-self was demonstrated in genetic high responder ($H-2^{k,a}$), but not in low responder ($H-2^{b,d}$) mouse strains. The activation of helper cells was found to be hapten-specific, but these helper cells (or the factors they generate) were able to help CTL responses for other non-crossreacting (at the effector cell level) haptens when the helper-cells were cultures with the specific hapten plus another hapten. Using this approach, we were able to by-pass the helper cell defect to a

particular hapten and demonstrate Ir gene defects at both the helper and CTL precursor or accessory cell levels.

The H-2 linked Ir gene control of CTL responses to hapten-self antigens was found to be independent of the hapten specificity of the CTL, but was found to be associated with whether the modifying agent was reactive with NH₂- or SH- groups. Thus, similar haptens exhibited different Ir gene patterns of response in congenic mouse strains, depending on whether they coupled with cell surface NH₂- or SH- groups. Modifications of SH- groups was associated with responsiveness in H-2^b but low responsiveness in H-2^{a,k} strains. In contrast, modification of NH₂- groups was associated with high responsiveness in H-2^{a,k} and low responsiveness in H-2^b mice.

Conjugation of B10.BR spleen cells with trinitrobenzene sulfonate (TNBS) resulted in an approximate 4-fold increase in detectable amount of H-2K^k antigen as detected by a number of K^k specific monoclonal reagents (e. g., 36.7.5). No changes were detected when the conjugated cells were exposed to monoclonal antibodies of different specificities. These results suggest that there is a molecular basis for functional observations that, in TNP-self CTL responses, there is preferential recognition of the hapten in association with K^k self determinants.

Significance to Biomedical Research and the Program of the Institute: The project is of fundamental immunological interest in that it describes a major histocompatibility linked immunological phenomenon involving self recognition. The recognition of self MHC-coded structures in association with foreign antigenic determinants raises the possibility for self recognition as important for autoimmunity and for virally-infected autologous cells. The finding that products of the MHC are important for the antigenic complex recognized as well as for determining immune response potential provides a working hypothesis for bifunctional MHC control of disease susceptibility in those examples of HLA-associated diseases in man. The intricate immunoregulatory phenomena identified in the hapten-self cytotoxic system permits us to look for similar patterns in the virally infected murine and human models, and to attempt to determine whether such mechanisms could be operating in the immune systems of the intact individual, in either an infectious or neoplastic state.

Proposed Course of Project: Investigation of this model will continue in order to determine: (a) whether different self MHC products are recognized by helper, and cytotoxic T cells; (b) whether these Ir genes are specific for the reactive groups; i.e., NH₂- vs. SH- rather than for the haptens themselves; (c) what components of the haptens are recognized as unique and crossreactive determinants by CTL; and (d) the role of accessory (antigen presenting) cells in Ir gene control and CTL specificity.

Certain preparations of olive oils have been found in Spain that cause immunological and neurological problems in humans. It is considered that contaminants of this oil are inducing "modified self" immune reactions in these patients. We have obtained a sample of this oil and are attempting to induce "modified self" types of reactions in mice that have been exposed to these oils by inhalation, skin painting, injection, and/or ingestion.

Publications:

Levy, R. B. and Shearer, G. M.: 1982. Can CTL recognize self determinants on molecules other than those expressing polymorphic MHC self determinants? Immunol. Today 3: 204-205.

Levy, R. B. and Shearer, G. M.: 1982. Cell-mediated lympholysis responses against autologous cells modified with haptenic sulphhydryl reagents. IV. Self-determinants recognized by wild-type anti-H-2K^b and H-2D^b-restricted cytotoxic T cells specific for sulphhydryl and amino-reactive haptens are absent in certain H-2 mutant strains. J. Immunol. 129: 1525-1529.

Hurtenbach, U. and Shearer, G. M.: 1983. Analysis of murine T lymphocyte markers during the early phases of GVH-associated suppression of cytotoxic T lymphocyte responses. J. Immunol. 130: 1561-1566.

Levy, R. B. and Shearer, G. M.: 1983. Cell-mediated lymphocytic responses against autologous cells modified with haptenic sulphhydryl reagents. V. H-2L^d self products are recognized by anti-AED-specific cytotoxic T cells. J. Immunol. 130: 1506-1511.

Hurtenbach, U., Shearer, G. M., and Levy, R. B.: Cross-reaction of allo-antigens and certain B6 mutant antigens by antigen-specific and polyclonally activated cytotoxic effector cells. Cellular Immunol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		201CB05050-09 I
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 on the Structure and Function of the Constant Portions of Immunoglobulins		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) David M. Segal, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 4.5	PROFESSIONAL: 3.5	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) (1) Cell:cell interactions between antibody-coated spleen cells and P388D1 cells have been examined using a novel flow microfluorometric technique. The mechanism of FcγR-mediated aggregation has been studied. (2) The distributions of FcγR have been studied in mouse T and B cells and in macrophages by dual parameter flow cytometry. Distributions in normal mice differ significantly from those in mice infected with different pathogenic agents. (3) A new type of effector cell has been generated by treating ADCC effector cells with anti-FcγR antibody cross linked to anti-target cell antibodies. (4) The appearance of neo MHC determinants has been detected on TNP modified spleen cells, using anti-MHC monoclonal antibodies. These results correlate with cross reactivities seen in CML reactions.		

Project Description

Other Professional Personnel Engaged on the Project:

B. Karpovsky, Clinical Staff Fellow, Immunology Branch, NCI
P. Perez, Visiting Fellow, Immunology Branch, NCI
F. D. Finkelman, Associate Professor, NMRI
I. Katona, NMRI
G. Shearer, Senior Investigator, Immunology Branch, NCI
R. Levy, Senior Staff Fellow, Immunology Branch, NCI
S. K. Dower, Immunex Corp., Seattle, Washington
J. F. Jones, Asst. Prof., Dept. of Microbiology, Thomas Jeff. Univ, Phil., PA

Objectives: (1) To investigate the molecular and cellular bases of the interactions of immunoglobulins with immune effector systems, (2) to study the relationship of antigenic recognition to these interactions, (3) to find methods by which these interactions can be artificially manipulated with the ultimate goal of increasing immune responses toward pathogens or neoplastic cells and decreasing autoimmune responses; and (4) to apply methods developed with the above studies to other immunologically related molecules and processes.

Methods Employed: Organic synthesis, gel filtration, ion exchange chromatography, polyacrylamide gel electrophoresis, complement fixation, radiolabeling of proteins, tissue culture, binding assays, antibody-dependent cytotoxicity assays, cell separations, flow microfluorometry, computer analyses.

Major Findings: I. Cell:cell interactions. The specific binding of one cell to another is a fundamental event in most processes of the immune system, for example in cell-mediated cytotoxicity, in antibody production, and in the regulation of responses. Specific interactions between cells most commonly arise because one cell type expresses ligand molecules on its surface which can bind to receptors, specific for that ligand, on the surfaces of other cells. The multiple interaction between ligands and receptors at the cell:cell interface leads to the formation of stable cell conjugates.

In order to continue our investigations into the role of multivalent interaction in the immune system, we have developed a rapid and accurate flow microfluorometric method for measuring the binding of one cell type to another. Our system is the Fc receptor (FcγR) mediated binding of antibody-coated mouse spleen cells to cells from the mouse macrophage line, P388D₁. The spleen cells are labeled with a red fluorophore and the P388D₁ with a green one, and the cells are mixed in suspension at 4°C. After various time intervals the cells are measured for both red and green fluorescence in a dual laser flow cytometer. Unconjugated cells appear either red or green while conjugates are detected as single particles which are both red and green.

In order to demonstrate the specificity of interaction between antibody-coated spleen cells and P388D₁, the two cells were mixed under conditions where (1) no antibody was on the spleen cells, (2) antibody was on the

red and green, and were subsequently mixed, no red:green particles were observed. These data demonstrate that conjugate formation is dependent upon both receptors on the P388D₁ cells and ligands (antibody) on the spleen cells, and that intercellular aggregation only occurs between P388D₁ and spleen cells.

In other control experiments we demonstrated that the flow system of the cytometer does not disrupt conjugates, and that the cytometer detects most, and probably all, conjugates as single particles.

Detailed analyses showed that most conjugates contained a single P388D₁ cell and multiple spleen cells, and that the ratio of spleen:P388D₁ increased as the ratio in the original mix increased. Using more sensitive methods, we were also able to show that a small percentage of conjugates contained two P388D₁ cells.

The rate of intercellular aggregation in cell suspensions increases hyperbolically with cell density, reaching a plateau when each cell type is at approximately 5×10^6 cells/ml. This suggests that an intermediate exists in the formation of stable aggregates, and that this intermediate reaches a maximal (saturation) value when each cell is at about 5×10^6 cells/ml. Next, we measured the rate of aggregation of cells at varying receptor densities (by partially blocking Fc γ R on P388D₁ with 2.4G2) and at varying antibody densities (by treating spleen cell with different antibody concentrations). These studies were done at high cell concentrations, so that the aggregation rate was independent of cell density. The results of these experiments showed that both the extent and rate of conjugate formation increased when either the ligand or receptor concentration increased. The extent of conjugate formation increased hyperbolically with receptor or antibody densities, while the rate of aggregation increased linearly with antibody density and exponentially with receptor density. These data are consistent with a mechanism in which ligand and receptor form an equilibrium at the cell:cell interface, and in which the rate limiting step in the formation of stable aggregates from the unstable intermediate, is the lateral diffusion of free receptors to unliganded antibody molecules on the spleen cells.

In other experiments we titrated free receptors on conjugated and unconjugated P388D₁ cells, using fluorescent 2.4G2 anti-receptor antibody. These experiments showed that only a small number of receptors was required to form cell conjugates. Finally we also showed that aggregation is not inhibited by azide, EDTA, or colchicine, but is inhibited by cytocholasin B. These experiments suggest that intercellular aggregation requires only a limited number of receptor-ligand interactions and that the cytoskeleton is somehow involved in aggregate formation.

II. The expression of Fc γ R on murine cells. The mouse is the most widely used experimental animal in the field of immunology, and in order to determine how Fc γ R function in mice it is necessary to know which cells express them. We have been approaching this problem using a flow cytometric analysis for Fc γ R which we developed several years ago. Basically Fc γ R on cells are saturated with oligomers of rabbit IgG, washed, and stained using a

FITC-labeled goat anti-rabbit antibody. The Fc γ R distribution is then measured quantitatively by flow cytometry. More recently we have been identifying the cells which bear Fc γ R by staining with a second antibody, labeled with the red fluorophore, Texas Red. The Fc γ R distributions within subsets of cells defined by these second antibodies are then determined by dual parameter flow cytometry.

In mouse spleen, B-cells were identified by their ability to bind antibodies against IgM, IgD, or I-A. Virtually all mouse spleen cells express Fc γ R ($1-3 \times 10^4$ receptors/cell), and the same levels of expression are observed on cells staining for sIgD, sIgM or I-A. In normal mice no relationship has been observed between Fc γ R expression and the expression of the three B-cell markers, suggesting that the synthesis of Fc γ R is distinct from that of I-A, sIgM and sIgD.

Fc γ R are difficult to measure on mouse macrophages, since these cells are present in low amounts in the spleen, and since a good macrophage specific marker is not available in the mouse. Nevertheless, MAC-1 appears to be specific for a subset of macrophages in mouse spleen, and by dual staining spleen cells for Fc γ R and MAC-1, we have found that all MAC-1 + spleen cells bear relatively high (8×10^4 receptors/cell) levels of Fc γ R. These same cells also stain somewhat less brightly than B cells for I-A. A better definition of Fc γ R on murine macrophages will require better anti-macrophage monoclonal reagents. A subpopulation of T-cells in the mouse also expresses Fc γ R. These T γ cells are present in relatively small numbers (an average of 5% of the total) and are heterogeneous in their expression of Fc γ R. The T γ cells stain positively for Thy and Lyt 1 while a subset stains for Lyt 2. This subset expresses lower levels of Lyt 2 than another Lyt 2⁺ subset which does not express Fc γ R. In general, dual staining experiments with mouse spleen cells for Fc γ R and Lyt 2, give similar results to studies with human peripheral blood leukocytes stained for Fc γ R and OKT8. In both cases the suppressor T cells probably lie in the FcR⁺, Lyt⁺ (or OKT8⁺) subsets.

There is no obvious strain dependence in the expression of Fc γ R, although BALB/c mice tend to express fewer Fc γ R on their spleen cells than do other strains, including DBA/2. In addition male and female mice express similar levels of Fc γ R, and in adult mice (6 weeks of age or greater) there is no obvious age dependence. In young mice (4 weeks of age or less), however, the numbers of Fc γ R expressed on splenic B cells are higher than in adults. In very young mice, B cells express high levels of Fc γ R and mainly sIgM. As they age, the levels of Fc γ R expressed on the spleen cells decrease, while the amounts of I-A and then sIgD increase. Fc γ R distributions in bone marrow cells from young or old mice are similar to those found on spleen cells from newborn mice.

The distributions of Fc γ R often change dramatically in response to disease. For example in trypanosome-infected mice the number of spleen cells markedly increases, with a concomittant increase in the numbers of cells expressing high levels of Fc γ R and sIgM. In CMV infected mice, the number of spleen cells drops, with an increase in the percentages of both B and T cells expressing high levels of Fc γ R. Finally in mice infected

with *Nippostrongylus brasiliensis*, there is little change in the Fc γ R expression on B cells, but the percentage of T γ cells increased markedly.

Franked ADCC

The high concentrations of monomeric IgG in the serum of normal individuals and high concentrations of immune complexes in the blood of patients suffering from autoimmune disease strongly inhibit all IgG-mediated effector functions. In order to override this inhibition in antibody-dependent, cell-mediated cytotoxicity (ADCC) we have previously developed a technique, termed "franking", in which the antibody is bound irreversibly to Fc γ R on the effector cells. Franked effector cells are much more resistant to inhibition by monomeric IgG and immune complexes than are normal ADCC effector cells. However in our original studies the franking technique was cumbersome, and in some cases resulted in effector cells which could still be partially blocked by immune complexes.

Recently we have developed a new method for producing franked effector cells which is easier, more reproducible, and requires less antibody than the earlier technique. The new franking procedure involves the covalent cross linking of antibodies with specificity for target cells with the 2.4G2 anti-mouse Fc γ R hybridoma antibody. Cross linking is done using the reagent SPDP, which specifically cross links anti-receptor with anti-target antibodies, but does not cross link either antibody to itself. When incubated at low concentrations (1-10 μ g/ml) with mouse effector cells, the cross linked protein binds to the effector cell, and converts it into a potent effector against the target cells. As before our model system has utilized P388D₁ cells as the effectors, and TNP-coated chicken erythrocytes as the targets, with anti-TNP antibody cross-linked to 2.4G2. P388D₁ cells franked with this reagent readily form conjugates with the target cells (as determined by flow cytometry), and when incubated with the targets, they cause lysis at relatively low effector:target ratios (10:1 or less). Both conjugate formation and lysis are readily blocked by free hapten (DNP- ϵ -aminocaproate), but neither is blocked by immune complexes or monomeric IgG. The blocking experiments are much more reproducible than when the original franking procedures were used.

We are currently screening tumor cells for expression of viral GP70, and are cross-linking 2.4G2 to a monoclonal anti-GP70 antibody, in hopes of testing franked effector cells against tumor cells in vivo.

The binding of anti-MHC class I monoclonal antibodies to modified spleen cells.

We are continuing our study of the expression of MHC antigens on cell surfaces using monoclonal antibodies. In one of these studies we assayed both TNP and MHC (K^k) expression on B10.A spleen cells modified with TNBS, as a control for CML experiments done in the laboratory of Dr. Gene Shearer. As expected, the TNP-modified cells bound high amounts of anti-DNP antibody, and binding was inhibited by TNP hapten. To our surprise, however, we also found that the K^k (but not D^d) expression (as measured by the binding of the 36-7-5 anti K^k monoclonal antibody) also increased as a result of hapten modification of the B10.A spleen cells. The binding of 36-7-5 was not inhibitable by TNP

hapten, nor by 2.4G2, showing that the increased antibody binding on the modified cells was not due to a cross reaction with the hapten nor binding to Fc receptors. Moreover at least two other anti K^k monoclonals also demonstrate the same effect, whereas two anti D^d antibodies show a slight decrease in binding upon hapten treatment. Even more interesting is the observation that B10 cells, cells which normally do not bind anti-K^k antibodies, do react with some anti K^k monoclonals when treated with TNBS.

The appearance of neo determinants on B10 and B10.A spleen cells after TNP modification parallels, and may explain, the Lemonier phenomenon, namely that B10 cells, when stimulated with irradiated B10.TNP spleen cells, will lyse B10.A target cells. Both antibody-binding and lytic studies are consistent with the interpretation that TNP modification of B10 or B10.A spleen cells causes antigens to appear on the cell surface which mimic K^k determinants.

Significance to Biomedical Research and the Program of the Institute: The recognition of foreign substances by antibodies elicits a number of reactions which normally lead to their elimination from the body. The purpose of this project is to examine the molecular events which occur as a result of antigenic recognition. It is hoped that these studies will enhance our understanding of these processes and allow us to better control immune reactions. An ultimate goal is to enhance the immune response toward neoplastic cells.

Proposed Course of Project:

1. We plan to continue our studies of cell:cell interactions with antibody-coated target cells and P388D₁ cells, by measuring interactions at 37°C. Subsequent to binding, P388D₁ cells can lyse antibody coated target cells at 37°, and we plan to study lysis, if possible, by flow cytometry.
2. We hope to finish the characterization of FcγR-bearing cells in the mouse, and to study the functions of FcγR in various subpopulations of cells.
3. We have begun a study of the class-I MHC antigen dependent aggregation of cloned CTL effector cells with appropriate target cells. These studies will be done with Dr. Jeffrey Bluestone, and are designed to probe the molecular events involved in MHC dependent cell:cell interactions.
4. We plan to test the franked effector system in mice, in vivo, against GP70-expressing tumor cells. We are also planning to use the 3G8 anti-human neutrophil FcγR monoclonal antibody to study franked human neutrophils as effectors against human tumor targets in vitro.

Publications:

Titus, J. A., Haynes, B. F., Thomas, C. A., Fauci, A. S. and Segal, D. M.: Analysis of Fc (IgG) receptors on human peripheral blood leukocytes by flow microfluorometry. I. Receptor distributions on monocytes, T_H cells and cells labeled with the 3A1 anti-T cell monoclonal antibody. (1982). Eur. J. Immunol. 12; 474-479.

Titus, J. A., Sharrow, S. O., and Segal, D. M.: Analysis of Fc (Ig) receptors on human peripheral blood leukocytes by dual fluorescence flow microfluorometry. II. Quantitation of receptors on cells that express the OKM1, OKT3, OKT4 and OKT8 antigens. (1983). *J. Immunol.* 130; 1152-1157.

Segal, D. M., Dower, S. K. and Titus, J. A.: The FcR-mediated endocytosis of model immune complexes by cells from the P388D₁ mouse macrophage line. I. Internalization of small, non-aggregating oligomers of IgG. (1983). *J. Immunol.* 130; 130-137.

Segal, D. M., Titus, J. A., and Dower, S. K.: The FcR-mediated endocytosis of model immune complexes by cells from the P388D₁ mouse macrophage line. II. The role of ligand induced self aggregation in promoting internalization. (1983). *J. Immunol.* 130; 138-144.

Otani, I., Conrad, D. H., Carlo, J. R., Segal, D. M., and Ruddy, S.: Phagocytosis by rat peritoneal mast cells: Independence of IgG Fc-mediated and C3-mediated signals. (1982). *J. Immunol.* 129; 2109-2112.

Sher, I., Titus, J. A., and Finkelman, F. D.: The ontogeny and distribution of B cells in normal and mutant immune-defective CBA/N mice: Two parameter analysis of surface IgM and IgD. (1983). *J. Immunol.* 130; 619-625.

Sher, I., Titus, J. A., Sharrow, S. O., Mond, J. J. and Finkelman, F. D.: Distribution of surface IgD on B lymphocytes of mice: Two parameter correlation with surface IgM and I-A. (1982). *Ann. N.Y. Acad. Sci.* 399; 204-220.

Istersky, C., Rivera, J., Segal, D. M. and Triche, T.: The fate of IgE bound to rat basophilic leukemia cells. II. Endocytosis of IgE oligomers and effect on receptor turnover. (1983). *J. Immunol.* in press.

Segal, D. M., Dower, S. K., and Titus, J. A.: The role of non-immune IgG in controlling IgG mediated effector functions. *Mol. Immunol.* in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05055-08 I
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.) Regulatory Influences of Cell-Mediated Immune Responses		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Richard J. Hodes Chief, Immunotherapy Section, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION Immunotherapy Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.1	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Previous investigations have described both antigen-specific and antigen-nonspecific T cell-mediated regulatory mechanisms capable of suppressing the in vitro generation of cell-mediated cytotoxicity. Further experiments have been directed at determining the cellular interactions involved in the in vitro generation of suppressor cells, and at analyzing genetic differences in the abilities of different inbred mouse strains to generate suppressor cells. Most recently, monoclonal anti-Lyt reagents have been employed to characterize the T cells responding in cell-mediated cytotoxicity as Lyt 1+2+. Two regulatory pathways have been described, one mediated by Lyt 1+2- T cells which nonspecifically suppresses the generation of cell-mediated cytotoxicity, and one requiring Lyt 1+2+ T cells which suppresses in an antigen-specific fashion.</p>		

Project Description

Objectives: In addition to the effector cells generated in response to antigen stimulus, there has been increasing data presented to suggest that cell-mediated regulatory influences are also activated by antigenic stimulus. It has been the object of these studies to define the regulating influence governing the generation of T cell-mediated responses.

Methods Employed: T cell-dependent suppression of cell-mediated lympholysis was generated by in vitro culture of normal murine spleen cells either (a) in the absence of allogeneic cells, or (b) in the presence of alloantigenic stimulating cells.

The cellular requirements for generation of T cell-dependent suppression were studied employing treatment with cytotoxic monoclonal anti-Lyt reagents to characterize the T cell subpopulations involved both as precursors and as effectors of suppression.

Major Findings:

In order to determine the identity of the T cell subpopulation(s) participating in the generation of T suppressor cells, the effects of treatment with cytotoxic anti-Lyt hybridoma reagents were studied. Spleen cells cultured alone, in the absence of alloantigen, generated a radiosensitive population of cells which nonspecifically suppress the induction of cell-mediated cytotoxicity. Both the precursors and the effectors of this suppression are Lyt 1⁺2⁻ T cells. Spleen cells cultured with irradiated alloantigenic stimulators generate radioresistant antigen-specific suppression. The generation of this specific suppression requires an Lyt 1⁺2⁺ precursor, and suppression is mediated by an Lyt 1⁺2⁻ T cell.

Significance to Biomedical Research and the Program of the Institute: States of in vivo tolerance or immune hyporesponsiveness have been associated with active suppressive populations in a number of systems. In addition, the possible role of specific or nonspecific immune suppression in the evaluation or progression of malignancy has been suggested. The studies described above may provide information concerning the mechanism of such in vivo immunosuppression and possible means of modifying the suppression as a therapeutic manipulation of the immune response.

Proposed Course of Project: Further studies are in progress to determine whether additional cells in responding populations participate in the mediation of suppression, and whether genetic restrictions exist among interacting populations in the regulatory process.

Publications:

Hathcock, K. S. and Hodes, R. J.: Regulatory mechanisms in cell-mediated immune response. V. Distinct Lyt subsets mediate antigen-specific and antigen non-specific suppression. Transplantation, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 201CB05058-08 I
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.) Immunoregulation of Antibody Synthesis and Secretion		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Howard B. Dickler, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.5	PROFESSIONAL: 2.5	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The goal of this project is to characterize mechanisms of immunoregulation of antibody synthesis and secretion, particularly Ir genes and idiotypic networks. Recently, a system has been developed in which, for the first time, soluble antibody responses to the synthetic polypeptide (T,G)-A--L can be generated and detected in vitro using antigen-primed lymph node cells. Responses are antigen dependent and specific, and H-2 linked Ir gene regulated. Antibodies specific for the idiotypes of anti-(T,G)-A--L antibodies induce antigen-independent anti-(T,G)-A--L antibody responses. These responses are specific at the levels of the anti-idiotypic reagent, the antigen-priming, and the antibody produced. The anti-idiotypic antibodies stimulate function from antigen-primed T lymphocytes in the form of soluble lymphokines, and function from both primed and unprimed B cells in the form of specific antibody secretion. Unprimed B cells, in addition to anti-idiotypic, require either primed T cells or idiotypic present in order to obtain function. Responses to anti-idiotypic antibodies, in contrast to those to antigen, appear not to be regulated by Ir genes.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

H. Weissberger, Postdoctoral Fellow, Immunology Branch, NCI
F. Uher, Postdoctoral Fellow, Immunology Branch, NCI

Objectives: Characterization of immunoregulation of antibody synthesis and secretion particularly by Ir genes and idiotypic networks.

Methods Employed: An in vitro microculture system using antigen-primed lymph node cells is utilized. Antibody responses are measured using an enzyme-linked immunoabsorbant assay (Elisa) with antigen-coated plastic plates. Immunoregulation is studied by manipulation of the conditions of interaction, and by immunologic and chemical modifications of both the lymphocytes and reagents. Anti-idiotypic antibody is prepared by immunization of Lewis rats with antigen-affinity purified murine anti-(T,G)-A--L antibodies with subsequent adsorption on normal mouse immunoglobulins. Anti-idiotypic activity can be measured by inhibition of binding of anti-(T,G)-A--L antibodies to the antigen, or by direct binding to the idiotypic. Genetic studies are carried out utilizing inbred, congenic, and/or recombinant mouse strains. Lymphocyte subpopulations are prepared by a variety of techniques including antibody and complement mediated lysis, antibody affinity plates, density gradients, and adherence columns. Other techniques employed include flow microfluorometry and production and purification of monoclonal antibodies.

Major Findings: Using a microculture system we have been able to obtain antigen-specific, T lymphocyte dependent responses to (T,G)-A--L in vitro. This is the first known success at obtaining a soluble antibody response in vitro to an Ir gene regulated antigen. The characteristics of the system are as follows: 1) In order to detect such responses it is necessary to wash the antigen out of the cultures after 3 days and use a very sensitive detection system (Elisa); 2) Primed T lymphocytes are required; 3) The response is antigen-dependent and specific; 4) The response is under Ir gene control and the Ir genes are phenotypically expressed by B lymphocytes and/or accessory cells; 5) T and B lymphocytes can cooperate to produce these responses if they are H-2 identical even if non-H-2 genes are different; 6) Responses are highly reproducible and levels of antibody are 100-500 ng/ml.

Recently, we have evaluated the effects of anti-idiotypic reagents in this system. It was found that antigen-primed lymph node cells responded to anti-idiotypic by the production of antibody in the absence of antigen. This antigen-independent response was specific at the level of the anti-idiotypic reagent in that other reagents including normal rat serum, rat anti-mouse IgG, rat anti-mouse IgM and rat anti-nuclease anti-idiotypic were not active. Moreover, idiotypic affinity-purified anti-idiotypic was active. The response was also specific at the level of priming in that cells primed to CFA or ovalbumin would not respond to anti-(T,G)-A--L anti-idiotypic. Finally, it was specific at the level of the antibody produced since antibodies against other antigens were not induced.

The cellular site of action of the antigen-independent anti-idiotypic induced anti-(T,G)-A--L response was evaluated. Rigorously B lymphocyte depleted

antigen-primed T lymphocytes together with equal numbers of unprimed B lymphocytes (unprimed lymphocytes, neither B or T, would respond to anti-idiotypic) responded to anti-idiotypic, suggesting that the anti-idiotypic was provoking function from T helper lymphocytes. This was directly shown by stimulating antigen-primed T cells for 3 days with anti-idiotypic and collecting the supernatant. This supernatant (but not controls) contained soluble helper lymphokines which allowed unprimed B cells to produce specific antibody when stimulated with anti-idiotypic (but not controls). Thus, the anti-idiotypic directly triggers both T and B lymphocytes. The T lymphocytes which respond to anti-idiotypic have been characterized as Lyt 1 positive, Lyt 2 negative. Responses to the anti-idiotypic do not appear to be regulated by I_r genes. Thus, mixtures of (Responder x Nonresponder)F₁ antigen-primed T lymphocytes with Nonresponder B plus accessory cells respond to anti-idiotypic but not to the antigen.

Antigen-primed B lymphocytes rigorously depleted of T cells specifically respond to anti-idiotypic by secretion of anti-(T,G)-A--L antibodies. This response was shown to be specific by a variety of criteria. This stimulation of function by anti-idiotypic appears to involve idiotypic-anti-idiotypic complexes because normal B cells respond to neither idiotypic or anti-idiotypic but do respond to a mixture of the two.

Significance to Biomedical Research and the Program of the Institute: An understanding of the regulation of the immune response could lead to new forms of therapy for human diseases including cancer. Specifically, the findings outlined here indicate it is possible to trigger antigen-specific immune function of lymphocytes in the absence of antigen using anti-idiotypic reagents. This raises the possibility of the use of such reagents themselves as therapeutic modalities.

Proposed Course of Project: Further characterization of antigen-independent response to anti-idiotypic as follows: 1) Phenotype subpopulations of lymphocytes which are responding. 2) Evaluate requirement for accessory cells. 3) Characterize mechanisms of activation. 4) Further evaluate role of H-2 restriction and I_r genes in these responses. 5) Determine if T cells which respond to antigen and anti-idiotypic are the same or different. 6) Determine specificity of helper lymphokines produced in response to anti-idiotypic. 7) Determine types of I_g complexes which will stimulate antigen-primed B cells to specifically secrete antibody.

Publications:

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Project Description

Other Professional Personnel Engaged on the Project:

J. Lunney, Staff Fellow, Immunology Branch, NCI
 B. Osborne, Staff Fellow, LCBGY, NCI
 D. Sachs, Chief, Transplantation Biology Section, Immunology Branch, NCI
 K. Ozato, Visiting Associate, Immunology Branch, NCI
 L. Glimcher, Res. Assoc, LI, NIAID
 R. Asofsky, Chief, LMI, NIAID
 W. Paul, Chief, LI, NIAID
 S. Korsmeyer, Senior Investigator, Metabolism Branch, NCI
 P. Leder, Chief, LMG, NCI
 T. Waldmann, Chief, Metabolism Branch, NCI
 J. Titus, Chemist, Immunology Branch, NCI
 D. Segal, Senior Investigator, Immunology Branch, NCI
 S. Epstein, Staff Fellow, Immunology Branch, NCI
 J. Bluestone, Senior Investigator, Immunology Branch, NCI

Objectives: The objective of this project is to utilize rapid flow microfluorometry (FMF) to study selected aspects of important problems which would be impossible or extremely difficult to pursue without such technical support.

Methods Employed: Cells have been both analyzed and separated by rapid flow microfluorometry (Fluorescent-Activated Cell Sorter (FACS), Becton-Dickinson Electronics Laboratory, Mountain View, CA). These studies involve preliminary treatment of suspensions of viable, dispersed cells with purified, well-characterized fluorescent reagents.

Major Findings: This report summarizes the major thrust of each overall project emphasizing those aspects most heavily supported by use of rapid flow microfluorometry (FMF).

Titus, Segal, and colleagues. Previous studies by this group demonstrated that human peripheral blood mononuclear cells fall into 3 distinct subsets based on the level of expression of cell surface receptors for IgG immunoglobulin (FcR). High and low levels of FcR expression were associated with T cells, whereas intermediate levels were associated with monocytes. This work has been pursued using dual laser FMF and monoclonal antibodies which define subsets of T cells. Five subsets of peripheral blood mononuclear cells have been identified by simultaneous analysis of expression of cell-surface FcR and OKT8 antigens. These serologic subsets have been associated with distinct biologic functions on the basis of previous studies by others and by further analysis with other monoclonal antibodies. The subsets are as follows: 1) Cell with negligible expression of FcR and OKT8 antigens. This group contains nearly all (99%) of cells reacting strongly with OKT4 antibody and has been associated with helper T cells. 2) Cells with negligible FcR expression which bind OKT8 monoclonal antibody. This group contains the majority (76%) of cells binding OKT8 antibody and has been associated with cytotoxic T cells and their precursors. 3) Cells with intermediate expression of FcR which also bind negligible levels of OKT8 antibody. This group contains the majority of monocytes and is relatively free of lymphocytes. The

association with monocytes is based on binding of high levels of OKM1 antibody, failure to bind antibodies which react with lymphocytes, adsorption by G10 sephadex, and relatively large cell size. 4) Cells with high expression of FcR which bind OKT8 antibody. This group has been associated with suppressor cell activity. 5) Cells with high expression of FcR which bind negligible levels of OKT8 antibody. This group probably includes most NK and ADCC effector T cells, which are known to express high levels of FcR and not to be depleted by anti OKT8 and complement.

Segal and Stephany. A quantitative method has been developed for measuring cell:cell binding by dual laser FMF. The model for this study involves the binding of a mouse macrophage cell line (P388D₁) to antibody-treated spleen cells. To measure conjugates of cells, macrophages have been directly labeled with FITC (green fluorescence) and spleen cells with X-RITC (red fluorescence). Following incubation at 4°C, unconjugated cells appear either red or green, while conjugates of cells are both red and green. The binding of macrophages to antibody-treated spleen cells is antibody dependent and requires free Fc receptors on the macrophages. Analysis of the reaction stoichiometry shows that conjugation follows first order kinetics and that at equilibrium most conjugates contain one macrophage and 1-3 spleen cells, with the number of spleen cells depending upon the ratio of macrophage and spleen cells in the original suspension. Shear forces generated by the flow cytometer have not affected the number of conjugates detected.

Korsmeyer, Leder, Waldmann, and colleagues. Understanding the process of B lymphocyte maturation has been aided in the past by FMF studies which helped show a correlation between cell function and the level of expression of cell-surface immunoglobulin (J. Immunol. 119:1938, 1977). The present study has utilized the concept that tumors represent arrested stages of normal cell maturation and within this context has demonstrated that one of the earliest changes in maturing human B lymphocytes is rearrangement of the germ-line genes which code for the immunoglobulin heavy chains. These cells also express Ia cell-surface antigens. As predicted, immunoglobulin genes in T cell tumors are not rearranged and are in the form of germ line genes. This effort has been supported by FMF, which has been used with monoclonal antibodies against cell-surface differentiation antigens to type the tumors (acute lymphocytic leukemia cells) as T cells, B cells, or non-T non-B cells. All of the non-T non-B tumors turned out to have rearranged immunoglobulin genes, indicating that they are transformed B-cell precursors, committed to differentiation in the B-cell lineage. The issue has been pursued using normal human peripheral blood B lymphocytes for which the final purification step involved positive FMF selection of B cells expressing Ig lambda chains. Over 95% of the Ig kappa chain genes in these cells were not in their germ line form. These observations are consistent with the interpretation of tumor cell data that kappa gene rearrangements precede those of lambda in the course of B-cell maturation.

Glimcher, Asofsky, Sachs, Paul and colleagues. Investigation of the role of I-region associated (Ia) antigens in MHC-restricted antigen presentation has also been supported by FMF typing and sorting of cells. In this study a hybridoma cell line capable of MHC-restricted antigen presentation to T cells was treated with mutagen (ethylmethane sulfonate) and subpopulations

eliminated with monoclonal antibodies or positively selected by cell sorting on the basis of expression of certain Ia determinants. The serologic phenotype of the isolated cells was verified by FMF analysis. Analysis of the Ia-restricted antigen presenting abilities of a panel of mutant cell lines showed a correlation between loss of functional antigen presentation to antigen-specific T cells and loss of specific Ia determinants. These observations support the view that Ia antigens are the products of immune response genes.

Lunney, Ozato, Sharrow, Sachs and colleagues. FMF has supported three separate studies involving analysis of shared serologic determinants. In a study by Lunney, et al., of Ia antigens shared between species, FMF data have contributed to the view that a variety of I-region associated (Ia) determinants which are expressed on I-E molecules in the mouse, are evolutionarily retained across species barriers even though these determinants are polymorphic. Thus, FMF data have contributed to the observation that mouse monoclonal antibodies which react with polymorphic determinants on mouse I-E molecules also bind to a subset of products of the Ia region in pig cells. In a separate study Ozato et al have analyzed idiotypes shared by antibodies in alloantisera against a subgroup of mouse MHC determinants, using an anti-idiotypic probe produced against a monoclonal IgM anti H-2L^d as the basic probe. FMF data have contributed to the observation that there is a widely shared idiotypic determinant among antibodies contained in alloantisera to L^d MHC determinants. Thus, FMF was used to measure inhibition of anti H-2L^d binding in the presence of anti-idiotypic. Because shared idiotypes have not been observed among antibodies against other mouse MHC regions (K^k) for which the basic probe was an anti-idiotypic produced against IgG monoclonal antibodies, the present results have raised the issue of whether these shared idiotypes are related more to the antibody class (IgM vs IgG) or the MHC region (K vs D). In a third study Sharrow and Sachs have identified two monoclonal antibodies against different Class I MHC determinants which cross-react with a determinant controlled by genes which map to the right of H-2^d. Further analysis by dual laser FMF has shown that the cross-reacting determinant is expressed in high amounts on peripheral T cells, weakly on Ia positive cells, and on small subpopulations of thymus and marrow cells.

Epstein, Bluestone, Sachs, and colleagues. FMF data have contributed to the observations a) that mice produce long-lasting titers of anti I-E antibody activity (most of which is the IgG1 subclass) following treatment with xenogeneic antibody against idiotopes on an anti I-E monoclonal antibody and b) that the genetic control differs for anti I-E antibody produced in response to alloimmunization versus that produced following treatment with the xenogeneic anti Id. Thus, FMF was used to detect the presence of anti I-E antibody in anti Id-treated or alloimmunized mice and to determine the antibody subclass.

Significance to Biomedical Research and the Program of the Institute:

Interest in FMF for analysis of subsets of human peripheral blood mononuclear cells is clear from the extensive literature reference to OKT4⁺/OKT8⁺ (helper/suppressor-cytotoxic) cell ratios, which appear to correlate with some forms of immunosuppression and enhanced immune activity (e.g., autoimmunity). Analysis of the quantitative expression of FcR and OKT8

cell-surface determinants on human peripheral blood mononuclear cells indicates that at least 5 subsets of cells can be measured in a single analysis: 1) helper cells, 2) suppressor cells, 3) cytotoxic T lymphocytes and their precursor cells, 4) monocytes, and 5) NK-ADCC effector cells. Analysis of additional FMF parameters, which is now technically feasible, should further sharpen the identity of these functional subsets.

Analysis of cell:cell conjugates is important because this process is one of the early possible steps in a variety of biologic events, including cell differentiation, proliferation, and target cell injury. FMF is particularly well suited to measurement of cell conjugation, because of the large number of cells rapidly processed and the fact that multiple parameters can be analyzed simultaneously. Thus, cellular events which may be closely linked to conjugation (e.g., membrane depolarization, calcium flux, cell death) and characterization of the interacting cell types by cell surface phenotyping may now be studied even if the interacting cell types represent only a small proportion of the original cell population.

The use of FMF for accurately identifying cell preparations and cell lines by their serologic phenotype has again been demonstrated in the study of B cell maturation, where it has been important to exclude other classes of cells and to determine the extent of B cell maturation by the expression of cell surface differentiation antigens.

The advantages of cell purification by FMF electronic cell sorting are well-demonstrated by the isolation of mutant cells expression modified Ia determinants. A small subpopulation of mutant cells have been isolated from a parent cell line within two hours under sterile conditions in sufficient numbers to establish a permanent subline for further study. This approach toward correlating mutation-induced alterations in cell surface determinants with changes in function should have a wide range of applications to other studies of cell structure and function.

FMF has provided 4 particularly useful features in studies of cross-reacting serologic determinants and for identification of anti I-E antibodies: 1) the ability to identify binding of antibody to a small portion of cells, 2) identification of antibody binding by a technique which doesn't depend on complement fixation (e.g., cytotoxicity) 3) identification of antibody-binding distribution histograms, and 4) multiparameter analysis which permits both detection of antibody binding and identification of antibody labeled cells at the single cell level.

Proposed Course of Project: As in the past, rapid flow microfluorometry will be used for selected, appropriate projects.

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Korsmeyer, S. J., Hieter, P. A., Sharrow, S. O., Goldman, C. K., Leder, P. and Waldmann, T. A.: 1982. Normal human B cells display ordered light chain gene rearrangement and deletions. J. Exp. Med. 156: 975-

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Kung, J. T., Sharrow, S. O., Mage, M. G. and Paul, W. E.: 1982. Detection of a common antigen on murine B cells and Lyt-2⁺ T cells by a rat monoclonal antibody, 14D10. J. Immunol. 129: 81-86.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05064-07 I
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 Control of the Immune Response In Vitro		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Alfred Singer, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 0.5	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The possibility that B cell-macrophage interactions are genetically restricted was assessed in vitro for responses stimulated by TNP-Ficoll. Under conditions in which TNP-Ficoll responses did not require T cells, it was observed that B cells from F1 --> parent and fully allogeneic (A --> B) radiation bone marrow chimeras were only triggered by macrophages expressing host H-2 determinants, and were not triggered by macrophages expressing donor H-2 determinants. This genetic restriction was not overcome by the addition of T cells. Indeed, it was observed that the activation of TNP-Ficoll responsive B cells by macrophages was genetically restricted requiring B cell recognition of macrophage H-2 determinants.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

R. J. Hodes, Chief, Immunotherapy Section, Immunology Branch, NCI

Objectives: The major objective of this project is the elucidation of the signals which are involved in the cell interactions which result in activation and/or regulation of the antigen-specific immune response.

Methods Employed: Chimeric mice are created by reconstituting lethally irradiated recipients with T cell depleted bone marrow stem cells.

The in vitro assay for antibody production involves dispersing single cell suspensions of murine spleens in microculture with TNO modified protein antigens for 4 days. The cells are then harvested and assayed for anti-TNP antibody-producing cells.

Spleen cell subpopulations are prepared as follows:

- a) T cells-nylon non-adherent spleen cells
- b) (B + accessory) cells - rabbit anti-mouse brain + C' treated spleen cells
- c) Accessory cells - 2 hour glass adherent, T cell depleted, irradiated spleen cells
- d) B cells - G-10 Sephadex passed, T cell depleted spleen cells
- e) (T+B) cells - G-10 Sephadex passed spleen cells.

Major Findings: For responses to TNP-Ficoll under conditions not requiring T cells, it was observed that B cells from F₁ --> parent and A --> B radiation bone marrow chimeras were only triggered by macrophages expressing host type H2 determinants. For responses to TNP-Ficoll under conditions which do require T cells, it was observed that both T_H cells and B cells were H2 restricted in their interaction with macrophages but were not H2 restricted in their interaction with each other. The addition of soluble helper factors activated TNP-Ficoll responsive B cells directly, obviating any requirement for macrophages. Thus, these experiments indicate that in the absence of exogenously added helper factors the activation of TNP-Ficoll responsive B cells involves a genetically restricted B-macrophage interaction.

Significance to Biomedical Research and the Program of the Institute: The regulation of the immune response by manipulation of the determinants actively recognized by cells as "self" promises to have a significant impact on our ability to regulate recognition of foreign antigen for the treatment of human disease.

Proposed Course of the Project: In the immediate future, the project will be directed at determining how self recognition by T and B cells is determined.

Publications:

Hodes, R. J., Shigeta, M., Hathcock, K. S., Fathman, C. G., and Singer, A.: 1982. Role of the major histocompatibility complex in T cell activation of B cell subpopulations. Antigen-specific and H-2 restricted monoclonal T_H cells activate Lyb5⁺ B cells through an antigen-nonspecific and H-2 un-restricted effector pathway. J. Immunol. 129: 267-271.

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Project Description

Other Professional Personnel Engaged on the Project:

W. E. Biddison, Neuroimmunology Branch, NINCDs
 D. L. Nelson, Metabolism Branch, NCI
 W. Tekolf, Immunology Branch, NCI
 D. Eckels, Georgetown University
 C. J. Lucas, Central Blood Lab, Amsterdam
 D. Monos/H. Cooper, Laboratory of Pathophysiology, NCI
 J. Coligan/A. Schaverdyk, Laboratory of Immunogenetics, NIAID

Objectives: The primary objectives of this laboratory are to investigate the function of T lymphocytes, the role of HLA antigens in T cell recognition, and the mechanism by which these HLA genes control immune responses. The hypotheses generated draw heavily from the precedents in animal models, particularly those in the mouse, but the experimental work is restricted to human studies. Because of the ethical and logistical considerations which limit in vivo studies in humans, it has been (and is) crucial to develop good in vitro models of human immune responses. The model systems used most extensively this year are cytotoxic and proliferative T cell responses to: 1) influenza and measles virus-infected cells and 2) the HLA-SB antigens on allogeneic cells.

Methods Employed: Human PBL are obtained from donors by phlebotomy or batch leukapheresis; mononuclear cells are prepared by density separation, and cryopreserved. For virus-specific responses, responder cells are exposed to infectious virus, incubated for 7 days and assayed for cytotoxicity in a short term ⁵¹Cr release assays using as targets T lymphoblasts or lymphoblastoid B cell lines which have been incubated with infectious virus. Responses to the SB antigens have been analyzed using T cells primed against those differences in combinations of selectively matched allogeneic donors. For higher resolution analysis of the specificity and function of individual T cells in some of the allogeneic studies, single cell cloning has been performed by limiting dilution and subsequent expansion in the presence of specific antigen and TCGF. Analysis of the HLA markers on the donors cells is performed by microcytotoxicity testing under contract NO1-CB04337.

Major Findings: We have extensive experience with T cell responses to the HLA-SB antigens, first described in our laboratory. This has proved to be a useful experimental system in which to dissect out functional components of the cellular interaction which occurs between antigen-specific T cells and antigen-positive cells with which they interact. It is plausible that many of the T cell differentiation antigens now being defined on T cells by monoclonal antibodies are functionally involved in T cell recognition of antigen. In collaboration with Dr. W. E. Biddison, we have demonstrated that the OKT4 molecule is present not only as a marker on the subset of T cells which interact with the SB molecules, but that the OKT4 molecule may be functionally involved in the T cell recognition of the SB molecule. Monoclonal antibodies directed against the OKT4 molecule (as well as antibodies directed against OKT3 and OKT11) inhibit the capacity of T cells to mediate SB-specific proliferative and cytotoxic responses. The inhibition was consistent, but never complete when analyzed on polyclonal populations of

T cells sensitized against alleles SB1, 2, 3, 4, and 5. It was postulated that interaction between the OKT4 molecule on the responding T cell and the HLA class II molecule on the stimulating cell might be necessary for the activation of some but not all clones. Progress has been made in cloning these SB-specific T cells by limiting dilution in the presence of allogeneic stimulator cells and TCGF. Although all the clones proliferate in response to antigen, heterogeneity exists among the clones with respect to their ability to mediate antigen-specific CML. All clones which mediate CML are also able to mediate NK-like killing on K562 targets. These two modes of killing appear to be activated in part by different recognition events since only CML not NK activity is inhibited by anti-OKT4 antibodies and anti-SB antibodies.

Previous studies under this topic have indicated functional heterogeneity between HLA molecules which are serologically indistinguishable. Structural comparisons between such molecules, inferred to have limited structural differences, promise to allow strong structure-function correlations. Collaborative projects are underway with the Drs. H. Cooper/ D. Monos (who are analyzing these molecules by 2-D gel) and Drs. T. Coligan/A. Schaverdyk (who are analyzing differences in primary amino acid sequence) to elucidate structural differences which can account for the observed functional differences between these molecules.

Collaborative studies were continued with Dr. D. Eckels at Georgetown University regarding the recognition of the SB antigens by viral antigen-specific T cells confirm the preliminary data in last year's report. Clones have been identified which recognize influenza virus antigens in conjunction with SB2 and herpes virus antigens in conjunction with SB4. These findings establish that SB functions in vitro as a restriction antigen.

Collaborative studies with Dr. C. J. Lucas on human measles-virus specific cell-mediated cytotoxicity expanded the information obtained in previous studies of influenza virus specific responses. Only 12% of donors produced significant responses measles virus-specific responses; the responses were mediated by OKT3⁺, 4⁻, 8⁺ T cells. Those responses were measles virus-specific and generally HLA-restricted, although some unexpected crossreactions were seen which may indicate the existence of additional undefined HLA determinants which are functionally important in the recognition of measles virus antigens.

Collaborative studies of patients with Ataxia-Telangiectasia were undertaken with Dr. D. L. Nelson in order to determine if this syndrome might include defects in virus-specific T cell responses. Peripheral blood cells from 11/12 of such patients failed to mount significant proliferative or cytotoxic responses to influenza virus-infected cells. Experiments to distinguish the site of the defect suggested that it reflected a defect in responding T cells.

Significance to Biomedical Research and the Program of the Institute: The problems addressed in this project are central to the understanding of how the immune system surveys the body to detect foreign antigen and to eliminate cells which express those foreign antigens. This surveillance system is thought to be important not only in dealing with microbial pathogens but also

in detection and destruction of neoplastic changes. Furthermore, these studies promise to clarify the relevance of genetic differences between individuals in their susceptibility to infectious, neoplastic and autoimmune diseases.

Proposed Course of the Project: Several different approaches will be pursued in the upcoming year. First, studies will be continued regarding the recognition events involved in activation of SB-specific proliferating and cytotoxic clones. We will continue to explore the ability of monoclonal antibodies to inhibit antigen recognition and the heterogeneity of distinct T cell clones with similar apparent antigen-specificity. In addition we will explore the possible role of carbohydrate determinants in this recognition by removal of carbohydrates from the target cell, introduction of potentially competitive levels of exogenous carbohydrate, or addition of anti-carbohydrate antibodies. Second, we will begin biochemical studies on the structures involved in this interaction. In particular, we will begin to purify cell surface molecules which are able to compete with the recognition event. Finally, it is apparent that even short term cellular assays like cell-mediated cytotoxicity are cumbersome tools for analyzing interactions. Therefore, we will try to develop binding assays which parallel this recognition and will allow more molecular analysis of interactions.

Publications:

Biddison, W. E., Rao, P. E., Talle, M. A., Goldstein, G., and Shaw, S.: 1982. Possible involvement of the OKT4 molecule in T cell recognition of class II HLA antigens: Evidence from studies of cytotoxic lymphocytes specific for SB antigens. J. Exp. Med. 156: 1065-1076.

Lucas, C. J., Biddison, W. E., Nelson, D. L., and Shaw, S.: 1982. Killing of measles virus-infected cells by human cytotoxic T cells. Infection and Immunity. 38: 226-232.

Eckels, D. D., Lake, P., Lamb, J. R., Johnson, A. H., Shaw, S., Woody, J. N. and Hartzman, R. J.: 1983. SB-restructured presentation of influenza and herpes virus antigens to human T-lymphocyte clones. Nature (Lond.). 301: 716-718.

Nelson, D. L., Biddison, W. E., and Shaw, S.: Defective in vitro production of influenza virus-specific cytotoxic T-lymphocytes in Ataxia-Telangiectasia. J. Immunol. In press, 1983.

Biddison, W. E., Rao, P. E., Talle, M. A., Goldstein, G., and Shaw, S.: Possible involvement of the T4 molecule in T cell recognition of class II HLA antigens: Evidence from studies of proliferative responses to SB antigens. J. Immunol. Submitted for publication, 1983.

Biddison, W. E., and Shaw, S.: Possible involvement of the T4 molecule in T cell recognition of class II HLA antigens. J. Diagnostic Immunol. In press, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05069-07 I
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 Ia Antigens on Functional Cell Subpopulations		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Richard J. Hodes Chief, Immunotherapy Section, Immunology Branch, NCI		
COOPERATING UNITS (if any) Centre d'immunologie, INSERM-CNRS de Marseille - Liminy Marseille, France		
LAB/BRANCH Immunology Branch		
SECTION Immunotherapy Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>It has been demonstrated that the T cell proliferative response to Con A and the T cell dependent antibody responses to the soluble antigens TNP-KLH, TNP-T,G-(A-L), and TNP-Nuclease require the participation of accessory cells. This subpopulation of spleen cells has been shown to be glass adherent, radioresistant, non-T, non-B and and to express Ia (I region associated) determinants encoded in I-A and I-E/C. In addition, I-A and I-E positive cells within the splenic adherent cell population are the predominant stimulators of the one way murine mixed lymphocyte response when responder and stimulator cells differ either at H-2 or the Mls locus. Studies designed to analyze the functional importance of specific determinants on Ia molecules have been initiated employing a battery of monoclonal anti-I-E reagents specific for different epitopes on the same I-E product molecule. Inhibition studies suggest that different domains on Ia molecules have differential functional importance.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

Barbara Needleman, Investigator, Immunotherapy Section, Immunology Branch, NCI

Patrick Dwyer, Investigator, Immunology Branch, NCI

David H. Sachs, Chief, Transplantation Biology Section, Immunology Branch, NCI

Suzanne Epstein, Investigator, Transplantation Biology Section, Immunology Branch, NCI

Objectives: Ia antigens are serologically demonstrable cell surface determinants which appear to play important roles in cell-cell interactions. The objective of this study is to investigate the role these Ia determinants play in cellular interactions involved in T cell-mediated or T cell-dependent responses. The T cell proliferative response to Con A and to soluble antigens, and T cell-dependent in vitro antibody responses are being studied. Studies employing monoclonal antibodies have been directed at defining the functional importance of different determinants on the same Ia molecules.

Methods Employed: Cell separation and reconstitution techniques are being employed and have established that accessory cells are required for Con A proliferative response, primary and secondary antibody responses and secondary T cell proliferative responses to soluble antigens. Cloned T cell populations have been generated by in vivo priming with soluble antigens, in vitro re-stimulation, and limiting dilution. These clones include I-A or I-E restricted T cells specific for antigens including KLH, GLPhe, and GLleu. Monoclonal anti-Ia antibodies have been employed including a panel of 15 anti-I-E^k antibodies specific for different domains on the I-E^k molecule as defined by competitive binding. Antigen-presenting cells have included both heterogeneous spleen cell populations and cloned B cell lines.

Major Findings: Secondary in vitro T cell proliferative responses to the synthetic polymer GLPhe were generated and were shown to be both antigen-specific and under the control of complementing immune response genes in the I-A and I-E subregions. The proliferative responses of B10.A(5R) T cells to GLPhe were selectively inhibited by anti-I-E monoclonal antibodies. More specifically, it was shown that antibodies specific for one epitope on the I-E molecule were uniformly more efficient inhibitors than were antibodies specific for a second and distinct epitope. These two groups of antibodies appeared otherwise comparable in terms of parameters such as Ig class and subclass and binding affinity.

Significance to Biomedical Research and the Program of the Institute: I region gene products play a significant role in regulation of immune responses. A basic understanding of the expression, and perhaps the functional role of one class of I region gene products should provide insight into the mechanisms of antigen presentation and T cell recognition in immune responses. The findings to date suggest that discrete sites on Ia molecules are differentially important in antigen presentation and/or T cell recognition. This may reflect antigen-specific association sites on Ia molecules, or sites involved in the overall integrity of Ia molecules for presentation of any antigen.

Proposed Course of Project: I-E controlled secondary in vitro T cell proliferative responses to a series of antigens are being established. In addition, both I-A and I-E restricted T cell clones have been generated to several antigens. The pattern of inhibition of T cell recognition by epitope-specific monoclonal anti I-E antibodies will be examined at the clonal level in order to determine whether antigen-specific and/or clone-specific differences exist in functional response inhibition by these antibodies. In addition, mutagenized Ia positive antigen-presenting cell lines will be derived and studied to further analyze the functional role of distinct determinants on Ia molecules.

Project Description

Other Professional Personnel Engaged on the Project:

D. H. Sachs, Chief, Transplantation Biology Section, Immunology Branch, NCI
S. Rudikoff, Senior Investigator, LCB, NCI
M. S. Satz, Visiting Fellow, Immunology Branch, NCI
R. Lifshitz, Visiting Fellow, Immunology Branch, NCI
L. Wang, Visiting Fellow, Immunology Branch, NCI
L. Abelson, Biologist, Immunology Branch, NCI

Objectives: This laboratory has undertaken to study the molecular biology of the major histocompatibility locus in the mouse and the miniature swine. In both species, it is known that the structural products regulated by this region of the genome are highly polymorphic and responsible for both transplantation rejection and regulation of immune responses. Biochemical studies have demonstrated that the MHC antigens of mouse and miniature swine are structurally homologous.

Genetic studies have shown that the genes involved in regulating the expression of the transplantation antigens, as well as those involved in regulating the immune response are all linked on a single chromosome. However, the organization of these genes varies between the two species. Therefore, this region is of considerable interest in studying the regulation of a coordinated set of functions. The objects of the studies are to characterize the genome organizations and regulation of these families of genes in each of the two species and to analyze the evolutionary relationship between them.

Methods Employed: The approach to studying the genomic organization of the MHC locus involves the direct isolation of the individual genes encoding the various structural products. Total DNA, isolated from the livers of inbred strains of mice and miniature swine, is enzymatically fragmented, and each fragment is inserted into a viral vector. These recombinant DNA libraries are then screened using a heterologous human MHC cDNA probe. Isolated genomic clones are initially characterized either by direct DNA sequence analysis or by the ability of these genes to direct the synthesis of MHC products. Once genomic clones are characterized as containing MHC genes, a detailed analysis of the DNA sequence organization of the clone is conducted. Segments of the isolated DNA are subcloned into plasmid vectors. Analysis of the genomic DNA and RNA for the presence of the subcloned sequences is conducted by established techniques of molecular biology. The nuclear organization of these DNA sequences is also examined by isolation of chromatin and characterization of its structure with respect to the sequences of interest. The isolation of such genomic MHC genes allows an analysis of the organization of a single constitutively expressed gene, as well as its relationship to other members of a multigene family.

Major Findings: It has been demonstrated that the swine genome contains 10-15 genes homologous to MHC DNA sequences. These can be divided into two subgroups, one containing 3-5 closely related sequences and one containing 5-10 more distantly related sequences. Porcine genomic clones containing MHC genes have been isolated by direct screening of a swine genomic library. Mouse L cells transformed with one of these clones stably and uniformly express swine MHC antigen as assessed by direct immunoprecipitation,

complement-mediated cytotoxicity and FACS analysis. These studies demonstrate that this clone contains a functional MHC gene. Studies on the chromatin organization of the swine DNA sequences in L cells suggest that they are actively regulated: the expressed SLA gene is DNase I sensitive, whereas flanking non-expressed sequences are relatively DNase I resistant. This notion is supported by the finding that interferon stimulates expression of SLA in transformed mouse cells but does not induce the transcription of other pig DNA sequences.

Another independent genomic MHC clone does not express SLA antigens when introduced into L cells. However, studies on MHC gene expression in pig tissue, using as assays DNase I sensitivity and methylation state, suggest that this gene is active in pig thymus. In contrast, the MHC clone which is expressed in L cells does not appear to be expressed in thymus. These data suggest that MHC genes are differentially expressed.

DNA sequence analysis of a swine MHC gene has revealed a structure similar to that observed in both mouse and man, namely separate exons encoding extracytoplasmic protein domains and transmembrane region and three exons encoding the intracytoplasmic regions. There appear to be alternative 3' splice sites for the final exon.

Further characterization of MHC-linked but non-coding sequences has revealed the non-random association of a series of moderate repetition sequence elements with MHC genes. The nature of these DNA sequences and their role in the evolution and regulation of MHC gene expression is under investigation.

Significance to Biomedical Research and the Program of the Institute: Genetic studies in a number of mammals, including man, mouse and guinea pig, have demonstrated the existence of immune response genes which control cellular interactions leading to both humoral and cellular immunity. The inability of an animal to respond to a given antigen or to reject foreign tissue probably represents a genetic defect. Despite the clear importance of the major histocompatibility locus in the immune response, nothing is known at the molecular level about the content, genetic organization or regulation of expression of this multigene family. An understanding of the molecular basis of the MHC may afford the possibility of treating various immunodeficiency diseases by appropriate genetic manipulations.

Proposed Course of Project: Characterization of the swine MHC will proceed along the following lines:

- (1) Construction of a cosmid library is in progress. Isolation and mapping of overlapping cosmid clones containing class I and class II MHC genes will reveal the overall organization of the swine MHC.
- (2) Clones will be analyzed to determine which MHC products they encode.
- (3) The nature of MHC linked sequences will be determined by direct DNA sequence analysis, and with respect to their sequence families and states of expression. The possible association of these elements exclusively with the MHC will be determined.

(4) The relationship between the organization of MHC sequences into chromatin and their expression will be further studied.

(5) The role of the flanking sequences in regulating gene expression will be studied.

Once these lines of research have been completed, it will be possible to determine the evolutionary relationships among the MHC genes. It should also be possible to begin to examine the regulation of expression of these genes.

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

PROJECT NUMBER
Z01CB05085-05 I

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 Syngeneic Tumor Immunity

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

(Name, title, laboratory, and institute affiliation)

Gene M. Shearer, Senior Investigator, Immunology Branch, NCI

COOPERATING UNITS (if any)

Department of Biology, Hebrew University, Jerusalem, Israel

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.3

PROFESSIONAL:

0.3

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

Mice of the BALB/c strain injected with a line of the syngeneic T cell lymphoma S-49 which grows in suspension accept the tumor and die within two weeks. BALB/c mice injected with a plastic adherent (7.3) line of the same tumor are not killed. Furthermore, mice injected first with the 7.3 line and subsequently challenged with TAS are protected from the syngeneic tumor.

Project Description

Other Professional Personnel Engaged on the Project:

J. Hochman, Department of Biology, Hebrew University, Jerusalem, Israel
L. Joseph, Medical Staff Fellow, Immunology Branch, NCI

Objectives: The aim of this project is to study the mechanistic aspects of acquired immunity of mice to syngeneic T cell tumors. The model chosen for this study is that of the S-49 T cell Lymphoma of BALB/c origin, for which tumorigenic (TAS) and immunogenic (7.3) lines have been developed. Since injection of BALB/c mice with 7.3 protects against development of the tumor when challenged with TAS, analysis of the mechanism(s) of protection is planned. A long-term objective of the project is to determine if immunogenic and tumorigenic lines of other murine T cell tumors can be developed.

Methods Employed: BALB/c mice were injected ip with $10\text{-}30 \times 10^6$ TAS or 7.3 tumor cells. Mice protected by injection with 7.3 were challenged with $10\text{-}30 \times 10^6$ TAS cells ip. Sera and splenic lymphocytes from the injected mice were collected and tested for antibody activity and cell mediated cytotoxicity, respectively, using 7.3 and TAS as target cells.

Major Findings: In confirmation of Hochman's original observations, we found that: (a) tumor growth followed by death of the animals was observed in BALB/c mice injected with TAS but not in the mice injected with 7.3; and (b) injection of mice with 7.3 followed 1 to 9 months later with TAS did not result in tumor growth nor in death of the mice.

Antibody activity was detected in the sera of mice injected either with TAS or 7.3. No distinct quantitative or qualitative differences in the antibody responses of 7.3-injected or TAS-injected mice have yet been identified.

No cell-mediated immune activity was detected in the splenic lymphocytes of mice injected with 7.3, TAS, or 7.3 followed by TAS, when potential effectors (either taken directly from the mice or "restimulated" in vitro with 7.3 or TAS) were assayed on ^{51}Cr -labelled 7.3 and TAS target cells.

Significance to Biomedical Research and the Program of the Institute: The model outlined above raises the possibility of an approach for developing immunity against autologous T cell lymphomas - possibly by their in vitro growth properties (suspension-growing TAS which results in tumor growth in vivo vs plastic-adherent 7.3 which protects against in vivo tumor growth).

Proposed Course of Project: More detailed studies of the antibodies produced as a result of injection of these cells will be performed to determine whether any differences can be detected in the antibodies resulting from TAS vs 7.3 injection. For example, these antibodies could be of identical or somewhat different specificities and/or affinities. If the antibodies are identical it may be that both cell lines are equally immunogenic, but that only TAS is metastatic in vivo.

Adoptive transfers of spleen cells and separated T and B lymphocytes from 7.3-injected, protected mice will be made into untreated BALB/c mice. These mice will then be challenged with TAS to determine if adoptive transfer of the cells confer protection against the tumorigenic line. If so, then the cell type (T or B) responsible for protection can be identified. At this point attempts will be made to clone the "protective" cells.

The demonstration of the above-outlined protective effect is of limited potential value if the phenomenon can be demonstrated for only one tumor. Therefore, one long-term goal of this project is to attempt to develop this system using other lymphoid tumors. Some 40 radiation-induced T cell lymphomas are available to this laboratory for such studies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05086-05 I
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.) Immune Response Gene Regulation of the Immune Response In Vitro		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and Institute affiliation) Richard J. Hodes Chief, Immunotherapy Section, Immunology Branch, NCI		
COOPERATING UNITS (If any)		
LAB/BRANCH Immunology Branch		
SECTION Immunotherapy Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.4	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (x) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The cellular expression of immune response (Ir) gene function was studied in both primary and secondary in vitro antibody responses to the TNP conjugates of (T,G)-A--L and (H,G)-A--L. It was demonstrated that the function of accessory cells in responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L is under the control of genes which also map to K or I-A. In contrast, the expression of Ir gene function by B cells is related to the B cell activation pathway; Ir gene function is expressed by Lyb5- B cells activated under appropriate conditions, while Ir genes do not influence Lyb5+ B cell function under different conditions. In vitro augmented primary and secondary responses to TNP-nuclease (TNP-NASE) have also been established and documented to be under the control of H-2 linked Ir gene(s) mapping to the I-B subregion. For these responses, accessory cell function was shown to be under Ir gene control. Recent data employing monoclonal anti-Ia reagents have suggested that genes in the I-A subregion may also be involved in regulating responses to TNP-NASE.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

Alfred Singer, Senior Investigator, Immunology Branch, NCI
 David H. Sachs, Chief, Transplantation Biology Section, Immunology Branch, NCI
 Yoshihiro Asano, Visiting Fellow, Immunology Branch, NCI
 Alison Finnegan, Investigator, Immunology Branch, NCI

Objectives: The major objective of this project is to investigate the mechanism of genetic regulation of antibody responses. Initial studies identified the cellular level of Ir gene expression for the in vitro responses to TNP-(T,G)-A--L, TNP-(H,G)-A--L and TNP-NASE. Current studies are assessing the Ir gene regulation of activation of defined B cell subpopulations.

Methods Employed: The methods employed have been described in detail. See project No. Z01 CB 05064-04 I.

Major Findings: The in vitro primary and secondary antibody responses to TNP-(T,G)-A--L and TNP-(H,G)-A--L are under strict H-2 linked gene control by genes mapping to the K or I-A subregions. The cellular level of Ir gene expression was examined under conditions which employ different pathways of B cell activation. Under all conditions, when Lyb5⁻ B cells are activated by a pathway requiring H-2 restricted T-B interaction, B cell function is found to be under Ir gene control. In contrast, in responses requiring Lyb5⁺ B cells, no requirement for restricted T-B interaction was observed, and B cell function was not under Ir gene control.

An in vitro system was established in which TNP-specific responses to TNP-conjugated Staphylococcal nuclease (TNP-NASE) were generated by spleen cells from NASE-primed mice. These responses were T-cell and accessory cell-dependent, and under H-2-linked Ir gene control, with strains of the H-2^a haplotype being responders and H-2^b strains nonresponders. Ir gene control mapped to I-B and was not explained by complementing genes in I-A and I-E/C. Cell fractionation experiments have shown that accessory cell function is under Ir gene control for the response to TNP-NASE. Experiments carried out with a hybridoma anti I-A^k reagent have demonstrated that this reagent is capable of inhibiting the response of (H-2^a x H-2^b)F₁ spleen cells to TNP-NASE. These findings suggest that a gene (or genes) in I-A, as well as genes in I-B may regulate the response to TNP-NASE.

Monoclonal T_H cell populations specific for NASE have been generated in BALB/c (H-2^d) and (B10x B10.A)F₁ (H-2^bxH-2^a) genotypes and will be examined for both subregion MHC restriction and antigen fine specificity (employing NASE fragments).

Significance to Biomedical Research and the Program of the Institute:

Genetic control of immune responses has been demonstrated in widely studied systems, including those responses to biologically "natural" antigens including allergens, viral determinants, and tumor antigens.

In order to understand the mechanism of differentially reactivity and susceptibility to these natural stimuli, the mechanism of Ir gene regulation of responses to defined stimuli may provide informative insights.

Proposed Course of Project: Conventional and monoclonal anti-Ia antibodies will be used to probe for the I region products which function in Ir gene expression. Cloned T cell populations will be analyzed for MHC restriction and antigen fine specificity.

Publications:

Singer, A., Asano, Y., Shigeta, M., Hathcock, K. S., Ahmed, A., Fathman, C. G. and Hodes, R. J.: 1982. Distinct B cell subpopulations differ in their genetic requirements for activation by T helper cells. Immunol. Rev. 64: 137-160.

Asano, Y., Singer, A. and Hodes, R. J.: 1983. Role of the major histocompatibility complex in T cell activation of B cell subpopulations. Ir gene regulation of the T cell dependent activation of distinct B cell subpopulations. J. Immunol. 130: 67-71.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CB05088-05 I
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 Graft Vs. Host Reactions on Cell-Mediated Immunity		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.)		
(Name, title, laboratory, and institute affiliation)		
Gene M. Shearer, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH		
Immunology Branch		
SECTION		
INSTITUTE AND LOCATION		
NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.5	2.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>The intravenous injection of F₁ hybrid mice with parental spleen cells resulted in a loss in the ability of the F₁ mice to generate T-cell mediated cytotoxic responses in vitro to TNP-self and alloantigens. The loss of response potential depended on the H-2 type of the parental cells, since H-2^K,^a spleen cells induced unresponsiveness, whereas H-2^b spleen cells did not. The phenomenon is dependent on recognition of F₁ I-A alloantigens by grafted parental cells (GVH), since loss of immune activity was associated with enlarged F₁ host spleens. Suppressor cells were found to be responsible for loss of immune potential. The failure of lymphocytes from parental strains was shown to be due to F₁ resistance to parental T cells, which mapped to H-2D^b. Protection against GVH-associated suppression could be achieved using anti-H-2 sera directed against specificities of donor or host. This antisera activated a counter-suppressor T cell which could be demonstrated in either host or donor spleen cell populations. Allogeneic combinations of donor and recipient strains also resulted in suppression. Suppression was observed in combinations of donors and hosts in which host-vs-graft reactions occurred. Chimerism was observed in some but not all parent-into-F₁ combinations.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

U. Hurtenbach, Visiting Associate, Immunology Branch, NCI
T. Tsuchida, Visiting Fellow, Immunology Branch, NCI
D. H. Sachs, Chief, Transplantation Biology Section, Immunology Branch, NCI
P. Lang, Visiting Fellow, Immunology Branch, NCI
R. Levy, Senior Staff Fellow, Immunology Branch, NCI
S. Sharrow, Technician, Immunology Branch, NCI

Objectives: The purpose of this project is to investigate the phenomenon of immunosuppression induced by or associated with a graft vs. host reaction, the immunogenetics associated with the resistance in some strain combinations of the graft vs. host associated suppression, and to attempt to establish whether this immunosuppression is correlated with autoimmune states and/or the development of acquired immune deficiency syndrome (AIDS) following a graft vs. host and/or host vs. graft reactions.

Methods Employed: F_1 hybrid mice of various strains were injected intravenously with from 1 to 40×10^6 F_1 , parental, or allogeneic spleen cells. Inbred mouse strains were also injected with allogeneic cells. At various times after injection, the spleens of the injected F_1 mice were sensitized in vitro against: (a) parental or F_1 syngeneic cells modified with TNBS; or (b) allogeneic spleen cells. The effector cell actively generated 5 days later was tested on the appropriate ^{51}Cr -labelled target cells. Mapping studies were performed using inbred and recombinant mice on the C57BL/10 genetic background, as well as strains of other genetic backgrounds. Mice were injected with anti-sera or monoclonal reagents specific for H-2 region and subregion gene products.

Major Findings: F_1 hybrid mice on the C57BL/10 genetic background injected intravenously with viable parental spleen cells lost their ability to respond by in vitro generated cytotoxic reactions to TNP-self and allo-antigens. The loss of cytotoxic potential was detected as early as four days after injection and persisted for at least 30 days after injection. Recovery from immunosuppression was detected 40-45 days after injection of parental cells. Immune reactivity against H-2 alloantigens consistently recovered before reactivity against modified self. The abolition of cytotoxic potential: (a) depended on a graft vs. host reaction by parental lymphocytes against host alloantigens; and (b) was dependent on the H-2 haplotype of the parental cells used, since the injection of B10.A or B10.BR but not C57BL/10 (B10) parental cells resulted in loss of immune reactivity. The abolition of cytotoxic potential by the GVH reaction is the result of an active suppressive mechanism, since the addition of spleen cells from parental-injected F_1 mice to normal F_1 spleen cells led to the inactivation of the cytotoxic potential of the normal cells. Protection against suppression was observed in F_1 mice injected with anti-H-2 antibodies specific for K, I, or D region gene products expressed either by the F_1 host or parental donor. Such protection was observed: (a) by using either anti-H-2 sera or monoclonal reagents, and (b) by injecting either F_1 host or parental donors. This protection was shown to be due to a counter-suppressor T cell, which could be activated either in the donor or recipient. It was also found that the

induction of suppression requires recognition of I region determinants expressed by the F_1 by the parental spleen cells, and that I-A is all that is required for this suppression to be activated. GVH-associated suppression was not limited to parent-into- F_1 combinations, since it was observed that some, but not all H-2 allogeneic strain combinations resulted in suppression. For example, C57BL/10 into B10.BR resulted in suppression. In contrast, B10.BR into C57BL/10 did not result in suppression. Furthermore, suppression could be induced in strain combinations in which host-vs-graft but not graft-vs-host reactions were possible.

Parental spleen cells injected into adult, intact F_1 mice resulted in chimerism as early as 30 days after injection in mice on the B10 genetic background. Parental chimerism could be detected for at least one year after inoculation. Chimerism was not detected in F_1 mice that expressed the C3H genetic background, e.g., B6C3F₁.

Some nine parallels have been established between AIDS in homosexual men and GVH-associated immunosuppression in our mouse model, including our recent findings that GVH mice exhibit levels of serum interferon and thymosin α 1 similar to that found in AIDS patients.

Significance to Biomedical Research and the Program of the Institute: The graft vs. host (GVH) reaction and possibly Hh-type reactions are important complicating factors which affect the success of hemopoietic transplantation. Furthermore, persisting GVH reactions may be associated with autoimmune disease and the development of tumors. The observations: (a) that GVH reactions can be elicited with low numbers of lymphocytes in immunocompetent adult mice (previous reports have been limited to the demonstration of GVH in neonates or immunosuppressed animals); (b) that these GVH reactions lead to severely impaired T-cells immune functions; and (c) that such GVH reactions can be overcome by host resistance mechanisms are potentially of fundamental relevance in: (1) understanding the possible complications resulting from hemopoietic grafting; (2) investigating the significance of a GVH-induced suppressed immune system in the development of autoimmune and neoplastic disease; and (3) understanding natural resistance systems as they may be relevant in surveillance against disease and neoplasms. It should also be noted that a certain proportion of children with severe combined immunodeficiency disease (SCID) are partial chimeras and carry circulating maternal T lymphocytes. It is possible that our murine model has genetic and mechanistic relevance for these SCID's. It is also possible that GVH-associated immunosuppression may be a cofactor in the development of AIDS among homosexual men. Etiologic considerations of this type are consistent with the Program of the Institute.

Proposed Course of Project: We shall continue to investigate all aspects of the phenomenon including: (a) the genetics of the F_1 and parental cells involved; (b) the determinants recognized on the F_1 cells; (c) the mechanistic aspects of both the GVH and the suspected Hh component involved; (d) other immune functions which may be impaired including antibody production, delayed hypersensitivity, skin graft rejection, T-cell proliferative responses, and natural killer cell activity; (e) the long-term effects of the GVH including survival and the development of autoimmune disease and tumors; (f) which

combinations of allogeneic cells and hosts can lead to GVH-associated immunosuppression; (g) analysis of different populations of cells involved in the induction of and protection by antibody against GVH-associated immunosuppression; and (h) whether GVH-associated suppression and/or HVG-associated suppression is a model for AIDS.

Publications:

Hurtenbach, U. and Shearer, G. M.: 1983. Analysis of murine T lymphocyte markers during the early phase of GVH-associated suppression of cytotoxic T lymphocyte responses. J. Immunol. 130: 1561-1566.

Shearer, G. M. and Levy, R. B.: 1983. Graft-vs.-host-associated immune suppression is activated by recognition of allogeneic murine I-A antigens. J. Exptl Med. 157: 937-946.

Shearer, G. M.: 1983. Natural resistance to parental T lymphocyte-induced immunosuppression in F₁ hybrid mice: Implications for acquired immune deficiency syndrome (AIDS). Immunol. Revs. 73: in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05090-05 I
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 Accessory Cells in B Cell Activation		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Shiro Ono, Visiting Fellow, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) It was demonstrated previously that macrophages specifically interact with a distinct B cell subpopulation which is characterized as Lyb5+. Current experiments have demonstrated that Lyb5- B cells can be stimulated by the mitogen LPS. To gain further insights into the activation requirement of B cells which comprise the Lyb5- B cell subpopulation, the ability of lipoprotein free (phenol extracted) and lipoprotein rich (butanol extracted) LPS to stimulate Lyb5- B cells was examined. It was shown that butanol extracted LPS stimulates all Lyb5- B cells. In contrast, phenol extracted LPS stimulates only a specific Lyb5- B cell subset which is present in normal mice, but which is absent in xid mice. Thus, these results define a new B cell subpopulation which is Lyb5-, appears early in ontogeny, and is absent in mutant xid mice.		

Project Description

Other Professional Personnel Engaged on the Project:

A. Singer, Senior Investigator, Immunology Branch, NCI

H. Golding, Visiting Fellow, Immunology Branch, NCI

Objectives: The major objective of this project is to use xid B cell mutant mice and thymic independent antigens as a probe for understanding the mechanism of B cell activation.

Methods Employed: Spleen cells from normal or immune defective xid mice were cultured with TNP-conjugated antigens (phenol extracted LPS, butanol extracted LPS, Ficoll) for 4 days. Cultured cells were then assayed for IgM anti-TNP antibody forming cells. T cell depletion was accomplished with cytotoxic T cell reagents. Macrophages were specifically removed by G-10 Sephadex passage. Lyb5+ cells were cytotoxically eliminated by treatment with anti-Lyb5 reagents + Complement.

Major Findings: B cells from normal mice responded to both TNP-LPS preparations, whereas xid mice responded only to TNP-LPS (butanol). The responses stimulated by both TNP-LPS preparations were independent of T cells and were relatively independent of macrophages. The failure of xid mice to respond to TNP-LPS (phenol) was shown neither to be due to the presence of suppressor cells nor to a shift in kinetics of their response. Rather, the defect of xid mice to respond to TNP-LPS(phenol) resided in their B cell population. The failure of xid B cells to respond to TNP-LPS(phenol) was not merely due to the absence of the Lyb5+ subpopulation in these mice because it was shown that TNP-LPS(phenol) stimulates Lyb5- B cells. Thus, B cells responsive to TNP-LPS(phenol) appear in normal neonates prior to the appearance of Lyb5+ B cells, and depletion of Lyb5+ B cells from normal adult mice does not affect their responses to TNP-LPS(phenol). Taken together, these results demonstrate that TNP-LPS (phenol) stimulates an early appearing Lyb5- B cell subpopulation which is present in normal mice but which is absent or markedly diminished in xid mice. Consequently, the Lyb5- B cells which are present in normal mice are not identical to the Lyb5- B cells present in xid mice, as previously thought.

Significance to Biomedical Research and the Program of the Institute:

Understanding B cell responses to bacterial LPS should provide information relevant to the treatment of septicemia in man. In addition, it should also provide insights into the mechanisms of B cell activation which can be utilized for manipulation of humoral immune responses in human disease.

Proposed Course of the Project: Future work will be directed toward delineating the different activation mechanisms utilized by distinct B cell subpopulations.

Publications:

Ono, S., Yayye, L. J., Ryan, J. L., and Singer, A.: 1983. Functional heterogeneity of the Lyb5⁻ B cell subpopulation. Mutant xid B cells and normal Lyb5⁻ B cells differ in their responsiveness to phenol extracted lipopoly-saccharide. J. Immunol. 130: 2014-2021.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05093-04 I
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.) Environmental Influences on Self-Tolerance		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Philip J. Morrissey, Staff Fellow, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NIC, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The induction of immunological tolerance in T cells can possibly occur prior to their entry into the thymus, during thymic differentiation or after the cells have emigrated from the thymus. Experimental systems have been constructed to investigate the susceptibility of T cells or their precursors to tolerance induction during the various phases of their differentiation. The basic model consists of murine thymus engrafted radiation bone marrow chimeras in which cell surface alloantigens can, in theory, be specifically localized in the extra-thymic or intra-thymic differentiation environments. Thus far, the induction of tolerance to two distinct cell surface alloantigen systems has been studied; they are those encoded by the MHC and the Mls loci. The results demonstrate that tolerance to MHC encoded alloantigens can occur both pre-thymically and intra-thymically and that tolerance to Mls encoded determinants can occur intra-thymically and post-thymically, but not pre-thymically. These results demonstrate that tolerance to various cell surface alloantigens may occur at different stages of T cell differentiation.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

A. Singer, Senior Investigator, Immunology Branch, NCI

Objective: These experiments will study the induction of tolerance in T cells at various stages of their development.

Methods Employed: Young adult mice were grafted subcutaneously with neonatal thymii. Three to five days later, they were lethally irradiated and reconstituted with bone marrow cells of the same genetic type as the thymus. In some cases, the host mouse was thymectomized prior to thymus grafting and irradiation. After allowing four weeks for the repopulation of the thymii with bone marrow derived cells, these mice were studied individually for tolerance to determinants unique to the host. Tolerance of thymocytes and spleen cells was assessed by in vitro proliferative assays (for the measurement of anti-I and anti-Mls reactivity) and by in vitro generation of cytotoxic T lymphocytes (for the measurement of anti-K/D reactivity). Additionally, immunofluorescence and flow microfluorometry was used to precisely identify the genetic origin of the thymocytes and to assess the possibility that host determinants may have infiltrated the thymus grafts.

Major Findings: Strain A thymocytes, which were maturing in a strain A thymus but which had pre-thymically been exposed to allogeneic strain B MHC determinants, were found to be specifically tolerant to the K/D and I determinants of the B haplotype. Immunofluorescence staining with strain-specific reagents did not reveal any determinants of the B haplotype present within the engrafted strain A thymus. Thus, these strain A thymocytes must have been tolerized to the allogeneic strain B MHC determinants prior to their entry into the thymus, suggesting that pre-T cells pre-thymically express receptors specific for MHC antigens.

In contrast, strain A thymocytes, which were maturing in a strain A thymus but which had pre-thymically been exposed to allogeneic Mls (non-MHC) determinants, were not tolerant to the allogeneic non-MHC determinants encountered pre-thymically. However, the strain A splenocytes from these same mice were tolerant to the allogeneic non-MHC determinants of the chimeric host. These findings indicate that tolerance to Mls determinants is mediated via a post-thymic, but not a pre-thymic, mechanism. Also, in similarly constructed non-thymectomized chimeras, thymocytes from the F₁ in situ thymus were tolerant to the Mls alloantigens of the F₁ host while those from the engrafted thymus were strongly reactive. This finding indicates that tolerance to Mls determinants first occurs intra-thymically. These findings also suggest that the pre-T cells either do not pre-thymically express receptors specific for non-MHC determinants or that pre-thymic recognition of non-MHC determinants does not induce tolerance. In either case, these experiments reveal a major difference between the expression of functional receptors specific for MHC antigens and the expression of functional receptors specific for non-MHC antigens.

Significance to Biomedical Research and the Program of the Institute: Insights into the mechanism of self-tolerance are important for our understanding

of the function of the immune system and the generation of the T cell receptor repertoire. Studies concerning the mechanism of development of self-tolerance should provide insights into auto-immune disease states, anergic states accompanying neoplasias, and into the development of clinical methods for organ transplantation.

Proposed Course of the Project: This project will continue to analyze the pre-thymic induction of tolerance to other cell surface alloantigens, such as the minor histocompatibility alloantigens and MHC mutant alloantigens, and will also assess the ability of the intrathymic and postthymic T cell compartments to induce tolerance.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201CB05094-04 I

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 the Thymus in Generation of the Self-MHC Specific T cell Repertoire

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

(Name, title, laboratory, and institute affiliation)

Ada Krulisbeek, Visiting Associate, Immunology Branch, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.1

PROFESSIONAL:

1.1

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

To determine the mechanism by which T cells are educated in the thymus, neonatal mice were chronically treated with monoclonal anti- $I-A^k$ antibodies in vivo. The results of these studies demonstrate that such mice are virtually devoid of Ia^+ cells. The T cells from these mice were found to be deficient in their recognition of either syngeneic or allogeneic class II MHC determinants, but were not deficient in their recognition of either syngeneic or allogeneic class I MHC determinants. The defect in Ia recognition correlated precisely with the intra-thymic suppression of Ia antigen expression but did not correlate with the extra-thymic suppression of Ia antigen expression. It is concluded that Ia -specific and K/D -specific T cells are educated on different intra-thymic elements.

Project Description

Other Professional Personnel Engaged on the Project:

A. Singer, Senior Investigator, Immunology Branch, NCI

Objective: The objective of this project is to assess the role of the thymus and non-thymic lymphoid compartments in the determination of the T cell repertoire.

Methods Employed: Thymocytes generally give low killer T cell responses to alloantigens and TNP-modified self H-2 antigens. Application of T cell growth factor within the cultures, however, allows thymocytes to express strong killer T cell responses with maintenance of specificity for the original stimulating signal. Thus, a method is available to study the specificity of T cells both intra-thymically and extra-thymically. Complicating allogeneic effects are avoided by the use of radiation bone marrow chimeras and thymus-engrafted nude mice. In addition neonatal mice were chronically administered monoclonal anti-I-A^k antibodies. After 3 weeks, T cells from these mice were functionally assessed.

Major Findings: Anti-Ia treated mice were found to be deficient in Ia bearing cells. T cells from these mice were found to be deficient in their ability to recognize Ia MHC determinants but were not deficient in their ability to recognize K/D MHC determinants. The failure to educate Ia-specific T cells was due to the intra-thymic suppression of Ia antigens and not due to the peripheral suppression of Ia antigens since T cells from mice which were peripherally suppressed, but not intra-thymically suppressed, were normal. Furthermore, the intra-thymic suppression was caused by the physical removal of Ia⁺ thymic cells since T cells from heterozygous H2^{b/k} mice treated with anti-I-A^k were defective whereas T cells from homozygous H2^b mice were not. Since T cell education of K/D determinants was normal in these mice, it can be concluded that education of K/D-specific T cells and Ia-specific T cells occurs on different intra-thymic elements.

Significance to Biomedical Research and the Program of the Institute: An understanding of how the T cell repertoire is generated will allow insights into the mechanisms responsible for the regulation of T lymphocyte responses to foreign antigens and thereby yield ways to manipulate immune disorders in humans which are a consequence of regulatory failure.

Proposed Course of the Project: The project will next investigate how extra-thymic T cell differentiation and education occurs.

Publications:

Bradley, S. M., Kruisbeek, A. M., and Singer, A.: Cytotoxic T lymphocyte responses in allogeneic radiation bone marrow chimeras. The chimeric host strictly dictates the self repertoire of Ia restricted T cells but not H-2K/D restricted T cells. J. Exp. Med. 156:1650-1654, 1982.

Kruisbeek, A. M., Sharrow, S. O., and Singer A.: Differences in the MHC-restricted self-recognition repertoire of intra-thymic and extra-thymic

cytotoxic T lymphocyte precursors. J. Immunol. 130:1027-1033.

Kruisbeek, A. M., Fultz, M. J., Sharrow, S. O., Singer, A., and Mond, J. J.:
Early development of the T cell repertoire. In vivo treatment of neonatal mice
with anti-Ia antibodies interferes with differentiation of I-restricted T cells
but not K/D-restricted T cells. J. Exp. Med. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05095-04 I
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 Cell-Mediated Immunity by Germ Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Gene M. Shearer, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any) Laboratory of Immunology, NINCDS		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.1	OTHER: 0.3
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Autologous mouse testicular cells derived from the seminiferous tubules activate suppressor T cells which inhibit mixed cell reactions in vitro. Generation of cytotoxic T cells in vitro is reduced in the presence of syngeneic germ cells (spermatozoa from the seminiferous tubules or epididymal sperm). Spleen cells from mice injected with syngeneic sperm show a nonspecifically suppressed potential to generate cytotoxic T cells in vitro. These observations may be relevant for the recently observed immunosuppressed condition seen among male homosexuals, and raises the possibility that the sperm is a contributing factor to this suppression.		

Project Description

Other Professional Personnel Engaged on the Project:

K. Tung, Associate Professor, University of New Mexico

Objectives: The objective of this study is to investigate the cellular and genetic aspects of the phenomenon in which mice injected with syngeneic or allogeneic sperm are immunosuppressed.

Methods Employed: Sperm cells were obtained from the epididymis. Spleen cells from normal mice and from mice previously injected with germ cells were stimulated in vitro against modified syngeneic or allogeneic spleen cells. Sperm and leukocytes were separated using Ficoll Hypaque. Lymphocyte reactivity was assayed by measuring the proliferative response by ^3H thymidine incorporation and by determining cytotoxic activity on ^{51}Cr -labelled PHA blast cells.

Major Findings: Some combinations of syngeneic and allogeneic sperm cell transfers in vivo were found to be suppressive, whereas other combinations were found to have no detectable effect or were observed to enhance immune potential to alloantigens. Since leukocytes were found to contaminate the sperm preparations, Ficoll-Hypaque separation was performed. Even after removal of all detectable leukocytes, some preparations of allogeneic sperm enhanced immune reactivity.

Significance to Biomedical Research and the Program of the Institute:

Antigens have been shown to be expressed on cells of the male germ line as well as on tumors which derive from embryonic cells. Both cell types induced immunosuppression. Therefore, this project may be of medical relevance, since it may help to understand the immune status of individuals if such antigens come into contact with the immune system; e.g. after vasectomy or during development of neoplastic cells.

Furthermore, a recent outbreak of opportunistic infections and Kaposi's sarcoma associated with suppressed cell mediated immunity has been reported among male homosexuals. It is possible that one component of this suppression is sperm, which may gain access to the bloodstream via intestinal lesions.

Proposed Course of Project: Efforts will be continued to elucidate the suppressive and stimulatory aspects of exposure of the lymphoid system to syngeneic and allogeneic sperm. Furthermore, studies will be initiated to determine whether human semen can be immunosuppressive when injected into mice. This may permit us to identify semen donors who might be high risk for transferring immunosuppression to homosexual partners. We shall also test seminal protein preparations for immunosuppressive potential.

Publications:

Hurtenbach, U. and Shearer, G. M.: 1982. Germ cell-induced immune suppression in mice. Effect of inoculation of syngeneic spermatozoa on cell-mediated immune responses. J. Exptl. Med. 155: 1719-1729.

Shearer, G. M. and Hurtenbach, U.: 1982. Is sperm immunosuppressive in male homosexuals and vastectomized men? Immunology Today 3: 152-153.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05096-04 I
PERIOD COVERED October 1, 1982 to July 29, 1983		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Identification and Function of Intracellular Calcium-Containing Organelles		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Maryanna P. Henkart, Expert, Immunology Branch, NCI		
COOPERATING UNITS (If any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 21205		
TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.2	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The purpose of this project is to determine the distribution of calcium in cellular organelles and the effects of physiologic stimuli on the distribution of calcium and the morphology of calcium-containing organelles. It is particularly concerned with the question whether the endoplasmic reticulum (ER) is a calcium uptake system and whether calcium can be released from the ER in response to surface membrane stimuli such as specific ligand-receptor interactions. Tissue is prepared by rapid freezing or by modified fixation procedures designed to retain Ca in situ. Ca is identified in sections by electron-probe x-ray microanalysis or by electron energy loss spectroscopy. A study of the distribution of calcium in BALB/c 3T3 cells as a function of the cell cycle has been initiated.		

Project Description

Other Professional Personnel Engaged on the Project:

C. E. Fiore, Physical Scientist, BEIB, DRS
 R. Leapman, Physical Scientist, BEIB, DRS
 P. J. Millard, Biologist, Immunology Branch, NCI
 R. W. Tucker, Asst. Professor, Dept. of Oncology, Johns Hopkins Medical School, Baltimore, Maryland

Objectives: The general objective of this project is to study the distribution of calcium within cells and to determine how its distribution is affected by physiologic stimuli. A working hypothesis underlying a part of this study is that the endoplasmic reticulum (ER) of all cells can function as a calcium sequestering system similar to the sarcoplasmic reticulum of muscle. An implication of this hypothesis is that there may also be a mechanism by which calcium can be released from the ER in response to surface membrane stimuli, providing for a trans-membrane signal in many cell types analogous to excitation-contraction coupling in muscle.

Methods Employed: Basic morphologic studies are done using standard techniques of electron microscopy. Identification of intracellular calcium-containing organelles require in addition: 1) Methods of tissue preparation that retain calcium *in situ*. Two approaches to this have been used: (a) Rapid freezing by various techniques followed by substitution in organic solvents in the presence of osmium. (b) Modified fixation procedures have also been used. These include fixation in aldehyde in the presence of oxalate followed by post-fixation in osmium in acetone. The results obtained by method (b) were compared with results obtained by method (a) using the squid giant axon and mouse skeletal muscle as test tissues. (2) Identification of calcium in the organelles of cells [prepared as in (1)]. This is done by electron-probe x-ray microanalysis or by electron energy loss spectroscopy using the analytic electron microscope facility in BEIB of DRS.

Various methods of tissue handling prior to freezing, variations of the freezing technique itself (different specimen stages, different shock-absorbing substances, liquid N₂ vs liquid He as coolants), and variations of freeze substitution protocols are being tested.

For studies of the distribution of calcium in BALB/c 3T3 cells as a function of their growth status we are comparing the ER structure and Ca distribution in cells rendered quiescent by serum deprivation with the same parameters in cells stimulated to enter the cell cycle by serum addition. As controls we have initially studied serum-deprived cells that have been calcium loaded for various periods of time under conditions that do not lead to growth stimulation.

Major Findings: In previous studies related to this problem I have shown that the endoplasmic reticulum of neurons and macrophages is a calcium-sequestering compartment. The ER of these cells forms morphologically specialized appositions (subsurface cisterns) with the surface membrane (or the membrane of newly internalized pinosomes) similar to the junctions between the surface membrane and sarcoplasmic reticulum of muscle at

"triads". In calcium-loaded BALB/c 3T3 cells the endoplasmic reticulum as well as conspicuous large inclusions resembling lysosomes contain calcium. Some cisterns and vesicles in the vicinity of the Golgi apparatus also contain dense deposits, but these are very small structures and it has not yet been possible to identify them in the images thus far obtained under conditions for x-ray analysis or energy loss spectroscopy.

Significance to Biomedical Research and the Program of the Institute: The role of calcium as a transmembrane signal or second messenger and its importance as a regulator of many intracellular functions is becoming increasingly apparent. Examples of calcium-regulated functions include: motility based on actin-myosin systems, secretion by exocytosis, control of membrane permeability to other ions, processes dependent upon polymerization of microtubules, the activity of many enzymes, and probably control of cell proliferation and differentiation. How calcium is distributed within cells and how its distribution is affected by physiologic stimuli are, thus, questions of fundamental importance for the understanding of normal cell function. Although many studies have suggested that calcium may be important in control of cell growth, no unified hypotheses have emerged about mechanisms. Identification of calcium in its morphologic context may help to clarify some of the intricacies of cellular control of calcium and, thus, lay the foundation for future studies directed at the role of calcium in the cell biology of cancer.

Proposed Course of Project: This project is being terminated in the Immunology Branch and will be the subject of research to be continued elsewhere.

Publications:

Henkart, M.: Identification and function of intracellular calcium stores in neurons. Introduction to Symposium. Fed. Proc. 39:2776-2777, 1980.

Henkart, M.: Downstream from the calcium channel: identification of intracellular calcium stores. In: The Physiology of Excitable Cells, A. Grinnell and W. Moody, eds. Alan Liss & Co. New York. 1983. pp.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05098-03 I
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.) Non-H-2-Linked Genetic Control of Cell-Mediated Cytotoxic Responses		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) <i>(Name, title, laboratory, and institute affiliation)</i> Gene M. Shearer, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Spleen cells from mice of different inbred strains sharing the same H-2 haplo-type but differing in their non-H-2 genetic background were compared for their ability to generate cytotoxic T lymphocyte (CTL) responses to syngeneic cells modified with the trinitrophenyl hapten (TNP-self). In both primary and secondary responses, high and low CTL strains were observed (i.e. non-H-2-linked Ir gene control). Among H-2^d strains the BALB/c was a high responder strain, whereas DBA/2 and B10.D2 were low responder strains. Among H-2^k mice, C3H, AKR/J and B10.BR were the respective high, intermediate, and low responders. Of the H-2^b strains studied C57BL/6 were high, whereas C3H.SW and C57BL/10 were low responder strains to TNP-self. By using different combinations of responding, stimulating and target cells, it was found that these non-H-2-linked differences were not attributable to stimulating or target cells. These studies raise some interesting issues concerning the role of non-major histocompatibility complex (MHC) genes in regulating CTL responses to foreign antigens recognized in association with self MHC gene products.</p>		
835		

Project Description

Objectives: It has been previously demonstrated that H-2 linked genes which map to the K and D regions regulate the CTL responses to TNP-self. The objective of this project was to determine whether non-H-2 linked genes also have an effect on regulating the CTL response to TNP-self. If so, it is also the objective of this project to determine whether the thymus (which is known to influence the expression of H-2 linked genetic control of H-2 restricted CTL response) will affect non-H-2 linked Ir gene control of CTL.

Methods Employed: For in vitro generation of CTL, mouse spleen cells were sensitized in vitro to syngeneic cells conjugated with trinitrobenzene sulfonic acid (TNP-self), and the effector cells generated were assayed on the appropriate ⁵¹Cr-labelled target cells. Mice whose spleen cells were to be used for in vitro sensitization to TNP-self were primed in vivo by skin painting with trinitrochlorobenzene.

H-2 matched allogeneic chimeras were prepared by irradiating recipient mice with 850R and by transferring bone marrow cells from donors, involving high responder recipients grafted with low responder stem cells and vice versa. Two-to-four months after cell transfer, the spleens of the chimeras will be tested in vitro for high and low response patterns to TNP-self.

Major Findings: Both in primary in vitro and in secondary in vitro (following in vivo priming) CTL studies the following non-H-2 linked high and low genetic control patterns were observed: among H-2^d strains--Balb/C, high responder; DBA/2 and B10.D2, low responders; among H-2^K strains--C3H, high responder; AKR/J, intermediate responder; B10.BR, low responder; among H-2^b strains--C57BL/6, high responder; C34H.SW and C57BL/10, low responders. These differences were observed to be more pronounced in the secondary than in the primary response, and radioresistant helper T cells were demonstrated to be involved in at least part of the differences among high and low responder strains. By varying the strains used for providing responding, stimulating and target cells, it was found that the low responder patterns could not be accounted for by stimulating or target cell defects. Therefore, these difference in high and low responder strains are likely to reside among the helper, CTL precursor, and/or accessory cells provided by the responding cell pool. By using H-2 matched allogeneic strains which differ at non H-2 loci as responder, stimulators and targets of CTL responses, it was found that the "defect" of this Ir gene control is expressed at the responder cell level.

Significance to Biomedical Research and the Program of the Institute: Over the last 15 years considerable emphasis has been placed on the importance of MHC linked Ir genes in the control immune responsiveness, and such regulation has been demonstrated both in experimental animal models and man. Based on the dramatic effects that the thymus has on both MHC restriction and on phenotypic expression of Ir genes, it has been postulated that Ir gene patterns of responsiveness are reflections of MHC restriction. The potential significance of the present project is that it demonstrates that Ir genes which are not linked to the murine MHC also have a dramatic effect on T cell immune responses--even those which are MHC restricted. Such studies underscore the fact that in considering the genetic regulation of immune

potential, heredity effects other than just those linked to the MHC must also be considered.

Proposed Course of Project: Among the strains thus far investigated, F₁ hybrids and backcross mice will be tested to establish whether high or low responsiveness is dominant and to obtain an estimate of the number of genes involved. Since these high and low responders are H-2 compatible, allogeneic irradiation chimeras are being prepared to determine whether high responsiveness is a characteristic of the host environment or of the donor stem cells. It may also be important to do thymic grafts in athymic nude mice to determine the role of the thymus in such Ir gene control. Congeneic mice differing at other known non-MHC markers will be compared to determine if there is linkage to other loci (e.g., allotype). CTL responses to other haptens plus self as well as to alloantigens will be investigated to determine how broad non-H-2-linked regulation of CTL responses are. Responding cell populations will be fractionated to attempt to define a particular cell population(s) which may express the genetic defect in low responder strains. This project has been terminated.

Publications:

Arora, P. K. and Shearer, G. M.: Non-H-2-linked genetic control of murine cell-mediated lympholysis of autologous cells modified with fluorescein isothiocyanate (FTC-self). J. Immunol. 129: 1200-1203, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05099-03 I
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.) Immunogenetic Effects of Murine Cytomegalovirus on Induced and Natural Immunity		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Gene M. Shearer, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.6	PROFESSIONAL: 0.5	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Mice injected with sublethal doses murine cytomegalovirus (MCMV) exhibit rapid and dramatic changes in their ability to generate in vitro cytotoxic T lymphocyte responses to hapten-self and to alloantigens. Within three days after intraperitoneal injection of (MCMV), the CTL responses to hapten-self and alloantigens are abrogated or severely reduced. This is followed by rapid recovery to a normal level of CTL potential. Injection of F ₁ hybrid mice with either MCMV or parental spleen cells resulted in rapid and severe immunosuppression. Inoculation of either the virus or parental cells were selected so that they would be below the threshold for severe immunosuppression. However, when these two inocula were combined, severe immunosuppression was observed. These studies permit the investigation of the immunosuppression of MCMV infection and the possibility consequences of CMV infection coupled with a graft-versus-host reaction (GVHR). Since CMV may be involved in the etiology of acquired immune deficiency syndrome (AIDS) in humans, such studies may provide a murine model for studying AIDS.		

Project Description

Other Professional Personnel Engaged on the Project:

P. Lang, Visiting Associate, Immunology Branch, NCI
J. Titus, Technician, Immunology Branch, NCI
D. Segal, Senior Investigator, Immunology Branch, NCI
S. Sharrow, Technician, Immunology Branch, NCI

Objectives: The purpose of this project is to investigate the immunological and genetic effects of MCMV infection and of MCMV infection plus a GVHR and for HVGR on (a) acquired T cell immunity to hapten-self antigens and allo-antigens; and (b) on natural resistance to MCMV infection and the GVHR. Since certain mouse strains are relatively resistant to MCMV and to parental T cell-induced GVHR, it will also be the purpose of this study to investigate the role of H-2-linked and non-H-2-linked genetic effects of resistance and susceptibility to MCMV, to GVHR and to a combination of MCMV and allogeneic cells. Since CMV may be a component in the etiology of AIDS in humans, combinations of MCMV and allogeneic leukocytes will be used to develop an AIDS-related model, including immune suppression, helper:suppressor cell ratios and possible appearance of Kaposi's-like lesion in skin, lung and gut.

Methods Employed: Sublethal doses of MCMV (prepared from salivary glands of infected mice) were injected intraperitoneally into various inbred and F₁ hybrid mouse strains. Also F₁ mice were injected intravenously with known concentrations of parental spleen cells, and F₁ mice were also injected with MCMV plus parental cells. Allogeneic leukocytes were also injected into involved mouse strains. The T cell immune potentials of injected and control mice were tested by in vitro sensitization to hapten-self and allo-antigens, and the CTL activity was determined 5 days later using the ⁵¹Cr-release assay. Changes in subpopulations of T and B lymphocytes were determined by Flow Microfluorometry.

Major Findings: The injection of sublethal doses of MCMV resulted in rapid suppression of CTL potential to both hapten-self and allogeneic antigens (within 3 days). This was followed by recovery (by around 7 days), and augmented CTL activity as detected by the hapten-self and not by the allogeneic CTL systems (days 9-13). The injection of F₁ mice with doses of MCMV plus parental spleen cells each of which alone did not drastically reduce CTL potential, resulted in synergistic effect which abrogated CTL potential. Combinations of allogeneic spleen cell transfers in certain but not all strain combinations resulted in suppression.

Significance to Biomedical Research and the Program of the Institute: Cytomegalovirus infection is one of the major problems currently facing human bone marrow transplantation, and may become critical in patients undergoing a chronic GVHR. An understanding of the genetic and mechanistic parameters involved in resistance and susceptibility to CMV in the murine model, the immunosuppression associated with CMV infection, and the possible synergistic effects of CMV infection and chronic GVH should be valuable for both basic and clinical purposes. Furthermore, since CMV may be an etiologic factor in the appearance of AIDS among homosexual men (especially Kaposi's patients), the development of a murine model involving MCMV and other suppressive factors,

e.g., allogeneic leukocytes, could be important in understanding the etiology of this complex syndrome.

Proposed Course of Project: A number of inbred, recombinant and F₁ hybrid mouse strains will be studied for their ability to be resistant or susceptible to immunosuppression resulting from MCMV infection and with the synergistic effects of MCMV HVGR and GVHR on immunosuppression. A number of complex protocols will be tested for the development of AIDS-related models, including: various combinations of doses and sequences of MCMV and allogeneic leukocytes; reactivation of latent MCMV by allogeneic leukocytes; possible selection of potent sublines of MCMV in mice immunosuppressed with allogeneic leukocytes or sperm, (this MCMV subline will be tested for increased infectivity and/or increased immunosuppressive potential and/or increased lethal effects on infant mice); and attempts to induce Kaposi's-like lesions in MCMV-infected and allogeneic-injected mice.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CB05100-03 I
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 Role of HLA Genes in Human Disease		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.)		
(Name, title, laboratory, and Institute affiliation)		
Stephen Shaw, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH		
Immunology Branch		
SECTION		
INSTITUTE AND LOCATION		
NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.1	0.2
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		A
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>We have defined a HLA locus (SB) which maps centromeric to the other known genes of the HLA complex. We have continued studies on the importance of the genetic region marked by this gene in three different human diseases: dermatitis herpetiformis, systemic lupus erythematosus, and kidney transplantation rejection. Previous studies of patients with dermatitis herpetiformis have been extended particularly with respect to family analysis of complexity related to the differentiation of DR3,3 from DR3,w6 phenotypes and statistical analysis of haplotype associations. The data are most consistent with the interpretation that the HLA-linked gene tends to occur on haplotypes with DR3 and SB1, and confers risk in an autosomal recessive manner. Studies of 4 pairs of HLA-A thru HLA-DR identical sibling kidney donor/recipient pairs in whom there had been rapid rejection failed to identify SB/DR recombinations, which would have suggested importance of SB region genes in kidney graft rejection. Pilot studies of 15 patients with SLE failed to show strong association of the disease with SB phenotype.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

T. Lawley, Investigator, Dermatology Branch, NCI
S. Katz, Chief, Dermatology Branch, NCI
W. Tekolf, Guest Worker, Immunology Branch, NCI
L. Ercolani, Veterans Administration Hospital, Iowa
J. Balow/G. Tsoukos, Arthritis and Rheumatism Branch, NIAMD

Objectives: Previous studies from many laboratories have demonstrated that there are associations between many specific diseases and particular HLA antigens. For virtually all of these associations it is not known: 1) exactly what gene product is involved in the disease pathogenesis (i.e., whether the HLA gene product identified is involved in the disease or some unknown allele with which it is in linkage disequilibrium); and 2) what the mechanism is for the association. The extraordinary number of HLA associated diseases and the overall importance of the HLA region in immune responses suggest that an understanding of these associations may be of rather general relevance. In addition, the HLA region is known to be crucial in an "iatrogenic disease", transplantation rejection. As we develop new markers of the HLA region (project Z01CB05101 I), we expect they will be useful in helping us to map more precisely the gene products involved in disease associations and transplantation rejection.

Methods Employed: Patients are selected by diagnostic criteria relevant to the particular disease. Patients peripheral blood lymphocytes are "typed" for SB antigen expression by the primed lymphocyte typing techniques outlined in project Z01CB05101 I; they are also serotyped for other HLA antigens under contract N01-CB-04337.

Major Findings: Previous findings had indicated that patients with dermatitis herpetiformis had increased frequency of DR3 and SB1. However two elements of complexity precluded easy interpretation. First inference of dominant or recessive inheritance can normally be made on the basis of heterozygosity of homozygosity for the most informative marker gene (DR3). However, because of technical difficulties with serologic assignment, it is impossible to distinguish with certainty the presence or absence of DRw6 by alloantisera when a donor carries DR3. Family studies have been possible for three patients in question, but have not been informative; therefore studies are underway to identify DR6 more definitively with cellular typing reagents and with a monoclonal antibody putatively specific for DRw6. These results should help distinguish the hypothesis of dominant or recessive inheritance. Second, it is difficult to be certain whether the increase in frequency is secondary to the increase in DR3 (with which it is associated in the population) or whether it indicates that the DR3/SB1 haplotype confers greater risk (statistically) than DR3 alone. Computerized statistical analysis has been implemented to distinguish these alternatives and indicates that the increase in SB1 is greater than that expected from linkage disequilibrium (for both the assumptions of rare autosomal dominant and autosomal recessive inheritance).

We had predicted that products of genes encoded in the HLA region around SB would function as histocompatibility barriers to allogeneic tissue transplantation. One specific context in which to test this prediction is in kidney transplantation. Since SB differences may not result in "positive" MLC results, as many as 2-5% of MLC identical siblings may have undetected DR/SB recombinations. If this group has a greater risk for transplantation rejection, then the group of "HLA-identical" recipient/donor pairs in which rejection has occurred may have an increased frequency of SB/DR recombination. Five pairs of "HLA-identical" donor/recipient pairs were identified in which kidney transplantation resulted in rapid rejection. However, SB typing did not identify recombination in any of these pairs. Since this sample is small, additional collaborative studies are underway to test this hypothesis on a larger sample of donor/recipient pairs.

Cells from 15 donors with systemic lupus erythematosus were SB-typed as a pilot study to determine if there were a strong statistical association of this disease with a particular SB allele. The results failed to indicate any such strong association.

Significance to Biomedical Research and the Program of the Institute: Many diseases are known to be HLA associated, including certain malignancies. Understanding of the role of HLA genes in the pathogenesis of these diseases might reasonably be expected to help in therapy and prevention of these diseases. Furthermore, if SB is important in transplantation, matching for SB would be expected to further improve the results of kidney and bone marrow transplantation.

Proposed Course of Project: In analysis of the DH patients, cellular and serologic reagents will be developed to reliably distinguish the presence or absence of DRw6 which will clarify inferences on the mode of inheritance of susceptibility conferred by the HLA-linked gene.

Additional pairs of "HLA-identical" recipient/donor kidney transplantation pairs s/p rejection will be tested as part of a National/International Histocompatibility Workshop to determine if any have detectable SB/DR recombination.

Twenty to thirty JOD families will be studied, particularly those with known DR/GLO recombination involving one of two affected HLA-identical siblings.

In general, the SB marker system may be informative in a number of other diseases which are known to be associated with DR3, such as the endocrinopathies. This should help resolve whether all of these diseases have in common a gene which predisposes to autoimmunity, or whether they have different disease genes each of which is in positive linkage disequilibrium with DR3.

Project Description

Other Professional Personnel Engaged on the Project:

W. Tekolf, Guest Worker, Immunology Branch, NCI
 W. E. Biddison, Investigator, Neuroimmunology Branch, NINCDS
 G. Pawalec/P. Wernet/A. Ziegler, Tubingen, Germany
 A. Termijtelen/J. J. van Rood, Leiden, Netherlands
 C. Mawas/D. Charnot, Marseille, France
 R. Duquesnoy, Milwaukee, Wisconsin
 R. DeMars, Madison, Wisconsin
 C. Hurley/J.D. Capra, Dallas, Texas
 L. Nadler, Boston, Massachusetts
 E. Long/B. Mach, Geneva, Switzerland
 J. Trowsdale/W. Bodmer, London, England

Objectives: Genes of the HLA region are important in tissue transplantation, immune regulation and regulation of susceptibility to a variety of diseases. Consequently, it is crucial to define the gene products of this region and to determine the function of these gene products. There has been a worldwide effort to do so, primarily by serologic techniques. We believe that T cells may be the most sensitive probe for defining functionally important details of the HLA region, since T cells seem to be uniquely committed to recognizing gene products of this region. Therefore we expect refined approaches to cellular typing to allow definition of new HLA gene products. As cellular reagents are found to define these antigens, we will investigate the genetics of these markers, the tissue distribution of the markers, and function of the markers in cellular immune responses.

In addition, we have begun investigation of human 'minor' histocompatibility antigens. Such antigens, like HLA molecules, are polymorphic cell surface molecules. Unlike HLA antigens, they must be recognized by T cells in association with HLA molecules. These antigens are of interest for several reasons: 1) they are important histocompatibility barriers to tissue transplantation, but until now have eluded investigation; 2) understanding of responses to such antigens is a useful area in which to generalize our understanding of HLA-restricted T cell recognition.

Methods Employed: Human PBL are obtained from donors by phlebotomy or batch leukapheresis; mononuclear cells are separated by density separation, and cryopreserved. Analysis of the serologically defined HLA markers on the donors cells is performed by microcytotoxicity testing under contract N01-CB 04337. Cells from carefully selected donors are sensitized in vitro in one way mixed lymphocyte culture, and generally the primed cells are restimulated after 10 days with the same stimulator to enhance weak responses. Primed cells are frozen in large batches, and thawed as necessary to provide a standard "reagent". Proliferation of these cells in response to stimulator cells is measured by ^3H -thymidine incorporation. Cytotoxic activity is analyzed by short term ^{51}Cr release assays using as targets T lymphoblasts or lymphoblastoid B cell lines. The assignment of specificities to a donor's cells is made on the basis of a statistical technique called centroid cluster analysis, which has been improved and adapted for this application. For inducing primary responses of normal donors to minor histocompatibility

antigen, lymphocytes are cultured in a limiting dilution assay in the presence of TCGF and the stimulating cells. Two monoclonal antibody binding assays used have been the "enzyme linked immunologically specific assay" (ELISA) and flow microfluorometry (FMF). Antibody inhibition of proliferation and cytotoxicity has been performed by adding antibody continuously to the culture system.

Major Findings: The discovery of a new gene "SB" in our laboratory has prompted a large number of collaborations both to optimize development of information about SB by utilizing the expertise of other laboratories, and to help others learn to define the SB gene products. As a result, rapid progress has been made by local and collaborative studies in defining the genetics, structure, and function of the SB antigens. Continued effort has been expended in systematically analyzing mouse monoclonal antibodies against Ia antigens. Studies described in last years report have been extended in several ways. First, a much wider variety of monoclonal antibodies have been analyzed from more than 20 different laboratories: including antibodies raised against both human and mouse Ia molecules. Second, systematic analysis of binding properties has included extensive studies on HLA-deletion mutant lymphoblastoid cells lines from R. DeMars. Analysis on these lines allows distinction of binding to determinants encoded by genes in three subregions of a single HLA haplotype: those linked to DR1, those linked to DC1 and those linked to SB2. In this way, complex patterns of binding can be distinguished which elude definition in the usual population studies. Third, more extensive studies have been performed on the ability of these monoclonals to inhibit SB-specific proliferative and cytotoxic responses.

Binding studies reveal extraordinary complexity. Careful analysis was necessary to identify technical artifacts which were encountered in the ELISA assay commonly used to measure binding. Careful analysis of binding in two assay systems (ELISA and FMF) indicated the following principles: 1) monoclonal antibodies commonly crossreact on products of more than one locus and most often recognize determinants shared by many or all alleles of a single locus; 2) many mouse monoclonals raised against alloantigens crossreact on human Ia molecules; 3) there is no close correlation between the locus specificity of such monoclonals in the mouse and their locus specificity for human Ia antigens; 4) in general such mouse allo-induced monoclonals recognize determinants that are more polymorphic on human Ia molecules than mouse xeno-induced monoclonals; 5) that there is closer serologic relationship between SB and DR than between SB/DR and DC is suggested by the high frequency of monoclonal antibodies which react with SB2 and DR1 relative to the virtual absence of antibodies detecting only DC1 and DR1 and the absence of antibodies binding only SB2 and DC1; 6) on the DC1 molecule the determinants which are polymorphic between alleles are immunodominant in mouse xenoimmunization in contrast to the DR1 (and probably SB2) molecules where the immunodominant determinants are widely shared between molecules.

Studies of monoclonal antibody inhibition of T cell responses is a potentially powerful approach to ascertain which antibodies recognize the SB molecule (i.e., the one defined by T cell recognition) and which antibodies may recognize molecules encoded by genes closely linked to SB. They also may distinguish different epitopes on the SB molecule. These expectations have been borne

out in the studies of the ability of about 70 monoclonal antibodies to inhibit cell-mediated cytotoxicity specific for SB. There is a correlation between those antibodies which bind to SB2-linked gene products and those which inhibit SB2-specific cell-mediated cytotoxicity: antibodies which do not bind do not inhibit and some (but not all) of those which bind do inhibit. Antibodies of the latter sort may be: 1) binding to another molecule encoded close to SB2; 2) binding to an epitope on SB2 which is not close to the predominant binding site of the majority of T cells; 3) binding to the relevant site on SB2 but be of too low affinity or concentration to result in strong inhibition. Comparisons between alleles of SB indicate that some antibodies (typically those recognizing determinants shared between loci) inhibit all SB responses, while most others inhibit in an allele dependent fashion (suggesting that their binding is affected by the polymorphism of the molecule). Similar studies of inhibition of proliferative responses has demonstrated less specificity, since, for example many monoclonal antibodies which do not bind to SB2 inhibit the proliferative response. Controls are being devised for such experiments to enhance the ability to interpret results of such inhibition studies.

Before embarking on such an ambitious program of studying monoclonal anti-Ia antibodies, we debated whether to generate our own or to screen the many available from other laboratories. We opted for the later, since we felt such cooperative sharing of information between labs would enhance the value of investigations being done in many laboratories. This has certainly been the case. Many laboratories now depend heavily on the information derived from our studies in the interpretation of the specificity of their monoclonal antibodies.

Another mode by which we have encouraged international collaboration is by providing other laboratories access to two kinds of reagents developed in our laboratory -- both of which are becoming world standards. The first is a panel of lymphoblastoid cell lines which express an optimal assortment of known SB alleles. These cells, distributed in part through contract NOL-CB 04337, are now an unlimited source of well characterized cells which allow preliminary evaluation of cellular and serological reagents for possible SB-reactivity in other laboratories. The other reagents are the primed cells we have developed as typing reagents; these reagents, when expanded with TCGF, can provide a more rigorous basis for international standardization. We have made these available to many labs, including Dr. Hartzman and coworkers who have expanded them for use in the International Histocompatibility Workshop (1984).

Structural studies of amino acid sequence of the SB glycoprotein molecule, by C. Hurley/D. Capra in collaboration with us, have progressed slowly because of the limited amounts of SB expressed on cell surfaces and the myriad cross-reactivities of the monoclonal antibodies against Ia molecules. Consequently, a more expeditious approach would be to identify the genes which encode the two polypeptide chains in this molecule. We have initiated collaborations with two labs (J. Trowsdale/ W. Bodmer and E. Long/B. Mach) to help identify which of the Ia genes correspond to SB. We have prepared Southern blots from a panel of our most informative donors which are matched for DR/DC but selectively mismatched for SB. These blots will be hybridized to locus-specific probes for the several alpha and beta chains to determine which identify fragments with site polymorphism among these donors, and thus good candidates for SB region genes.

Other collaborative studies include: 1) Those aimed at identification of allo-antisera which detect SB molecules; collaborators in Leiden have identified such antisera which correlate with SB4 reactivity; 2) characterization of commonly used homozygous typing cells for their SB antigens. 3) Analysis of the complexity of T cell clones which are specific for SB; surprising homogeneity of T cell clones has been observed for SB2, SB3 but not SB4. 4) Further populations studies in Europe and the Orient to broaden the understanding of polymorphism, population distribution and linkage disequilibrium; striking differences are seen in SB allele frequency between populations particularly between Japanese in whom SB5 is most common and Caucasians in whom SB5 is least common of the five defined alleles.

A new area of major emphasis this year relates to analysis of human minor histocompatibility antigens made in two systems: first by the observation of an unusual donor combination in whom we observed minor antigen-specific responses using routine culture conditions; second by the development of technology to elicit minor antigen-specific responses from other normal donors. The original observation was of a normal female donor whose cells generated strong CML against completely HLA-A,B,C-matched donor's T cells after secondary in vitro stimulation. The CTL derived were shown to recognize predominantly a single human minor histocompatibility antigen (i.e., a polymorphism not inherited with HLA), and their capacity to recognize this antigen was HLA-B7 restricted. Genetic data on this minor antigen (designated Hm1) is consistent with the interpretation of autosomal dominant inheritance of a molecule encoded by an allele with gene frequency of about 25%. We suspect that priming to this antigen during pregnancy facilitated her cytotoxic T cell response to minor antigens since: 1) she has no known exposure to allogeneic cells other than pregnancy; 2) her offspring are both HLA-identical to her except for SB and 3) one carries the minor antigen of interest. Careful analysis of other cytotoxic responses of her cells indicates that she was also sensitized to other minor antigens: one additional antigen recognized in association with HLA-B7 and one with HLA-A2. These responses are mediated by T cells with the phenotype characteristic of cells interacting with class I antigens: $OKT3^+$, $OKT4^-$, $OKT8^+$.

The foregoing findings, although provocative, were of limited general applicability because of the special circumstances of priming. Therefore we sought to find a technique to explore, with greater freedom, cellular recognition in vitro of minor histocompatibility antigens. It was observed that minor antigen-specific responses could be generated between HLA-identical siblings under conditions of limiting responder cell number and added T-cell growth factor. Several clones of $OKT8^+$ T cells have been derived from two separate combinations of HLA-identical siblings; since these clones appear to define new minor antigens, the technique promises to be one which is generally applicable in studying recognition of minor histocompatibility antigens.

Studies of diversity of the HLA-A3 and HLA-Bw44 molecules, described in last years reports, have been pursued collaboratively. Exchanges of cells conducted with the Central Blood Laboratory have confirmed our findings regarding Bw44 and allowed clarification of their data. Collaborative studies in progress with Dr. Helse are testing the hypothesis that heterogeneity of Bw44 as defined by CML will correlate with heterogeneity he has detected with alloantisera.

Significance to Biomedical Research and the Program of the Institute: Because genes of the HLA region are crucial in controlling immune responses, transplantation, and increasing the risk of a large variety of diseases, therapeutic intervention related to these phenomenon may depend on further understanding of the genes in this region. The SB gene defined already in this project promises to be a very informative one. Since it maps quite a distance from the other known HLA markers, it will provide an important new marker for population studies. Furthermore, the SB gene product itself may be important in immune regulation and disease. Its similarities to HLA-DR and the murine Ia antigens suggest that it may be involved as an Ir gene (controlling immune responses).

Studies of minor histocompatibility antigens are of great potential importance for several reasons. First, these antigens are clinically important barriers to tissue transplantation. The ability to type for the immunodominant minor antigens might thus be useful. More important, understanding of the cellular basis for these responses should allow better therapeutic intervention to overcome these responses. Second, responses to minor antigens may be a good model of tumor antigen recognition -- since minor antigens may, like tumor antigens, be weak immunogens present on cell surfaces. Thus, understanding of regulation of minor antigen responses may be very relevant to tumor immunology.

Proposed Course of Project: We will continue collaborations to elucidate the structure of SB at the level of the genome. We plan to pursue functional studies of the SB antigens with respect to their role in cellular immune responses to foreign antigens and their possible function as Ir genes (probably under project Z01-CB-05067 I). We will continue studies of the relevance of this new marker system in disease (project Z01-CB-05100).

This work has attracted considerable attention from the world community of scientists interested in HLA. Of necessity, we will be the world reference laboratory for defining the SB antigens, until other laboratories are prepared to assume this function.

The development of a general system in which to generate minor antigen-specific human T cell responses opens many possibilities. We shall investigate regulation of this response, particularly as to why the response does not occur in bulk culture but does so readily in limiting dilution culture. We shall explore the genetics of individual minor antigens recognized and begin to ascertain whether or not there are a limited number of immunodominant minor antigens. For studies of genetics, it would be desirable to have a system in which to "type" every donor for the antigen. Using the readout of cell-mediated lysis, expression of the relevant HLA antigen becomes limiting. Therefore, we will consider developing methodologies for transferring in the relevant restriction antigen to the target cell. In addition, however, we are interested in defining these antigens biochemically. To do so we will develop monoclonal antibodies specific for such markers. Once we have developed general typing methods, we shall explore collaboratively the potential importance of these antigens in allograft rejection.

Publications:

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Pawelec, G., Shaw, S., Schneider, M., Blaurock, M., Frauer, M., Brackertz, D., and Wernet, P.: 1982. Population studies of the HLA-linked SB antigens and their relative importance in primary MLC-typing: analysis of HLA-D homozygous typing cells and normal heterozygous populations. Hum. Immunol. 5: 215-223.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05102-03 I
PERIOD COVERED October 1, 1982 to July 29, 1983		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Morphologic Studies of Cellular Interactions in the Immune System		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Maryanna P. Henkart, Expert, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 0.9	OTHER: 0.6
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues * <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Interactions of positively identified human natural killer (NK) cells with appropriate target cells were studied in serial thin sections with the electron microscope. These studies were extended to human effectors of ADCC rat NK cells and rat tumor cells with NK and ADCC activities. All these cells contain prominent cytoplasmic granules and in all cases the granule contents are secreted by exocytosis under conditions which activate the effectors to kill. The material secreted by all effector types is heterogeneous and has as a major component membrane vesicles which bear ring-shaped profiles on their surfaces. Granules have been isolated from cells of several rat leukemias which have NK and ADCC activities. The isolated granule material is cytotoxic to a variety of target cells. Ultrastructural studies of the isolated granules show that membrane vesicles bearing ring shaped profiles are elaborated from the granules during the time and under the conditions in which the granule material is cytotoxic. Ultrastructural studies on cloned T cells of known functions have been continued. The morphology of inclusions in NK and suppressor clones have some similarities, while in proliferative or helper clones the inclusions are quite different.		

Project Description

Other Professional Personnel Engaged on the Project:

P. A. Henkart, Chemist, Immunology Branch, NCI
 P. J. Millard, Biologist, Immunology Branch, NCI
 A. Shekhtman, Visiting Fellow, Immunology Branch, NCI
 C. Reynolds, FCRC
 M. Schneider, Immunology Laboratory, Medi, Univ., Klinik, II Tubingen,
 W. Germany

Objectives: (1) To study the ultrastructure of interactions between identified cell types involved in various aspects of immune responses and (2) To correlate morphologic observations with functional studies. The first system being approached in this way is the interaction of cytotoxic lymphocytes with appropriate target cells. The ultrastructure of a number of T cell clones of various defined functions is also being studied as the basis for detailed studies of other specific cell interactions in immune responses.

Methods Employed: Basic morphologic studies are done using standard techniques of electron microscopy. For studies of particular cellular interactions study of serial thin sections is necessary. Special stains and extracellular tracers are used as required. Immunocytochemistry using fluorescent labelled antibodies for light microscopy and ferritin or colloidal gold labelled antibodies at the EM level are used for identification of cellular constituents.

NK: Cells are obtained from peripheral blood of normal adult human donors. Fractions of lymphocytes from discontinuous Percoll gradients are prepared by published techniques (Timonen & Saskela, J. Immunol. Methods 36: 285, 1980). Killer-target conjugates are made by centrifuging together and rediluting the the natural killer cell enriched lymphocyte fraction with NK susceptible targets, the human leukemia cell line, K-562. Rat leukemias having natural killer and/or ADCC activity have been obtained from Dr. Craig Reynolds of FCRC and are being used for combined studies of the morphology of the killer cells and for isolation of the granules which appear to be involved in the killing mechanism (see below).

ADCC: For studies of antibody-dependent effector cells, monocyte-depleted peripheral blood lymphocytes are allowed to attach to plastic surfaces coated with antigen-antibody complexes or are allowed to settle onto monolayers of TNP-modified, anti-TNP antibody-coated red blood cells or red cell ghosts for varying lengths of time in medium or balanced salt solutions in which the ionic composition could be modified. Parallel ⁵¹Cr release assays are done on each batch of lymphocytes and targets, or the release of fluorescein from resealed red cell ghosts is used as an indicator of cytotoxic activity. Serial sections are examined in the electron microscope.

T-cells clones: The morphology of a number of T-cell clones of defined functions is being studied.

Granule isolation: Cells from the spleens of Fischer rats having leukemias with NK activity are disrupted by nitrogen cavitation; the lysates are filtered through nucleopore filters (5 μ m followed by 2 μ m), and the filtrates are then separated on Percoll density gradients. The gradients are fractionated and the fractions assayed for enzymatic activities, studied by gel electrophoresis, and are also studied in negative stain and thin sections by electron microscopy.

Assay for granule cytotoxicity: Fractions from the gradients are tested for cytotoxic activity by mixing with ^{51}Cr labelled target cells (erythrocytes or various nucleated targets), incubating for 20 min at 37°C, and counting the ^{51}Cr released into the supernatant. Granule material and target cells are studied in negative stain and thin sections by electron microscopy under conditions in which the granules are or are not cytotoxic. Antibodies to isolated NK granules: Polyclonal antibodies are prepared by injecting rabbits with the granule-containing fractions from the Percoll gradients in complete Freund's adjuvant.

Major findings: NK cells and effectors of ADCC from human and rat contain prominent cytoplasmic granules. Under conditions which activate these effectors to kill, the granules are secreted by exocytosis. The material secreted by the killer in close proximity to the target cell membrane has as a major component membrane vesicles which often bear ring-shaped profiles. The images seen in the EM suggest further that the membranous material released from the killer cell may fuse with the target cell.

Granules have been isolated from rat leukemia cells with NK and/or ADCC activities by separation of cell lysates on Percoll density gradients. The fractions which by EM are found to contain granules are essentially free of other organelles, although there may be more than one population of granule on the basis of size and detailed morphology of the granule contents. (For details of the enzymatic activities and protein contents as indicated by polyacrylamide gel electrophoresis see Annual Report # 05018-13.)

The isolated granule material has been found to cause ^{51}Cr release from (be cytotoxic to) a variety of target cells including erythrocytes and a number of nucleated cells. (For other aspects of the cytotoxicity experiments see Ann. Report #05018-13.) The cytotoxic activity of the granule material has a strict requirement for calcium. The morphology of the isolated granules can, therefore, be studied under conditions in which they are or are not cytotoxic. Within two minutes after the addition of calcium to isolated granules, membrane vesicles are elaborated from the granules, and ring-shaped profiles can be found on their surfaces. The number of ring-bearing vesicles as well as the number of rings per vesicle increases with time in the presence of calcium for roughly 20 min. This corresponds to the time of calcium pretreatment of the granule material that results in complete loss of the capacity of the pretreated granules to lyse target cells. Thus, ring-bearing vesicles are generated in the presence of calcium with the same time course as the cytotoxic activity of the isolated granules.

The possibility that the ring-forming material released from killer cell granules may be transferred to the target cell membrane is being pursued by immunocytochemistry with polyclonal antibodies raised against the isolated granule material. Results thus far at the light microscope level indicate that the antibodies recognize material in the granules of rat NK cells.

Examination of six lines of cytotoxic T-cells from the mouse has shown that they contain characteristic granules. The morphology of the granules in mouse CTL lines differs from that of the granules in human and rat NK and ADCC effectors, but they appear to be secreted under conditions in which the CTL are stimulated to kill. Four T-cell lines or clones from the mouse which have no cytotoxic function but proliferate in response to antigen or IL-2 did not have comparable granules. Nine clones isolated from allo sensitizations in mixed lymphocyte cultures of human peripheral blood lymphocytes have been examined. Of these, 4 had NK-like cytotoxic activity, and 4 were suppressors of various immune functions. The inclusions in all these cell types were heterogeneous but had in common granules containing a gray, grainy-textured core material. One clone that proliferated in response to antigen lacked this type of inclusion.

Significance to Biomedical Research and the Program of the Institute:

Ultrastructural studies of cellular interactions in the immune system promise to contribute much to the understanding of mechanisms of immune functions. Until recently, however, this has not been profitable approach because of the heterogeneity of cell types involved in immune responses. With the recent development of techniques for isolation, characterization, and, in some cases, cloning of lymphocyte populations with specific functions it has become feasible to study cellular interactions at the ultrastructural level.

Attack of tumor cells by cytotoxic lymphocytes: Natural killer cells, antibody-dependent effectors, or cytotoxic T-cells is probably important in normal defense against neoplasms. Understanding of the mechanisms by which these cytotoxic effector cells kill their targets is fundamental to the potentially useful ability to manipulate cytotoxic lymphocyte function for prevention or therapy of neoplasms.

Proposed Course of Project: This project is being terminated in the Immunology Branch.

Publications:

Henkart, M. and P. Henkart: Lymphocyte-mediated cytolysis as a secretory phenomenon. In: Clark, W. R. and Golstein, P. (Eds.) Mechanisms in Cell Mediated Cytotoxicity. Adv. Exp. Biol. Med. 146: 227-242, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05103-02 I
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 and Function of Cloned Lymphocytes		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Pierre A. Henkart, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 0.5	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Cloned cytotoxic T lymphocytes were maintained with stable cytotoxic phenotype for over six months. By subcloning and selection, variants were obtained which expressed >10X lower levels of cytotoxicity. In order to test the feasibility using molecular genetics approaches to analyze the small differences between cytotoxic and non cytotoxic cells, a preparation of mRNA from the T cell lymphoma EL4 was made, yielding .08 pg undegraded poly A+ RNA/cell. This suggests that it is feasible to use cytotoxic T cell lines as a source of mRNA to create a cDNA library. Rabbit antibodies against rat NK tumor cytoplasmic granules show some inhibition of cloned CTL cytotoxicity; these antibodies detect cytoplasmic granules in the CTL by immunofluorescence.		

Project Description

Other Professional Personnel Engaged on the Project:

T. Soares, Microbiologist, Immunology Branch, NCI

Objectives: We proposed to define the molecular differences which are directly responsible for the functions of these lymphocytes, particularly cytotoxicity. This will be done in two ways: 1) By making a cDNA library from cloned cytotoxic CTL and identifying those clones which correspond to genes not expressed in cytotoxic clones; and 2) by examining the cytoplasmic granules of cloned CTL for molecular and functional similarities to ADCC/NK lymphocyte granules.

Methods employed: Cloned alloreactive cytotoxic T cells are grown in *in vitro* culture with IL2 from EL4 and ConA stimulated spleen cell supernatants. They are cloned by limiting dilution methods and assayed for cytotoxicity by the ⁵¹Cr release method. mRNA was prepared from cytoplasm by phenol extraction and oligo-dT columns, and analyzed by denaturing agarose gel electrophoresis. Anti-NK cell granule antibodies are prepared by injecting purified rat NK tumor cytoplasmic granules into rabbits in complete Freund's adjuvant.

Major Findings: Variants of cloned CTL lines with reduced cytotoxicity have been isolated so that they can be analyzed for biochemical differences. Using CTL clones which are stable in their cytotoxic phenotype, variants deficient in cytotoxicity have been isolated by subcloning and selection in the absence of mutagens. Culturing in the absence of antigen does not produce a permanent loss of cytotoxic function in these clones, but by repeated subcloning and selection, lines which have lost greater than 90% of their cytotoxic capacity have been produced. Since previous results showed that analysis of total proteins of various cloned lymphocytes did not readily show differences correlating with function, the feasibility of using molecular biology approaches to this problem has been investigated. mRNA from the easily grown lymphoid tumor EL4 has been isolated with a yield of .08 pg undegraded poly A⁺ RNA/cell. At this level it is possible to use mRNA from cloned CTL to create a cDNA library or to make labeled probes but not as a source of mRNA for cascade hybridization. Preliminary results suggest that rabbit antibodies against rat NK tumor cytoplasmic granules can inhibit cytotoxicity of various CTL, and that these antibodies detect cytoplasmic granules in cloned CTL by immunofluorescence. Some non cytotoxic cloned T cells do not show this staining.

Significance to Biomedical Research and the Program of the Institute: It is clear that the cell-mediated immune system is responsible for many fundamental properties of the body's overall defense system against foreign organisms. This also appears to be true of the body's natural defenses against tumors. We are bringing a new means of analysis to bear on the cells which mediate these activities so that they can be understood on a molecular basis. This knowledge should be of great benefit in designing new therapeutic modalities.

Proposed Course of Project: Various sources of non cytotoxic variants of cloned CTL will be developed so that these can be used for biochemical analysis. A cDNA library from cloned CTL will be established and clones

representing mRNA expressed uniquely in cytotoxic lines will be identified by hybridization selection. The possibility that cytoplasmic granules in these CTL are involved in cytotoxicity will be further investigated by more extensive immunofluorescence studies and use of more potent and specific sera against NK granule components.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05104-02 I
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.) Detection and Analysis of H-2 Variant Cell Lines from Murine T Cell Lymphomas		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Gene M. Shearer, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Various lines of the S-49 T cell lymphoma of BALB/c origin are being studied for the expression of H-2 antigens. Normal BALB/c lymphocytes express H-2K^d, H-2D^d, and H-2D^d antigens. We have found that the five lines of the S-49 lymphoma thusfar studied do not express all of these cell surface H-2 antigens. The patterns of expression of H-2 antigens using these cells as targets for: (a) antibody and complement; and (b) cytotoxic T lymphocytes (CTL) exhibit four different patterns of H-2^d expression in the five lines tested. This system may be of value for investigating regulation of expression of major histocompatibility complex (MHC) antigens, and raises the possibility of a relatively high rate of modulation of these antigens among tumor cell lines of the same origin.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

Loren Joseph, Medical Staff Fellow, Immunology Branch, NCI

Objectives: The purpose of this project is to study the expression of H-2 antigens on different lines of a T cell lymphoma, to determine the mechanism(s) of differential expression of these antigens on the cell lines and to establish whether we can detect changes occurring in H-2 expression as we carry these lines in vitro and in vivo.

Methods Employed: The various tumor lines as well as "wild type" BALB/c cells were tested for expression of K^d, D^d and L^d antigens by CTL, monoclonal reagents plus complement, and monoclonal reagents using FACS. Internal labelling using ¹⁴C-leucine for immunoprecipitation studies were performed to determine whether the lack of expression of these H-2 antigens are a problem of cell surface antigen expression or intra-cellular synthesis.

Major Findings: Of the five lines of S-49 thusfar examined, at least four different patterns of H-2^d antigen expression has been observed: the "7.3" and "TAS" lines (actually splits of the same line) K^d(-), D^d(+), L^d(-); "100.0" K^d(-), D^d(-), L^d(-); S-49.1 K^d(+), D^d(+), L^d(-); S-49-Thy-K^d(+), D^d(-), L^d(-). Using BALB/c anti-pool sera as well as BALB/c anti-pool CTL, both of which should detect antigens of any other known H-2 haplotype, no other H-2 antigens have been detected. This is compatible with the hypothesis that these cell lines are not mixed up or contaminated with any other murine tumor cell lines that would express other H-2 antigens.

Significance to Biomedical Research and the Program of the Institute: The modulation of major histocompatibility complex (MHC) antigens by tumor cells provides an interesting model for investigating expression of these antigens on cell surfaces as well as gene expression. Furthermore, since autologous tumor antigens appear to be recognized in association with syngeneic MHC antigens by T lymphocytes, the modulation of MHC antigens may be a mechanism by which tumors could escape rejection. The tumor lines we have identified that have "lost" certain H-2 antigens could represent lines which have been selected to grow in host mice by such an "escape" mechanism.

Proposed Course of Project: Biochemical analysis at the levels of cell surface expression and intracellular synthesis will be performed in order to understand this antigenic modulation. Other S-49 cell lines will be studied in an attempt to identify additional lines which exhibit differential H-2^d antigen expression. An extensive panel of anti-H-2^d monoclonal reagents will be employed to elucidate the fine specificity of the antigens expressed by these tumor cell lines. The tumor lines will be injected into syngeneic BALB/c and H-2 allogeneic mice in an attempt to recover cell lines that exhibit additional differences in H-2^d antigen expression. If a number of the above experiments are interesting it will be important to determine if the phenomenon is more general, and could be demonstrated for other murine T cell lymphomas. Some 40 lines are available for these studies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CB05105-02 I
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.) Specificity of Human Cytotoxic Effector Cells Generated by Stimulation with ConA		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and Institute affiliation) Gene M. Shearer, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any) Surgery Branch, NCI		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.1	PROFESSIONAL: 0.0	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Peripheral blood leukocytes (PBL) from normal donors stimulated with Concanavalin A (Con A) generate cytotoxic effector cells (EC) which lyse allogeneic PBL from sarcoma patients but not PBL from normal donors. These EC also lyse allogeneic Epstein-Bar virus (EBV)-transformed cell lines, but not T cells from the same donors. They also lyse Daudi cells, which do not express Class I but do express Class II MHC antigens. These findings raise the possibility that Con A activated EC are detecting unique antigens expressed by virus-transformed cells and found in cancer patients but not normal leukocytes. These antigens could be modified Class II MHC antigens. Note: Due to recent emphasis on AIDS-related research, there has been no progress on this project during the past year. However, note Proposed Course of Project for AIDS-related work in future.		

Project Description

Other Professional Personnel Engaged on the Project:

S. Payne, Technician, Immunology Branch, NCI
S. Rosenberg, Branch Chief, Surgery Branch, NCI

Objectives: The purpose of this study is to determine whether human PBL can be stimulated with mitogens such as Con A to generate cytotoxic effector cells (as has been reported in the murine system), and if so, to determine whether these EC recognize a particular class of foreign and/or self antigens.

Methods Employed: Human PBL from normal volunteers were stimulated with different concentrations of Con A (0.1 - 10.0 ug/ml) for 1-5 days and the effectors generated were assayed by ⁵¹Cr-release assay on a panel of targets, including self and allogeneic T cell blasts, EBV-transformed cell lines, PBL from allogeneic sarcoma patients, and the Daudi line (which does not express HLA Class I, but does express Class II antigens).

Major Findings: Con A-stimulated EC lysed PBL from allogeneic sarcoma patients, EBV-transformed allogenic cells and the Daudi cell line. These EC, however did not lyse self or allogeneic T cell blasts, nor self EBV-transformed lines.

Significance to Biomedical Research and the Program of the Institute: The selective lysis of viral-transformed targets and PBL targets from tumor patients by Con A-stimulated EC raises the possibility that these EC recognize tumor antigens or viral antigens. Alternative, these target cells may express a particular class of HLA antigens (Class II), which could be relevant for recognition of tumor antigens.

Proposed Course of Project: Experiments will be performed using monoclonal antibodies and a variety of target cells to determine whether the antigens recognized by the Con A EC are Class II (D/DR antigens), and to understand why these EC does not appear to recognized Class I antigens (HLA,-A,-B,-C), which are the usual HLA antigens recognized by cytotoxic EC. Studies will be undertaken to establish whether these EC have any potential relevance in tumor cell recognition.

Since these Con A-stimulated EC lysed PBL from HLA-mismatched sarcoma patients, it may be that they will also lyse PBL targets from AIDS patients or from donors who are at high risk for AIDS. We plan to test this possibility, which may have predictive value for diagnosing AIDS patients.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 201CB05106-02 I
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.) Analysis of the T Cell Alloreactive Repertoire		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Richard J. Hodes Chief, Immunotherapy Section, Immunology Branch, NCI		
COOPERATING UNITS (if any) Surgery Branch, NCI		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:	PROFESSIONAL: 0.5	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The alloreactive T cell repertoire has been analyzed for responses to two categories of alloantigens: mutant K^b determinants and non MHC-encoded Mls^c antigens. It was demonstrated by limiting dilution techniques and slope analysis that proliferating F₁ T cell populations contain distinct subsets capable of recognizing Mls^c encoded determinants in the context of parental MHC determinants. These findings demonstrate that MHC Mls^c determinants are recognized by responding T cells in association with MHC encoded determinants. T cell clones specific for Mls determinants have been generated, and the MHC restriction of recognition by these clones is being evaluated. Responses to K^b mutant determinants were evaluated employing radiation bone marrow chimeras, neonatal tolerization, and cold target inhibition in assays of cell mediated lympholysis (CML). The results of such studies demonstrated that the generation of the T cell repertoire to these mutant MHC determinants was not the result of T cell genotype alone or of maturation environment alone, but rather represented the outcome of unique interactions between these two variables. This T cell repertoire appears to reflect, at least in part, the activation requirements of cytotoxic T cell precursors, and to be regulated by genes in the K region of the MHC.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

Ronald Gress, Investigator, Immunology Branch, NCI
David Lynch, Investigator, Immunology Branch, NCI

Objectives: T cell responses to Mls or MHC encoded alloantigens appear to be unique in the strength of these primary responses and in the correspondingly high precursor frequency of responding T lymphocytes. For both these conceptual reasons, and because of the extreme importance of these loci in transplantation biology, a more complete understanding of response mechanisms to these determinants is both interesting and important.

Methods Employed: Responses to Mls^c determinants were assayed by proliferation in mixed leukocyte response (MLR) cultures. Conditions were established in which stimulating cells were in demonstrable excess, such that response magnitude was proportional to the number of responding T cells in culture. The slope of response, calculated as the ratio of (cpm): (responding cell number) as established by linear regression analysis represented an index of the number of responding T cells capable of recognizing a given stimulating cell population. T cell clones were generated by in vitro culture and limiting dilution techniques, employing stimulator populations expressing Mls^a determinants.

Studies of responses to the mutant K^b products employed a series of mutants including K^{bm1} and the K^{bm6}. Responses to these determinants were studied in assays of CML in which responding spleen cells were cultured with irradiated stimulators for 5 days, and specific cytotoxicity assayed at the end of this period by ⁵¹Cr release. Responding populations included normal spleen cell populations, radiation bone marrow chimeric spleen cell populations, and spleen cells from neonatally tolerized animals. Neonatal tolerization was performed by the intravenous injection of parental type spleen cells into newborn F₁ mice. Cold target inhibition studies were carried out by the addition of unlabelled target cells to cultures of cytolytic effector T cells and ⁵¹Cr labelled targets.

Major Findings: Responses to Mls^c determinants were studied in F₁ responding T cell populations which were homozygous for non-H-2 background but were MHC heterozygous. Stimulating cells were selected which were of one or the other parental MHC type but which presented alloantigenic Mls^c encoded determinants. Responses to mixtures of stimulating cells which presented Mls^c in association with both parental MHC haplotypes generated responses which were approximately additive of those responses to Mls^c in the context of only one parental MHC type. The implication of these findings is that responding F₁ T cell populations are composed of distinct subpopulations capable of responding to identical Mls^c products in the context of specific MHC encoded determinants; and therefore, that the recognition of Mls^c determinants by T lymphocytes is MHC-restricted. Cloned T cells have recently been generated which are specific for Mls^a determinants, and studies are currently in progress to determine whether such clones are MHC restricted in their recognition of Mls^a.

Wild type B6 T cells generate strong CML responses to the B6 mutant strains bml and bm6. In addition, the H-2^d strain B10.D2 also can respond to these mutant determinants, as demonstrated by cold target inhibition studies and by responses of B10.D2 mice neonatally tolerized to H-2^b B10.D2 --> B10 chimeric cells were similarly able to respond to bml and bm6 determinants. In contrast, however, B10 --> B10.D2 chimeric T cells had a markedly reduced response to bml and were entirely unresponsive to bm6, in spite of their normal alloreactivity to third party B10.BR. The same selective unresponsiveness to bm6 was observed in T cells from B6 --> bml chimeras, indicating that K region differences in maturation environment were sufficient to cause this effect. These findings demonstrate that the alloreactive T cell repertoire to these mutant K region determinants is not the product of T cell genotype alone (since either H-2^b or H-2^d T cells can respond) or of the T cell maturation environment alone (since T cells which have matured in either an H-2^b or H-2^d environment can be responsive). Rather, the alloreactive T cell repertoire appears to be the unique outcome of interaction between T cell genotype and maturation environment, paralleling the phenomenon previously demonstrated for MHC-restricted T cell recognition of conventional antigens. Cell mixing experiments indicated that the restricted repertoire observed in these chimeric populations is a property of the cytotoxic T cell precursor population.

Implications: It remains a central question whether the mechanisms of immune repertoire generation are identical for conventional antigens and for MHC encoded antigens. In this respect, the results of the current studies have demonstrated that in several respects the strong T cell responses to both Mls^c and mutant K^b determinants parallel response mechanisms studied for MHC-restricted responses to conventional antigens. The most immediately apparent implications of such findings include their application to situations such as clinical bone marrow transplantation, in which complex influences can be anticipated upon the ultimate host response repertoire to transplantation as well as conventional antigenic challenge.

Future Plans: Results to date have demonstrated the interaction of T cell genotype and host environment in the generation of the alloreactive repertoire to mutant K^b determinants. Future studies will be directed toward an analysis of the precise mechanism by which such influences are exerted. In particular, the function of both helper T cells and of cytolytic T cell precursors will be independently analyzed.

In collaboration with Dr. S. Rosenberg of the Surgery Branch of the NCI, studies are in progress to define the effect of Mls-specific T cell clones in skin graft rejection, and the role of MHC restriction in this function.

Publications:

Gress, R. E. and Hodes, R. J.: 1982. Generation of the alloreactive T cell repertoire: Interaction of T cell genotype and maturation environment. Proceedings of the National Academy of Science 79: 4728-4732.

Publications

Gress, R. E., Wesley, M. and Hodes, R. J.: The role of H-2 in T cell recognition. J. Immunol. 127: 1763-1766, 1981.

Gress, R. E. and Hodes, R. J.: Generation of the alloreactive T cell repertoire: Interaction of T cell genotype and maturation environment. Proceedings of the National Academy of Science, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05107-02 I
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 Cell Responses to Minor Histocompatibility Antigens		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Eric C. Groves, Medical Staff Fellow, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.1	PROFESSIONAL: 1.1	OTHER:
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The ability to generate cytotoxic T lymphocyte responses to minor H antigens offers a potent tool for the study of self-tolerance and self-recognition. Results obtained in this system have thus far demonstrated 1) that the self + X T cell repertoire is highly cross-reactive for allogeneic MHC determinants suggesting that the response to allogeneic MHC antigens is comprised of multiple self + X specificities and 2) that self minor H determinants tolerize T cells only in association with self MHC determinants so that tolerance induction to non MHC self components is restricted by MHC encoded products. Thus, these results demonstrate that the induction of T cell tolerance to self components is genetically restricted by MHC gene products.		

Project Description

Other Professional Personnel Engaged on the Project:

A. Singer, Senior Investigator, Immunology Branch, NCI

Objective: These experiments will study the mechanism of the development of tolerance and reactivity to minor histocompatibility antigens.

Methods Employed: Normal and chimeric mice are primed with spleen cells which express allogeneic minor H antigens. After 2.5 weeks, recipient thymus or spleen cells are restimulated in vitro and the generation of cytotoxic T cells specific for allogeneic minor H antigens is assessed.

Major Findings: Cytotoxic T lymphocyte responses to minor H antigens were elicited from both the spleen and thymus of primed mice. These responses were antigen specific and H-2 restricted. However, these responses were highly cross-reactive for allogeneic MHC determinants, demonstrating that the T cell repertoire for allogeneic MHC determinants is, to a large extent, composed of anti-Self + X specificities.

The MHC dependence of T cell tolerance induction to minor H antigens was investigated using parent \rightarrow F₁ radiation bone marrow chimeras of the form C3H.SW \rightarrow (B10xBR)F₁. In these chimeras, the donor and host differ by the minor H antigens associated with the C3H and Black strains, share the H-2^b MHC and the host bears additionally the H-2^k MHC. These chimeras were found to be specifically tolerant to the minor C3H antigens of the donor in the MHC context of donor H-2^b determinants. However the chimera was reactive to the donor minor H antigen in the context of the non-donor MHC (i.e., they were reactive to C3H [H-2^k]). The MHC-minor H antigen combination H-2^k and C3H minor, do not occur in this chimera although both H-2^k and C3H minor do appear on the animal cells. Hence the tolerance pattern observed in this animal demonstrates that T cells are not tolerant to self minor H antigens alone but are only tolerant to these antigens in the MHC context in which they appear in the animal.

Significance to Biomedical Research and the Program of the Institute: Insights into the mechanism of self-tolerance are important to our understanding of the function of the immune system. As the underlying principles become clarified, it is anticipated that they will have significant impact on human transplantation and the immunological approach to cancer treatment.

Proposed Course of the Project: The spectrum of antigens demonstrating H-2 tolerance induction will be investigated and chimeric mice bearing one or more transplanted thymuses will be used to define the point in T cell ontogeny at which tolerance induction occurs.

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

PROJECT NUMBER

Z01CB05108-01 I

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 Cell Regulation of B Cell Activation

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

(Name, title, laboratory, and institute affiliation) Yoshihiro Assano, Visiting Associate,
Immunotherapy Section, Immunology Branch, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

Immunotherapy Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.1

PROFESSIONAL:

1.1

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

A series of experiments were carried out to investigate the role and function of regulatory T cells in modulating the activation of B cells to antibody production. It was first demonstrated that high concentrations of free carrier inhibit the MHC-restricted activation of B cell responses. This inhibition was shown to be mediated by regulatory T cells functioning through two distinct pathways distinguishable by the involvement of different Lyt-defined T cell subpopulations. Both pathways were MHC-restricted and antigen-specific in their activation requirements. An Lyt 1+2- population functioned through an antigen non-specific effector pathway requiring the participation of an Lyt 1-2+ unprimed T cell. An Lyt 1-2+ T cell functioned through an antigen-specific and MHC restricted effector pathway without requirement for participation of additional T cells. Monoclonal T cell suppressor populations have recently been derived and are currently being evaluated.

Project Description

Other Professional Personnel Engaged on the Project:

Richard J. Hodes, Chief, Immunotherapy Section, Immunology Branch, NCI

Objectives: This project is designed to evaluate the existence and mechanisms of regulatory T cell networks controlling B cell activation and antibody response.

Methods Employed: The response systems studied is a T cell and accessory cell dependent secondary antibody response in vitro. Suppressor populations are generated by in vivo carrier priming of mice, followed by isolation of T cells by B cell and accessory cell depletion employing respectively treatment with a cytotoxic anti T cell reagent and passage over G-10 Sephadex columns. In vitro activation of suppressor cell activity is accomplished by culturing of T cells with accessory cells (T cell depleted irradiated spleen cells) and appropriate antigen. Following such activation, putative suppressor populations are assayed for their ability to affect the response of carrier primed T cells and hapten primed B cells to appropriate carrier-hapten soluble conjugates in vitro. Evaluation of T cell subpopulation requirements was accomplished by negative selection employing treatment with monoclonal anti-Lyt 1 or anti-Lyt 2 reagents plus complement. Assays of antigen-specific plaque forming cells were carried out.

The generation of monoclonal T cell populations was accomplished through repeated in vitro stimulation of in vivo primed T cells in the presence of antigen presenting cells, antigen, and a source of T cell growth factor. Cloning was accomplished through limiting dilution techniques, and cloned cells propagated through repeated in vitro stimulation.

Major Findings: The optimal response of carrier primed T cells and hapten primed B cells to carrier-hapten conjugates was found to be profoundly inhibited by the addition of high doses of free carrier. It was demonstrated that this inhibition is mediated through the activation of suppressor (T_S) cells by high concentrations of carrier. The activation of suppressor cells requires in vivo priming, followed by in vitro re-exposure of T cells to the specific priming antigen in the presence of appropriate antigen presenting cells. Two apparently distinct suppressor pathways are activated by this procedure. $Lyt\ 1^{+}2^{-}$ T cells are activated in vitro to generate $Lyt\ 1^{+}2^{-}$ T_S cells. Once activated, these cells are capable of suppressing responses in an antigen non-specific manner, and require for their activity the presence of an unprimed $Lyt\ 1^{-}2^{+}$ population. In a second pathway, $Lyt\ 1^{-}2^{+}$ T cells from carrier primed animals are activated in vitro by specific carrier to generate $Lyt\ 1^{-}2^{+}$ T suppressor cells which then function in an antigen-specific effector pathway which does not appear to require the participation of additional T cell subpopulations.

The activation of both suppressor pathways identified above requires an MHC-restricted interaction between T_S precursors and accessory cells. The MHC restricted repertoire of T_S cells is influenced both by the maturation environment of these T cells and the environment in which they were primed.

Both suppressor populations are MHC-restricted in their effector mechanisms as well. The unique finding of these studies was that the suppressor populations are capable of suppressing only those antibody responses in which the functioning T helper cells expressed both the same antigen specificity and the same MHC restriction specificity as the suppressor cells regulating these responses.

Through in vitro stimulation and limiting dilution, cloned populations of both helper and suppressor T cells have been generated. Cloned suppressor cells have been shown to function in an antigen specific and MHC restricted fashion, suppressing responses mediated both by heterogeneous T cell populations and by monoclonal T helper populations. Further studies are currently in progress to analyze the mechanism of suppression by these cloned populations.

Significance to Biomedical Research and the Program of the Institute: It has become increasingly clear that the immune response to both experimental antigens and to naturally occurring antigens of infectious agents or of tumors is determined in large part by the regulatory influences of helper and suppressor T cell populations. It has been possible through the studies outlined above to analyze the regulatory effects on antibody responses in vitro. This has in turn permitted the analysis of T cell subpopulation interactions, and most recently the identification of monoclonal regulatory cells. It is anticipated that through the further study of such populations, cellular as well as molecular analysis of immune response regulation can be accomplished. It is further intended that such principles will be applied to models of immune response to tumor antigens.

Proposed Course of Project: Studies are now in progress analyzing the mechanism of suppression mediated by cloned T suppressor populations. In addition, an analysis is being made of soluble factors produced by both conventional and cloned regulatory cells in an attempt to define at a molecular level the active regulatory principles.

Publications:

Asano, Y. and Hodes, R. J.: 1982. T cell regulation of B cell activation. T cells independently regulate the responses mediated by distinct B cell subpopulations. J. Exp. Med. 155: 1267-1276.

Asano, Y. and Hodes, R. J.: 1983. T cell regulation of B cell activation: Antigen-specific and antigen-nonspecific suppressor pathways are mediated by distinct T cell subpopulations. J. Immunol. 130: 1061-1065.

Asano, Y. and Hodes, R. J.: T cell regulation of B cell activation. I-A restricted T suppressor cells inhibit the major histocompatibility complex-restricted interactions of T helper cells with B cells and accessory cells. J. Exp. Med. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 201CB05109-01 I
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.) Cyclophosphamide Effects on Murine T Cell Responses		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation)		
Gene M. Shearer, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.2	OTHER: 0.2
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p> F₁ mice undergoing GVH-associated immunosuppression as a result of parental T cell inoculation were treated with cyclophosphamide (Cy) at the time of parental cell inoculation. Such treatment prevented the development of immunosuppression. Furthermore, mice injected 37 days earlier with parental spleen cells were "rescued" in that their immune response potential was restored by injection of Cy. Inbred strains of mice injected with spleen cells from allogeneic strains and Cy exhibited antigen-specific reduced response potential to the H-2 antigens expressed by the strain used for injection. </p>		

Project Description

Other Professional Personnel Engaged on the Project:

R. Levy, Senior Staff Fellow, Immunology Branch, NCI

M. Miller, Technician, Immunology Branch, NCI

Objectives: The purpose of this project is two-fold: The first is to determine whether mice injected with allogeneic or parental cells under conditions that induce immunosuppression can be prevented from being suppressed, and/or can be "rescued from suppression" by injection of Cy. The second purpose is to determine whether in vivo negative selection for allogeneic or modified self antigens can be achieved by injection of Cy and cells expressing foreign antigens.

Methods Employed: F₁ mice were injected intravenously with parental spleen cells, and intraperitoneally with Cy at various time intervals and different doses. The immune response potential of the treated mice was tested by in vitro MLR and CTL testing at various times after treatment.

Negative selection experiments were performed by injecting allogeneic or hapten-modified cells intravenously or intraperitoneally and by injecting Cy at various doses and time intervals after cell inoculation. Multiple injections were also performed.

Major Findings: Two major findings have been made thus far:

- 1) F₁ hybrid mice injected with parental spleen cells do not develop immunosuppression if they are injected with Cy at the same time that the parental cells are inoculated. Cy treatment was effective if administered to recipient for up to three days after parental cell injection, but it was not effective if given before parental cell inoculation nor when administered between days four and 16 after suppression induction. However, we were able to rescue parental suppressed F₁ mice when the Cy was given 37 days after suppression induction.
- 2) The injection of inbred mice with H-2 allogeneic spleen cells and Cy resulted in drastically reduced CTL response potential of spleen cells from the treated mice. This reduced responsiveness appeared to be specific for the alloantigens expressed by the strain used for inoculation. The maintenance of reduced responsiveness appeared to be time-dependent, and initial studies indicated that a state of prolonged unresponsiveness required additional injections of Cy.

Significance to Biomedical Research and the Program of the Institute: The prevention and/or rescue of mice from GVH-associated immunosuppression by chemotherapeutic manipulation has as potential relevance for similar protocols in the treatment of humans undergoing GVH reactions. If allogeneic leukocytes contribute to a state of immunosuppression leading to AIDS, then these murine studies may provide a basis for design of human protocols that could be therapeutic for this syndrome. If negative selection by antigen exposure combined with Cy treatment is successful in depleting or suppressing specific immune responses, similar approaches could be used to selectively reduce immunity in foreign tissue transplantation.

Proposed Course of Project: Studies will continue to establish the optimal antigen and Cy doses, as well as optimal time intervals for loss of immunity. More extensive studies will be performed to elucidate antigen specificity of loss of immune reactivity. Experiments will be performed to determine whether loss of immune potential is due to negative-selection or active suppression or both.

More experiments will be performed to determine whether GVH immunosuppressed mice can be "rescued" by Cy, and if successful, optimal conditions for such restoration of immune potential will be established.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05110-01 I
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.) Immune Studies in Homosexual Men at Risk for Acquired Immune Deficiency Syndrome		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Gene M. Shearer, Senior Investigator, Immunology Branch, NCI		
COOPERATING UNITS (if any) Susan Zolla-Pazner, Ph.D., New York University, New York, New York Kenneth Tung, M.D., University of New Mexico, Albuquerque, New Mexico Allan Goldstein, Ph.D., George Washington University, Washington, DC		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.1	PROFESSIONAL: 1.1	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Peripheral blood leukocytes (PBL) were drawn from a number of age matched heterosexual and homosexual men from the Washington, DC area, New York City and Albuquerque, New Mexico. The PBL were sensitized in vitro to influenza virus and to HLA alloantigens. These sensitized cultures were tested for proliferation and for the generation of cytotoxic T lymphocytes specific for influenza virus and alloantigens. Assays were also run for OKT4:OKT8 ratios (i.e., helper:suppressor cell), for thymosin α 1, and for interferon in sera as well as for interferon production in culture in the presence of influenza virus. In the Washington group, approximately 50% of anti-influenza CTL responses were reduced without any detectable loss in reactivity to HLA alloantigens. Abnormalities were also detected in interferon in this group, although all of these donors exhibited normal OKT4:OKT8 and thymosin α 1 levels. A higher proportion of suppressed individuals was observed in the New York group and some of the Albuquerque group was also suppressed. Heterosexuals from all three cities generated CTL responses within the normal range.		

Project Description

Other Professional Personnel Engaged on the Project:

S. Payne, Technician, Immunology Branch, NCI
W. Biddison, Senior Investigator, NINDS
S. Jacobson, Postdoctoral Fellow, NINDS

Objectives: The purpose of this project is to investigate various immune parameters of individuals at high risk for AIDS. The immune parameters of individuals will be followed for at least two years to determine whether any of them develop AIDS symptoms. It is also the objective of this project to attempt to identify immunological abnormalities that may be predictive for AIDS development.

Methods Employed: Peripheral blood leukocytes were separated on Ficoll-Hypaque and sensitized in vitro to influenza self and HLA alloantigens. Proliferative and cytotoxic T lymphocyte (CTL) assays were run under optimal conditions. Suboptimal sensitization conditions were also tested by sensitizing with lower concentrations of influenza virus. OKT3, OKT4 and OKT8 analyses were run on the PBL using flow microfluorometry. Sera were tested for interferon, thymosin α 1 and for antibodies that inhibit mitogenic responses of PBL from heterosexuals. PBL from donors were also cultured for the generation of interferon and possibly suppressive factors in vitro. HLA typing was performed on contact.

Major Findings: CTL responses to influenza were consistently strong in all heterosexual controls. However, these responses were much reduced 4-to-40-fold in approximately 30% of the Washington homosexual group. This group also exhibited elevated serum interferon levels, but reduced potential for producing interferon in culture in the presence of virus. All heterosexuals and homosexuals had normal helper:suppressor T cell ratios. A similar proportion of homosexuals from the New York group exhibited reduced CTL response potential to influenza, and many of these had helper:suppressor T cell ratio reversals. Of the few homosexuals studied from Albuquerque (an area where AIDS has not yet been diagnosed), approximately 40% exhibited reduced responses to influenza. All donors exhibited either normal or elevated CTL responses to HLA alloantigens. Helper:suppressor T cell ratios and other parameters have not yet been evaluated in this latter group. It should be noted that some of the immunosuppressed Washington donors have been suppressed for at least six months in repetitive bleeds and tests.

Significance to Biomedical Research and the Program of the Institute:

Understanding the etiology of AIDS is a high priority of the biomedical research community and particularly of the NCI. The evaluation of immune parameters of high risk groups may be predictive for AIDS development and may elucidate the mechanism(s) of suppression leading AIDS and genetic factors of susceptibility.

Proposed Course of Project: The donors from all three locations will be followed for a period of up to two years with immune evaluation every three months. Individuals whose immune status appears to be of interest will be

studied more frequently and more extensively. PBL from individuals who may be chimeric (one donor has been identified who may be in this category) will be expanded and cloned. HLA typing will be performed to determine whether abherent haplotypes are present. Immune potential of the cloned cells will be evaluated. Attempts will be made to reconstitute immune function in vitro with biologically active derivatives of cyclophosphamide.

Publications:

Shearer, G. M.: 1983. Allogeneic leukocytes as a possible factor in induction of AIDS in homosexual men. N. Engl. J. Med. 308: 223-224.

Shearer, G. M.: Immunosuppression and recognition of class II (Ia) antigens: A possible factor in the etiology of acquired immune deficiency syndrome. Proc. New York Univ. Symp. on Epidermic Kaposi's Sarcoma and Opportunistic Infections in Homosexual Men. A. Friedman-Kien (ed) Masson Publish., Inc. N.Y., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 201CB05111-01 I
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.) Generation of Allospecific CTL		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Hana Golding, Visiting Fellow, Immunology Branch, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Immunology Branch		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The role performed by macrophages during activation of alloreactive cytotoxic T lymphocytes was investigated. Using an experimental approach in which both stimulator and responding populations were depleted of accessory cells, no cytotoxic activity could be induced across H-2 differences. Reconstitution of the response could be achieved using accessory cells of either stimulator or responder origin, but the mechanisms of activation differed fundamentally depending on the H-2 type of macrophages used. Using the lysosomal disruptive drug chloroquine it was found that activation via responder macrophages required antigen processing. In addition this activation pathway was extremely sensitive to blocking by monoclonal anti-Ia antibodies. Reconstitution by stimulator macrophages was both chloroquine insensitive and completely resistant to blocking by anti Ia mAb. We have thus identified two CTL activation pathways (Ia-dependent vs Ia-independent) and demonstrated that macrophages play central yet different roles in initiating these alternative pathways.</p>		

Project Description

Other Professional Personnel Engaged on the Project:

A. Singer, Senior Investigator, Immunology Branch, NCI
T. Muziochi, Visiting Fellow, Immunology Branch, NCI

Objectives: The major objective of this project is to identify the regulatory cell interactions involved in the generation of allospecific CTL.

Methods Employed: Spleen cells from H-2 disparate congenic mouse strains were cultured for 5 days in a 24-well tissue culture plate. Macrophages were removed by passage of responder and stimulator populations over Sephadex G-10 columns. Accessory cells for reconstitution were obtained from spleen cells treated with rabbit anti mouse brain (RoMB) and C' to remove T cells and irradiated with 2000R. Macrophages of stimulator or responder origin were treated with chloroquine (30 μ M:3 μ M) for 30 min at 37°, washed extensively, irradiated (2000R) and were then added to culture together with G10-passed stimulator and responder cell populations. This treatment interferes with processing by macrophages. Lymphocytes were suspended in Hanks balanced Salt solution containing 0.1-0.5% freshly prepared paraformaldehyde and were incubated for 20 min at room temperature. Fixation was stopped by addition of cold HBSS-10% FCS. Cells were washed twice and irradiated (2000R) and then added to cultures as stimulator cells.

Major Findings: Treatment of stimulator macrophages with chloroquine had only minimal effect on their ability to reconstitute T CTL induction in cultures of G10-passed cell population. In contrast, treatment of responder macrophages with as little as 3 μ M of chloroquine completely abrogated their ability to reconstitute the response. These findings strongly indicated that antigen processing is an essential step in the Ia-dependent pathway which is initiated by responder macrophages.

Fixation of stimulator cells with paraformaldehyde prevented reconstitution of T CTL responses by responder macrophages but had no effect on the ability of stimulator macrophages to reconstitute the response. The same fixed stimulator cells were capable of triggering CTL responses if exogenous TCGF (12.5%) was added to culture.

Taken together, these experiments support the hypothesis that activation of precursor CTL may proceed via two alternative pathways and involving different mechanisms for activation of helper T cells. If syngeneic macrophages are the sole accessory cells in culture, activation of T_H cells involved reprocessing of Shed Class I antigens from the stimulator populations and their representation in the context of self-Ia. In the alternative pathway (Ia-independent) no processing is required since the reconstituting accessory cells bear the allo-class I determinants which provide the antigenic stimulus to both the T_H and precursor CTLs. It is important to emphasize that the T_H cells which play a role in this pathway have the unique property of being non Ia restricted.

Significance to Biomedical Research and the Program of the Institute:

Understanding the regulatory mechanism involved in the generation of allo-specific CTL is necessary to devise modalities for increasing CTL activity in carcinogenesis and decreasing it during graft transplantation.

Proposed Course of Project: Our immediate goal is to characterize the T_H cells population involved in these two cell interaction pathways as to their repertoire, surface phenotype, and frequencies.

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

PROJECT NUMBER

Z01CB05112-01 I

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.)

Analysis of Recognition Structures on T and B Cells

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

(Name, title, laboratory, and institute affiliation)

Jeffrey A. Bluestone, Lab Head, Transp. Biology Section, Immunol. Br., NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

Transplantation Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

B

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

The recognition structures of both B cells and T cells have been examined using anti-receptor antibodies prepared against monoclonal anti-H-2 antibodies (mAb) and cytotoxic T cell (CTL) clones. Anti-idiotypic antibodies (anti-Id) were prepared against several monoclonal anti-H-2 antibodies. In one case, anti-idiotypic antibodies prepared against an anti-H-2K^b mAb detected a public idio type expressed on a majority of anti-H-2K^b alloantibodies. In contrast, no reactivity could be detected between these anti-idiotypes and a series of cytotoxic T cell clones of a similar specificity. The results suggested that either the recognition structures of T cells and B cells are substantially different or the allo-determinants recognized by these cells are not the same. Current efforts have been devoted to examining the nature of the allo-determinants recognized by cloned T cell populations as compared to those determinants recognized by alloantibodies. In addition, anti-receptor antibodies specific for the CTL clones have been produced and attempts are being made to generate mAbs that recognize the T cell receptor.

Project Description

Other Professional Personnel Engaged on the Project:

David H. Sachs, Chief, Transplantation Biology Section, Immunology Branch, NCI

Objectives: T cell responses to alloantigens encoded by the major histocompatibility complex (MHC) are extremely important in the immune responses associated with organ transplantation. Anti-receptor antibodies which react with alloreactive T cells could be useful in examining the recognition structures for alloantigens and might be employed in vivo to modify transplantation reactions. In addition monoclonal CTL populations would be useful for a more complete understanding of the activation requirements of T cells, the fine specificity of antigen recognition and the relationship between the recognition structures of T and B cells.

Methods Employed: Anti-idiotypic antibodies have been generated against several anti-MHC monoclonal antibodies directed at class I antigens. Xenogeneic anti-idiotypic antibodies were produced in miniature swine and rabbits immunized with affinity purified antibodies from hybridoma culture supernatants. Immune sera were extensively absorbed with myeloma immunoglobulin (Ig) or normal Ig-coupled to Sepharose beads to remove all anti-normal Ig activity. Anti-idiotypic antibodies were then absorbed to and eluted from idio-type-bearing Sepharose columns. In all systems studied, this absorption procedure has led to specific anti-idiotypic antibodies as evidenced by the loss of reactivity with normal Ig but retention of very high levels of binding to the specific immunogen.

Cross-reactive idiotypes were detected on other monoclonal antibodies and in alloantisera using two types of assays. The first assay, the Enzyme-linked immunosorbent (ELISA) inhibition assay, is a non-binding-site specific assay which examines the ability of test sera or mAb to inhibit the binding of anti-idiotypic to the original idiotypic antibody. The other assay used in these studies is an antigen-binding inhibition assay which requires that the anti-Id antibodies bind idiotopes within the combining site in order to inhibit the binding of anti-MHC antibodies to MHC antigen-bearing cells. The binding of the anti-MHC antibodies is then assessed by flow microfluorometry. Crossreactive idiotypes were examined on cloned CTL populations by direct binding of anti-idiotypic to the T cell clones and inhibition of cytolytic T cell activity by anti-idiotypic.

Cloned CTL were isolated from alloreactive or modified-self-specific mixed lymphocyte cultures. CTL were cloned by limiting dilution at 1 cell/well in the presence of stimulator cells and 10% T cell growth factor (IL-2), screened for cytolytic activity in a 4 hour ⁵¹Cr Release assay and subcloned at 0.3 cells per well. Fine specificity of the cloned CTL was examined using a panel of MHC congenic mouse strains and in some cases MHC mutant mice.

Major Findings: Although we were able to generate anti-idiotypic antibodies against monoclonal anti-MHC antibodies it was unclear whether these reagents, directed to only a limited number of possible receptors, would detect determinants expressed by a significant portion of anti-MHC antibodies or

alloreactive T cells of similar specificities. We therefore examined the expression of idiotypes in alloantisera, on a large panel of hybridoma anti-MHC antibodies and alloreactive T cell populations. Our results have shown that the prevalence of Id among antibodies in alloantisera varies depending on the idiotype system studied. Predominant idiotypes were detected in two idiotype systems; one an anti-H-2K^b mAb (28-13-3); the other, an anti-H-2L^d mAb (23-10-1). The 28-13-3 Id was expressed in all C3H mice immunized with H-2K^b tissue in an allotype-linked manner. In the 23-10-1 Id system, xenogeneic anti-Id inhibited the binding of anti-L^d antibodies produced by BALB/c-H-2^{dm2} mice by hyper immunization with BALB/c tissue. The predominant Id was present on both IgG and IgM antibodies representing approximately one-quarter to one-half of the total anti-L^d humoral response.

These two anti-idiotypes, which detected a public idotype in alloantisera, were also screened on a series of cytotoxic T cell clones generated from similar strain combinations. In no case did anti-idiotypes against either the public H-2K^b or H-2L^d mAb react with CTL of similar specificity. In fact, in screening well over 100 CTL clones no binding or inhibition of cytolytic activity was detected. The implication of these findings is that the predominant idotype expressed on alloantibodies of a given specificity is not shared among cloned CTL populations. However, it remains possible that cytotoxic T cell clones recognize different antigenic determinants than the monoclonal antibodies and therefore might be expected to use a different receptor structure. Current efforts are being directed at addressing this question by examining CTL clone activity on in vitro generated H-2 mutant cell lines which have lost the 23-10-1 or 28-13-3 serological determinants. Preliminary results suggest that an overwhelming majority of the CTL detect different determinants than the mAbs.

More recently, alloreactive CTL clones have been used to immunize mice to generate antibodies against the T cell receptor molecules. Antibodies have been identified in the sera of hyperimmunized mice which react specifically with the immunizing clone and not other CTL clones of similar specificity.

Implications: A central problem in Transplantation Biology is the identification of specific methods to alter immune responses to transplantation antigens and thus effect allograft rejection. Since T cells are thought to be responsible for these immune reactions, reagents must be employed which affect the relevant T cell populations. One approach, to this problem has been the use of anti-idiotypic antibodies against monoclonal anti-MHC antibodies to modify T cell responses to alloantigens. To date this approach has been unsuccessful perhaps since T cells and B cells do not use homologous receptors and may detect very different determinants on the target tissue.

Future Plans: Future studies will be designed to examine the precise nature of the antigenic determinants recognized by the alloreactive cloned CTL. In addition, newly derived monoclonal anti-T cell antibodies will be examined to determine if any of these antibodies detect the T cell receptor and might be useful in modifying transplantation responses.

Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1982 to September 30, 1983

INTRODUCTION

The Laboratory of Immunobiology has undergone several changes during the past year. The major event affecting personnel and operations was the move to the Frederick Cancer Research Facility. The move and renovations caused an estimated loss of working time of about 2 months; one senior person left the Laboratory (and NIH), his leaving representing a major loss to the program of the Immunopathology Section. Coordination and close cooperation among members of the three sections continued to be encouraged; advantage is being taken of the new environment in that cooperative projects with FCRF members have been initiated by members of all three sections. In completing consolidation and tightening of the program of the Laboratory all scientific activities formerly in the Office of the Chief were transferred to the appropriate Sections or were phased out.

Studies in the Humoral Immunity Section centered on elucidating cytotoxic mechanisms of humoral immune factors and the analysis of behavior of cells under immune attack. Investigations continued into the nature of the initial steps in the sequence of activation of complement (C) at cell surfaces. Fundamental questions being asked are: how do immunoglobulin (Ig) molecules acquire the capability of interaction with the first component of complement (C1); is interaction invariably followed by activation and finally, is activation followed by cytotoxic activity. From studies conducted in this section during the past few years it has become clear that at cell surfaces the acquisition of complement fixing property by Igs is a function of density and distribution of antigen epitopes on the cell. While it was obvious that a minimal number of epitopes must be present to bind sufficient numbers of Igs for C fixation to occur, it was not clear what effect changes in density of epitopes would have on C fixation by Igs when there were large numbers of epitopes on the surface. Results of studies with cells with large numbers of epitopes/cell (average distance between epitopes 5-20 nm) demonstrated that under conditions when density had no effect on the ability of Igs to fix complement (i.e., the same amount of C1 was fixed by a given amount of Ig regardless of epitope density) activation of C1 was directly dependent on density. These results clearly differentiated between binding (fixation) and activation of C1 by Igs. Based on these observations it was proposed that activating properties of Igs was governed by the angles of the Fab arms as determined by the average distances between epitope molecules. Activation of C1, however, was not always followed by the appearance of cytotoxic activity. Analysis of the blockage in the sequence leading to cytolysis confirmed old observations that the manner of binding C4 (the fourth component) was critical. In analyzing the relation between binding and chain structure of C4 it was discovered that binding of C4 to a cell surface made C4 totally resistant to the action of mercaptoethanol and partially resistant to the action of dithiothreitol. The latter compound destroyed C4 activity without removing the alpha' and beta chains but removed the gamma chain from about 70 % of C4 molecules. This observation implied that the gamma chain is important in the generation C3 convertase (a bimolecular enzyme generated from C4 and C2 by the action of C1). Additional complexity was introduced by the finding

that homologous C2 (i.e. C2 from the same species as C4) generated C3 convertase which was marginally effective with homologous C3. Studies on the structural, biochemical and immunological properties of C4 and C2 are in progress to better understand the molecular basis of generating C3 convertase, which is the key enzyme in the classical pathway of C activation.

In studies concerning the sensitivity of nucleated cells to immune attack, emphasis was placed on antibody dependent C-mediated killing of in vivo grown guinea pig hepatoma cells, L1 and L10. It was demonstrated with cells pretreated with selected metabolic inhibitors, hormones or x-ray that cellular lipid and/or fatty acid composition played a major role in influencing the sensitivity of the cells to C killing. Cells rendered sensitive to C killing had increased content of unsaturated fatty acids. No correlation between susceptibility and the synthesis of other cellular macromolecules was observed. When studies were expanded to include cells from human (Raji) and mouse (P815) it was found these cells removed at different stages of asynchronous cell growth in vitro varied in their sensitivity to C killing. The human lymphoid cells (Raji) were resistant to C killing at stationary phase of growth whereas the mouse mastocytoma cells (P815) were resistant at log phase of growth. Cells in S, G₁, and M phase of their cell cycle were equally susceptible to killing by C. Analysis of macromolecular synthesis and composition of the human and mouse cells supported observations made with the guinea pig hepatoma cells that sensitive cells contained increased amounts of selected unsaturated fatty acids. However, unlike the hepatoma cells no correlation between sensitivity to C attack and the synthesis and composition of cellular lipids was observed. In addition no correlation could be made with membrane fluidity and sensitivity to C killing.

In experiments similar to those with the hepatoma cells it was demonstrated that the sensitivity of Raji and P815 cells could be manipulated by pretreating the cells with selected metabolic inhibitors, hormones, or by changing cell number or replacing culture medium with fresh medium. With regard to the last two points, preliminary evidence indicated that changes from resistance to sensitivity occurred within a few hours of manipulation. All of the above studies have been performed using C as a probe to determine the properties of nucleated cells which influence their susceptibility to immune attack. In the past year studies have been initiated which include cellular mechanisms of immune attack (i.e. NK cells, CTL and ADCC). Preliminary experiments with P815 cells have indicated these cells vary in their sensitivity to CTL attack but are resistant to NK. Unlike the observations with antibody dependent C mediated cytotoxicity P815 cells were resistant to CTL in their stationary phase of growth. These observations suggest that resistance of tumor cells to immune attack is not restricted to humoral immune mechanisms.

The Immunopathology Section has continued to pursue two main projects, one on macrophage activation and the other on chemotaxis and inflammation. Studies on macrophage activation were on factors that activate mouse peritoneal macrophages for tumor cytotoxicity. The prototype material is found in antigen or mitogen stimulated murine spleen cell culture supernatants. Of great practical interest is the fact that culture fluids from a phorbol myristate acetate stimulated E1-4 thymoma cell line also activate macrophages to become non-specifically tumoricidal. This will provide virtually unlimited amounts of starting material for purification of the active principle. However, it is important to determine whether or not the spleen cell and cell line activities are the same. By four functional criteria, activity from the two sources was

comparable: (1) separation into priming and triggering steps; (2) kinetics of activation and decay; (3) responsiveness of exudate but not resident macrophages; and (4) the pattern of mouse strain responsiveness. New physicochemical studies on the spleen cell activity were made. They established measurable size heterogeneity of the active factor, but a single isoelectric point of pH 5.8 with no sign of charge heterogeneity. The number of criteria by which this lymphokine has now been characterized should suffice to distinguish it from other lymphokines evaluated by similar methods.

It was shown previously that human blood monocytes comprise 2 subpopulations: one migrates to the chemoattractant, fMet-Leu-Phe, and has saturable binding sites for this peptide; the other does not migrate, and exhibits little peptide binding. To determine if expression of binding sites was a function of monocyte maturation, human subjects were depleted of blood monocytes by leukapheresis so that the circulation was repopulated by monocytes released from the bone marrow. Pre- and postleukapheresis monocytes were then compared for fMet-Leu-[³H]Phe binding, superoxide generation and chemotactic responses. No significant differences in peptide binding curves were found, suggesting that receptor expression was stable over the maturational span represented by these two groups of cells. This supports the hypothesis that there are two distinct lineages of monocytes with respect to expression of receptors for fMet-Leu-Phe. An additional finding of interest was that the number of chemotactically responsive cells immediately post-leukapheresis was half the control. This was a transient state; monocyte responses were normal 3 hours after termination of leukapheresis, suggesting that they rapidly become functionally mature. Studies are in progress to determine if this apparent maturation process occurs in vitro.

Two developmental efforts were pursued, as a basis for future experimental work. One was on macrophage stimulating protein (MSP), a mammalian serum protein discovered by this laboratory that renders mouse peritoneal macrophages responsive to chemoattractants. An ELISA assay for human MSP was developed. This will be used as a screening assay for monoclonal anti-MSP. The objective is an assay for surveying serum MSP concentrations in search for MSP deficiency and for cellular studies to find the source of production. For future studies on basophil chemotaxis, a one step purification of human blood basophils on discontinuous Percoll gradients was developed; within an hour, suspensions with up to 50% basophils can be obtained by this method.

The Cellular Immunity Section has three major areas of investigation: evaluation of methods for augmenting host response to nonimmunogenic tumors, analysis of the basis of rejection of virus-infected tumor cells and development of a small animal model for plasma therapy of mammary cancer.

Most naturally-arising rodent tumors are nonimmunogenic. Three methods were evaluated for ability to alter host response to nonimmunogenic tumors: superinfection of tumor cells with murine retroviruses, selection of nontumorigenic variants after chemical mutagen treatment and alteration of tumor cell membrane fluidity with cholesterol esters. Strain 2 guinea pigs and several nonimmunogenic 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.

Infection of guinea pig fibrosarcoma cells with murine retroviruses altered the transplantability of these tumor cells; MuLV-infected tumor cells grew and regressed in syngeneic animals (4 fibrosarcoma cell lines). Evidence was obtained to indicate that the basis of this altered transplantability was a T-dependent immunological reaction to viral antigens expressed on fibrosarcoma cells. Animals that rejected virus-infected tumors had no detectable immunological response to uninfected fibrosarcoma cells (either antibody or transplantation protection).

A series of investigations was begun to determine whether it was possible to produce, *in vitro*, tumor cell variants with potent transplantation protection antigens and whether immunization with such variants provided 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 in liquid medium, in soft agar and in immunosuppressed and conventional animals. The frequency of isolation of stable, nontumorigenic variants from a guinea pig fibrosarcoma was 30 %. One cloned line (M 48) grew and regressed in conventional animals but grew progressively and metastasized in "B" guinea pigs. This experiment provided evidence that this clone had acquired a potent transplantation protection antigen(s). Immunization with M 48 did not consistently produce resistance to challenge with the parent tumor, 107C3.

Alteration in tumor cell membrane fluidity by treatment with cholesterol esters may be associated with increased immunogenicity. This approach was evaluated with a tumor known to contain a tumor rejection antigen (line 10) and with two nonimmunogenic tumors (lines 104C1 and 107C3). Tumor cells were treated with cholesterol hemisuccinate (CHS). After CHS treatment, the fluidity of tumor cells membrane decreased. Immunization of guinea pigs with CHS-treated line 10 cells or with CHS-treated fibrosarcoma cells failed to produce transplantation resistance to untreated tumor cells.

The basis of rejection of MuLV-infected guinea pig fibrosarcoma cells has been analyzed. Evidence was obtained to indicate that the host response was directed to viral antigens and required an intact T-cell system. MuLV-infected tumor cells grew and regressed in conventional animals but grew progressively in "B" guinea pigs. Occasionally, tumors recurred in conventional animals at sites of injection of 107C3/4070A. The incidence of recurrent tumors was increased and the time of recurrence decreased in guinea pigs immunized with virus-infected tumor cells. In related experiments, tumor recurrences have invariably been 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 expression 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. A monoclonal antibody to a subset of guinea pig T-cells has been prepared. This antibody may facilitate analysis of the T-cell subsets mediating rejection of virus-infected tumor 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 the factors involved in this new form of antitumor therapy. Buffalo/N rats were given a single injection of NMU. When

animals developed a single 5-10 mm mammary tumor they were entered into the treatment protocol. Antitumor responses were evaluated by measurement of breast tumor weight 50 days after onset of treatment. Animals received 4 intravenous injections of plasma. Before infusion, plasma from tumor bearing or control rats was absorbed with Sepharose, CNBr Sepharose, or Sepharose protein A. Antitumor effects occurred with transfer of plasmas from tumor bearing rats after adsorption with Sepharose, CNBr Sepharose, or Sepharose Protein A. This model may be of value in identifying the factor(s) responsible for this antitumor effect.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB08525-07 LIB
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.) Immunotherapy of Primary Autochthonous Cancer		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) PI: B. Zbar Chief, Cellular Immunity Section LIB NCI		
COOPERATING UNITS (if any) John Langone, Department of Medicine, Baylor University School of Medicine, Houston, Texas		
LAB/BRANCH Laboratory of Immunobiology		
SECTION Cellular Immunity Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.7	PROFESSIONAL: 0.7	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Rats with experimentally-induced primary autochthonous mammary cancer are being studied as guides to the immunotherapy of human cancer. Rats with primary mammary adenocarcinomas have been treated by intravenous injection of plasma from tumor bearing rats. Before injection, plasma was absorbed with protein A Sepharose, Sepharose, or CNBr-Sepharose.		

OTHER: S. Sukumar	Visiting Associate	LIB NCI
N. Terata	Visiting Fellow	LIB NCI

Project Description

Objectives: The primary objective of this project is to evaluate biologic response modifiers in animals with primary cancer as a guide for treatment of human cancer.

Methods Employed: Breast cancers were induced in rats by a single intravenous injection of N-nitroso N-methyl urea into 50 day old animals. Malignant tumors developed in about 30 percent of the animals; a majority of animals with breast cancer developed only a single lesion. Animals with a single primary autochthonous breast cancer (5-12mm in diameter) were treated by 4 intravenous injections of plasma absorbed with protein A-Sepharose, Sepharose, or inactivated CNBr-Sepharose. Plasma donors were either untreated, control rats or rats with mammary tumors. Rats were killed 50 days after initiation of treatment; each mammary tumor was weighed. Comparisons between treatment groups were made with the Mann Whitney U test.

Major Findings: Three experiments have been performed. The conclusions can be summarized as follows: in untreated animals, primary breast tumors grew progressively. At the end of 50 days of observation, the median weights of primary tumors was 3.5, 4.2 and 5.5 grams. No inhibition of tumor growth was observed in animals treated with normal plasma or plasma from tumor bearing animals (median tumor weights, 3.7 and 3.8 grams, respectively). Plasma from normal animals after absorption with Sepharose did not inhibit tumor growth (median tumor weights, 2.7 and 4.4 grams). Plasma from normal animals after absorption with inactivated CNBr Sepharose did not inhibit tumor growth (median tumor weight, 2.0). Plasma from tumor bearing animals after absorption with Sepharose inhibited tumor growth in one experiment (median tumor weight, 0.4 grams) but not in a second experiment (median tumor weight, 1.8 grams). Plasma from tumor bearing rats absorbed with inactivated CNBr Sepharose inhibited tumor growth (median tumor weight 0.1 grams). Plasma from tumor bearing rats after absorption with protein A Sepharose inhibited tumor growth in 3 consecutive experiments (median tumor weights, 0.4, 0.6, and 0.8 grams).

Significance to Biomedical Research and Program of the Institute: Recent clinical studies indicated that administration of plasma absorbed with protein A can lead to partial regression of human mammary cancers. Little is known about the mechanism of this antitumor effect or the method for optimal treatment. The development of a small animal model that demonstrates inhibition of tumor growth after treatment with absorbed plasma may facilitate investigation of these questions. The results of the investigation to date suggest that regression of rat mammary cancer following infusion of absorbed plasma may not require protein A. One requirement is that plasma donors should be tumor-bearing rats.

Proposed Course of Project: Experiments will be performed to determine reproducibility of the antitumor effects observed with plasma from tumor-bearing rats after absorption with inactivated CNBr Sepharose. Tests for endotoxin in some of the plasma samples indicate the presence of 0.04-0.1 ng/ml endotoxin.

Rats will be injected with endotoxin to determine whether ng amounts of endotoxin can produce inhibition of tumor growth in this animal model. Experiments will be performed to determine the minimal dose of absorbed plasma that can produce antitumor effects and to determine when animals develop plasmas that after absorption produces tumor regression.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CB08528-07 LIB
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.) Mechanisms of Delayed Hypersensitivity and Tumor Graft Rejection		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) PI: B. Zbar Chief, Cellular Immunity Section LIB NCI		
COOPERATING UNITS (if any) Janet Hartley, Laboratory of Viral Diseases, NIAID Stephen J. Galli, Department of Pathology, Beth Isreal Hospital, Boston, MA Matthew Gonda, Program Resources Inc., Frederick, Maryland		
LAB/BRANCH Laboratory of Immunobiology		
SECTION Cellular Immunity Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.0	PROFESSIONAL: 2.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) It is the purpose of this project to develop methods for modification of host response to nonimmunogenic tumors and to analyze the cellular and molecular basis of tumor rejection. The current areas of interest are the biologic and immunologic consequences of superinfection of cavine tumors with murine leukemia viruses and the induction, by in vitro treatment of tumor cells with mutagens, of new tumor transplantation rejection antigens.		

OTHER:	A. Nagai	Visiting Fellow	LIB NCI
	N. Terata	Visiting Fellow	LIB NCI
	Y. Tanio	Visiting Fellow	LIB NCI
	S. Shu	Expert	LIB NCI

Project Description

Objectives: The goals of this project are to develop methods to modify host response to nonimmunogenic tumors and to study the basis of rejection of retrovirus-infected tumor cells.

Methods Employed: Animals. Strain 2 guinea pigs were obtained from the Experimental Animal Breeding Facility of the National Cancer Institute, Frederick Cancer Research Facility, Frederick, Maryland. C3H/HeN MTV-mice were obtained from Charles River Laboratories, Wilmington, Mass. and the Experimental Animal Breeding Facility of the NCI-FCRF, Frederick, MD.

Cavine tumors. Fibrosarcomas 104C1 and 107C3 were obtained from Dr. Charles Evans, Laboratory of Biology, National Cancer Institute. These cell lines lacked detectable tumor rejection antigens and serologically detectable tumor-associated antigens. Fibrosarcoma cell lines were maintained in tissue culture flasks. The biologic and immunologic characteristics of the line 10 hepatoma have been described in previous annual reports.

Murine tumors. Fibrosarcoma 1769 was obtained from Dr. Gerald Bartlett, Pennsylvania State University, Hershey Medical Center, Hershey, PA. This tumor developed, in the absence of carcinogen or virus treatment, in tissues of a C3H/HeN mouse.

Infection of guinea pig cell cultures. Cell cultures were infected 24 hours after tumor cells were seeded by replacement of the existing nutrient medium with medium containing polybrene and murine leukemia virus (MuLV) 4070A or NFS-Th-2. Infected cell lines were maintained by serial passage.

Viruses and assays. MuLV 4070A, present in supernatant fluids, was detected and quantitated with the mink cell immunofluorescence test and the mink S+L- assay. Expression of MuLV antigen(s) by infected guinea pig tumor cells was monitored with a direct immunofluorescence test.

Treatment of tumor cells with chemical mutagens. Tumor cells were treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Surviving cells were harvested and seeded in 60-mm petri dishes at 200-400 cells per dish. Colonies, isolated with cloning cylinders, were expanded and then tested for growth by injection into conventional and immunodepressed syngeneic guinea pigs or nude mice (5×10^6 cells, 3 animals per clone). Nude mice were injected by the intradermal or intracerebral routes.

Immunosuppressed guinea pigs. T-cell depleted guinea pigs were prepared by thymectomy of adult guinea pigs followed by lethal whole body irradiation and bone marrow transplantation ("B" guinea pigs). To reduce bacterial infection animals received acidified (pH 2.5) drinking water. Syngeneic bone marrow cells (10^8) were injected into the left ventricle.

Histologic observations. Specimens from tumor-bearing animals were collected and prepared for observation with the electron and light microscopes.

Treatment of tumor cells with cholesterol hemisuccinate (CHS). Tumor cells were treated with CHS, 40-250 ug/ml for 1 1/2 to 2 hours at 37 C.

Major Findings: Mechanisms of rejection of retrovirus-infected cavine tumor cells. Retrovirus-infected tumor cells grew progressively in "B" guinea pigs, but grew and regressed in conventional animals. This result indicated that regression of retrovirus-infected fibrosarcoma cells was dependent on the existence of an intact T-cell system. Although this result suggested that regression of retrovirus-infected tumor cells was mediated directly by T-cells, this conclusion must be tempered by the observation that "B" guinea pigs did not form detectable protein A-fixing antibodies to retrovirus-infected tumor cells. Formation of protein A-fixing antibodies to retrovirus 4070A was T-cell dependent.

Retroviral antigens were the targets of the host immunological response to lines 104C1/4070A and 107C3/4070A. This conclusion was based on the results of experiments which showed that animals immunized to lines 104C1/4070A or 107C3/4070A had no detectable immunological response to uninfected tumor cells.

The specificity of the immunological rejection of retrovirus-infected cells was investigated. This investigation was facilitated by the observation that in an occasional animal tumors recurred at sites of injection of 107C3/4070A. Growth of retrovirus-infected cells was evaluated in guinea pigs with different abilities to respond immunologically to virus-infected cells and by analysis of the antigenic and virologic properties of the tumors formed in these animals. Three distinct patterns of tumor growth, regression and recurrence were observed. In unimmunized, conventional animals, line 107C3/4070A grew temporarily and then regressed; in 10-20 % of animals tumors reappeared at sites of injection 50-60 days after inoculation. In guinea pigs immunized to virus-infected tumor cells, regression of line 107C3/ 4070A occurred more rapidly than in unimmunized animals; tumors recurred more frequently and in a higher proportion of virus-immunized animals than in unimmunized animals. In "B" guinea pigs, line 107C3/4070A grew progressively. Recurrences from conventional animals differed antigenically and virologically from tumors of immunosuppressed animals. Recurrent tumors from conventional animals had diminished or absent expression of retrovirus. Progressively growing tumors from "B" animals expressed viral antigens and released infectious MuLV. Clonal analysis of the parent population showed marked heterogeneity of virus expression with one of 21 clones showing neither virus antigen nor release of infectious virus. The data suggested that reappearance of tumors in conventional animals injected with 107C3/4070A reflects a T-dependent selection and rejection of virus-positive cells. Tumor cells that have the potential to form tumor recurrences preexist in the parent population. Immunization to virus-infected cells favors the emergence of virus-negative tumors in conventional animals.

The inflammatory response to retrovirus-infected tumor cells was analyzed by light and electron microscopy. Basophils formed a prominent part of the cellular infiltrate at sites of rejection of retrovirus-infected tumor cells. Approximately 80% of the inflammatory cells at sites of rejection were basophils.

Cells which resembled activated T cells were observed by electron microscopic examination at sites of rejection of retrovirus-infected tumor cells. A basophilia accompanied tumor rejection. "B" guinea pigs had markedly impaired cutaneous basophil hypersensitivity to keyhole limpet hemocyanin.

Isolation of tumor negative variants from cavine and murine tumors after treatment with chemical mutagens. Tumor 107C3. Exposure of Line 107C3 cells to MNNG (3 ug/ml) for 60 minutes at 37 C led to a marked reduction in number of viable cells. Surviving cells were successfully cloned by seeding 200-400 cells in 60 mm petri dishes. The colony forming efficiency of the parent tumor population was 50 %; the colony forming efficiency of the tumor cells present in cultures on day 10 was 2 %. Injection of tumor cells into syngeneic animals (5×10^6) produced two growth patterns. Some variants grew temporarily and then regressed; other variants did not form detectable tumors. The cloning procedure did not alter the transplantability of tumor cells since clones isolated from cultures not treated with mutagen grew progressively in syngeneic animals. Changes were observed in the transplantability of some of the mutagen treated clones; after 4-6 passages in vitro, some clones derived after mutagen treatment regained the ability to grow progressively in vivo. This result suggested that the lack of transplantability of some clones might be due to toxicity of mutagen exposure. Growth and regression has been a stable property (12-14 passages) of three clones (M 32, M 33 and M 48). Experiments were begun to determine whether the growth and regression of these clones is attributable to immunological mechanisms. Intracerebral inoculation into nude mice of M 33 and M 48 led to progressive tumor growth. M 48 grew progressively in "B" animals but grew and regressed in conventional animals. Taken together the data indicate that mutagen treatment has not altered the ability of clones M33 and M 48 to grow progressively. The data suggest that the failure of M 48 to grow progressively in conventional animals is a T-dependent phenomenon. A large number of experiments were performed to determine whether immunization with clones derived after mutagen treatment produced resistance to challenge with the parent tumor. Some clones produced resistance to challenge with the parent tumor; this result was not consistent. In some cases inversion of the dose response to tumor challenge was observed. Experiments in progress deal with the possibility that immunization with clones derived after mutagen treatment in admixture with adjuvant may provide more consistent protection against challenge with the parent tumor. Mycoplasma or retroviruses were not detected in the parent tumor or variant clones.

Tumor 1769-2 This naturally occurring murine tumor was selected for studies on induction of altered transplantability after mutagen treatment. Transplantation protection experiments indicated that tumor 1769 was not immunogenic. The parent tumor was cloned and one clone 1769-2 was selected for further study. The clone was homogeneous as evidenced by the fact that subclones derived from the 1769-2 tumor grew progressively in syngeneic animals. Conditions of mutagen dose and duration of mutagen exposure was explored and correlated with the development of clones with altered transplantability. A total of 120 clones were isolated after MNNG treatment in 4 separate experiments. Fifty-seven percent of clones tested did not grow progressively at 10^4 cells; 59 % of clones tested did not grow progressively at 10^5 cells; 17 % of clones tested did not grow at 10^6 cells.

The frequency of obtaining clones which failed to grow progressively at an inoculum dose of 10^4 or 10^5 cells was a function of the MNNG dose in the range from 0.5 to 3 ug/ml. The frequency of obtaining tumor negative clones at an inoculum dose of 10^5 cells was 8 % at a mutagen concentration of 0.5 ug/ml and 43 % at a mutagen concentration of 2 ug/ml. Two patterns of tumor growth were observed. Some clones failed to grow at a given dose; other clones grew temporarily and then regressed. Alteration of transplantability was a relative characteristic. Tumors with reduced transplantability at 10^4 or 10^5 cells generally grew at a dose of 10^6 cells. Growth and regression of clones 3-9 and 3-39 were observed after injection of 10^5 tumor cells; growth and regression of clones 3-22 and 3-23 was observed after injection of 10^6 tumor cells. Experiments are in progress to determine whether acquisition of new antigens can account for the altered transplantability of these clones. To date, one clone, 3-17 has been shown to possess new transplantation rejection antigens. Line 1769-2 and variant clones did not release infectious ecotropic or amphotropic MuLV.

Modification of cell cholesterol content of guinea pig tumor cells.

Experiments were designed to determine whether alteration of cell membrane fluidity by treatment with CHS led to increased immunogenicity of the tumor cells. Three tumors were selected for study: the line 10 hepatoma, the fibrosarcomas 107C3 and 104C1. The line 10 tumor was selected for study since this tumor is known to contain a tumor rejection antigen that can be recognized by animals when presented in the context of a mycobacterial adjuvant. The lines 107C3 and 104C1 were not detectably immunogenic. CHS treatment altered microviscosity of each tumor cell line. Animals were immunized with three injections of cholesterol hemisuccinate treated, irradiated tumor cells. As a positive control, animals were immunized with line 10 cells admixed with an oil-in-water emulsion of Mycobacterium bovis strain bacillus Calmette Guerin cell walls. Animals were challenged with 10^4 - 10^6 tumor cells. No evidence of induction of immunity was evident in animals immunized with cholesterol-treated line 10, line 107C3 or 104C1 tumor cells.

Significance to Biomedical Research and the Program of the Institute: Recent work in experimental tumor immunology has focused attention on the lack of detectable tumor rejection antigens on spontaneously arising tumors of mice and rats. The results of these investigations, in agreement with results from other laboratories, indicates that it is possible, by in vitro treatment of nonimmunogenic tumor cell populations with chemical mutagens, to produce immunogenic tumor cell clones. This finding is of considerable theoretical and possible practical importance. Since the tumors studied were nonimmunogenic, the observations indicate that tumors can arise without formation of immunogenic tumor transplantation rejection antigens. Since exposure of these tumor cells in vitro to chemical mutagen (carcinogen) leads to the isolation of some clones that are immunogenic, the data suggest that formation of immunogenic transplantation protection antigens represents a secondary event in the neoplastic process. Activation of endogenous retroviruses present in murine cells by mutagens might account for the observed immunogenicity. The guinea pig is a species that does not contain a large number of endogenous retroviruses. The observation that it is possible to produce immunogenic variants from nonimmunogenic guinea pig tumor cells suggests that the acquisition of new antigens after chemical mutagen treatment is independent of retrovirus expression. A central question

is whether immunization with clones, derived after mutagen treatment and with reduced transplantability, will offer protection against challenge with the parent tumor. Results in other laboratories suggest that this occurs for some naturally-occurring mouse tumors. Results from our laboratory should be forth coming. We have failed, to date, to provide evidence of protection after immunization with CHS-treated tumor cells or retrovirus-infected tumor cells. These results indicate that CHS treatment or retrovirus infection are not generally applicable methods for augmenting host response to nonimmunogenic tumors.

Proposed Course of the Project: We will continue our studies on modification of host response to weakly immunogenic tumors. Studies will be pursued with three tumors: line 107C3 (guinea pig), 1769-2 (mouse) and L2C (guinea pig). With the line 107C3, mutagen-treated clones that grow and regress are now available in the laboratory. Studies will be performed in "B" and conventional guinea pigs to determine whether these clones have acquired new transplantation protection antigens. Studies will be pursued to determine whether reproducible protection against challenge with the parent tumor can be achieved by immunization with mutagen-treated cells in admixture with mycobacterial adjuvant. With the line 1769-2, mutagen-treated clones that grow and regress are also available in the laboratory. As for the guinea pig line 107C3, studies will be performed in immunosuppressed (adult thymectomized and sublethal irradiated) and conventional animals to determine whether these clones have acquired new transplantation protection antigens. Animals immunized with the variant clones will be tested with threshold doses of the parent tumor cell to determine whether resistance can be demonstrated to the parent tumor. A subline of a leukemia of strain 2 guinea pigs (L2C-BZ) is known to be antigenic and not immunogenic. A combination of in vitro mutagen treatment and in vivo passage is being used in an effort to produce an immunogenic population. Studies will be performed to compare the DNAs of parent and mutagen-derived immunogenic clones.

Studies of the histopathologic characteristics of rejection of retrovirus-infected tumor cells will be performed in conventional and "B" guinea pigs. Attempts will be made to correlate the content of basophils at sites of injection of 107C3/4070A with tumor growth or regression and to determine whether basophilia occurs in "B" animals. Reconstitution studies will be performed with serum and selected cell populations and measurements will be made of tumor growth and tumor basophil infiltration.

The characteristics of tumors that recur in animals immunized with 107C3/4070A will be further characterized. Attempts will focus on evaluation of the DNA of the recurrent tumors to determine whether this DNA contains fingerprints of MuLV 4070A infection. Attempts will be made to characterize the DNA alterations that accompany the production of tumor cells variants with reduced transplantability.

Publications

Galli, S.J., Bast, R.J., Jr., Bast, B.S., Isomura, T., Zbar, B., Rapp, H.J., and Dvorak, H.F.: Bystander suppression of tumor growth: Evidence that specific targets and bystanders are damaged by injury to a common microvasculature. J. Immunol., 129: 890-899, 1982.

Sukumar, S., Hunter, J.T., Terata, N. and Rapp, H.J.: Eradication of hepatic metastases by active specific immunization. Cancer Immuno. and Immunother., 14: 151-154, 1983.

Yarkoni, E., Ashley, M.P., Zbar, B., Sugimoto, T. and Rapp, H.J.: Eradication by active specific immunotherapy of established tumor transplants and microscopic lymph node metastases. Cancer Res., 42: 2544-2546, 1982.

Yarkoni, E., Hunter, J.T., and Sukumar, S.: A specific vaccine effective against stage I and stage II malignant disease in guinea pigs: Effect of variations in preparations and storage. Cancer Immunol. Immunother., 14: 92-95, 1982.

Zbar, B., Nagai, A., Terata, N. and Hovis, J.: Tumor rejection mediated by an amphotropic murine leukemia virus. Cancer Res., 43: 46-53, 1983.

Zbar, B., Nagai, A., Hovis, J. and Terata, N.: Host response to guinea pig tumor cells infected with murine leukemia viruses. Cancer: Etiology and Prevention, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB08530-04 LIB
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.) Mechanisms of Immune Eradication of Tumors		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) PI: B. Zbar Chief, Cellular Immunity Section LIB NCI		
COOPERATING UNITS (if any) Ethan Shevach, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases		
LAB/BRANCH Laboratory of Immunobiology		
SECTION Cellular Immunity Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.0	PROFESSIONAL: 2.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Adoptive transfer of tumor immunity was investigated in guinea pigs treated with carrageen, trypan blue or lethal irradiation. Evidence for the participation of recipient components other than T cells was obtained using the inhibitors carrageenan and trypan blue. Monoclonal antibodies were produced to guinea pig T cells. One monoclonal antibody was obtained that reacts with a subpopulation of guinea pig T cells.		

OTHER:	S. Shu	Expert	LIB	NCI
	L. Fonseca	Guest Worker	LIB	NCI
	H. Kato	Visiting Fellow	LIB	NCI
	A. Nagai	Visiting Fellow	LIB	NCI

Project Description

Objectives: The principal goal of this project is to study the mechanisms of adoptive immunity to the guinea pig line-10 hepatoma. Evidence was sought for recipient participation in eradication of tumor after adoptive transfer of immune lymphoid cells. The possible contribution of the recipient's primary immune response and T cells to adoptive transfer of immunity were analyzed.

Methods Employed: Adoptive transfer of antitumor immunity. Nucleated cells from spleens of guinea pigs immunized to line-10 hepatoma were injected intravenously into recipient strain 2 guinea pigs. Animals were challenged with ascites line-10 cells intradermally. Growth of the inoculated tumor was monitored for evaluation of tumor immunity.

Treatment of guinea pigs with carrageenan or trypan blue. Carrageenan was dissolved in saline by boiling. Carrageenan (3 mg/ml, 10 ml) was injected intraperitoneally for four consecutive days starting one day before adoptive transfer and tumor challenge. Trypan blue (20 mg/ml) was dialyzed in deionized water for 7 days with water replacement twice each day. Non-dialyzable material was concentrated by boiling and samples were lyophilized to estimate the dry weight content. Trypan blue was adjusted to a concentration of 10 mg/ml and sterilized in an autoclave. Trypan blue was given at a initial dose of 160 mg/kg and a maintenance dose of 40 mg/kg three times each week. This dose schedule was lethal to 30 to 50% of treated guinea pigs.

Bioassays for the presence of line-10 at skin inoculation sites. Two bioassays were designed to detect the presence of tumor cells and immune effector cells at sites of tumor rejection. At various time intervals after adoptive transfer, tumor inoculation sites were biopsied and minced. These materials were injected into the peritoneal cavity or subcutaneous space of weanling strain 2 guinea pigs. Development of ascites line-10 tumor after i.p. injection is evidence for the presence of viable line-10 in the transferred tissue; this assay does not detect immune effector cells. The subcutaneous bioassay detects both tumor cells and immune effector cells.

Major Findings. To investigate the contributions of recipient components, other than T lymphocytes, in the successful elimination of tumor after adoptive transfer, we used two known inhibitors of the development of tumor immunity and delayed type hypersensitivity skin reactions in guinea pigs. Treatment of immune cell donors or isolated immune spleen cells with carrageenan or trypan blue did not appear to be functionally toxic for lymphocytes capable of conveying passive sensitization. Therefore, we performed adoptive transfer experiments in animals treated with carrageenan or trypan blue. Treatment of recipient guinea pigs with a total of 120 mg carrageenan starting one day before adoptive transfer and tumor inoculation blocked the expression of adoptively transferred immunity. Trypan blue treatment of recipient guinea

pigs starting one day before adoptive transfer and tumor inoculation did not seem to affect the expression of immunity to line-10 tumor. Treatment with each chemical resulted in death of some guinea pigs before the end of the experiments (30% and 40% for carrageenan and trypan blue, respectively). Surviving animals were emaciated and losing weight. Because of this toxicity, we developed bioassays to evaluate tumor eradication by transferring tumor inoculation sites to normal animals. The intraperitoneal (ip) bioassay detects the tumor cells despite the presence of immune lymphoid cells. The ip bioassay of tumor inoculation sites taken from carrageenan-treated, immune cell transfused guinea pigs revealed the presence of viable tumor cells. This result is consistent with the finding that carrageenan inhibited the expression of adoptive immunity. Interestingly, the ip bioassay of trypan blue-treated, immune cell transfused guinea pigs also revealed the presence of viable tumor cells despite the fact that there was no detectable tumor in the donor animals. This finding indicated that trypan blue's effect on the adoptive immunity was transient and quantitatively less potent than that of carrageenan. The subcutaneous (sc) bioassay detects tumor cells and may be influenced by the presence of immune effectors. The sc bioassay of tumor inoculation sites from carrageenan and trypan blue treated, immune cell transferred guinea pigs resulted in no tumor growth in secondary host despite evident tumor presence detected by ip bioassay. This result is interpreted as evidence of the presence of immune cells at the sites of tumor inoculation sites.

The development of bioassays allowed us to evaluate radiosensitivity of the host component(s) that participated in tumor eradication after adoptive transfer. Lethal irradiation (500 rads) of recipient guinea pigs before adoptive transfer did not affect the efficacy of tumor suppression. Therefore, we concluded that host components sensitive to carrageenan as well as trypan blue but not to lethal irradiation are essential for successful expression of adoptive antitumor immunity in intact animals. Considering the characteristics of the host non-specific component(s) that are defined by our studies, it seems likely that mature macrophages may be the targets for the effects of carrageenan and trypan blue.

Monoclonal antibodies were produced to guinea pig T cells and the pattern of reactivity of the antibodies to a panel of guinea pig lymphoid cells was determined. The functional characteristics of the antibodies were also studied. One monoclonal antibody reacted with guinea pig T cells and did not react with bone marrow cells, or the L2C leukemia. Studies with the Fluorescent Activated Cell Sorter suggested that this antibody reacted with a subpopulation of guinea pig T cells. This antibody inhibited antigen-specific proliferation of guinea pig T cells but did not inhibit proliferation induced by T cell mitogens.

Significance to Biomedical Research and the Program of the Institute: Interest in the possible use of adoptive immunity as a means of specific immunotherapy of cancer in man has been renewed since the discovery and characterization of interleukin 2. Findings summarized in our experiments provide additional insight into this potentially powerful approach to control tumor growth. Our finding of noncytolytic nature of immune lymphoid-tumor cell interaction led us to characterize host primary immune response, T lymphocytes as well as non-committed host component(s) in the elimination of tumor by transferred immune lymphoid cells. While immunocompetence at T cell level was apparently not significant, we found

evidence for the participation of host component(s) sensitive to carrageenan and to a lesser degree, trypan blue but resistant to 500 rad irradiation. The availability of a monoclonal antibody to a subpopulation of guinea pig T cells may facilitate investigation of a number of problems of immunologic interest.

Proposed Course of Project: The major emphasis will be on further characterization of the monoclonal antibodies to guinea pig T cells and possible use of these antibodies in analysis of the cell populations that mediate adoptive transfer of tumor immunity.

Publications

Shu, S., Fonseca, L.S., Hunter, J.T., and Rapp, H.J.: Mechanisms of immunologic eradication of a syngeneic guinea pig tumor. II. Effect of methotrexate treatment and T cell depletion of the recipient on adoptive immunity. Transplantation, 35: 56-61, 1983.

Shu, S., Fonsca, L.S., Kata, H, and Zbar, B. Mechanisms of immunological eradication of a syngeneic guinea pig hepatoma: participation of a component(s) of recipient origin in the expression of systemic adoptive immunotherapy. Cancer Res., 43: 2637-2643, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB08550-09 LIB
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.) Modification of Tumor Cells and Immune Cytolysis		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) PI: S. H. Ohanian Research Microbiologist LIB NCI		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Immunobiology		
SECTION Humoral Immunity Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Pretreatment of tumor cells with chemotherapeutic agents, metabolic inhibitors, enzymes or hormones modifies the susceptibility of the cells to killing by immune attack. The purpose of this investigation is to determine the attributes of cells which influence the cells' ability to modify or resist cellular and humoral cytotoxic mechanisms.		

OTHER: S. Gupta Visiting Fellow LIB NCI

Project Description

Objectives: 1) To study the mechanism whereby enzymes, metabolic inhibitors and anti-lipidemic agents effect the sensitivity of tumor cells to immune attack; 2) to study mechanisms whereby hormones effect sensitivity of cells to immune attack; 3) to determine metabolic pathways and the physical and chemical property of the cell that may be modified following such treatment; 4) to determine the cellular processes that may be modified following or during immune attack.

Methods Employed: Antibody is quantitated by the C1 fixation and transfer test and immune cytolysis. Sensitivity of cells to antibody-C attack is measured by uptake of trypan blue and/or release of $^{125}\text{IUdR}$. Incorporation of radioisotopically-labeled precursors of DNA, RNA, protein, complex carbohydrate, glycoproteins, glycolipids and lipids are being used to measure general metabolic properties of the cells. Thin layer chromatography is being utilized to analyze and identify specific lipid moieties synthesized by the cells. Sucrose density gradient ultracentrifugation is used to prepare plasma membrane and intracellular membrane fractions of tumor cells. High pressure liquid chromatography (HPLC) is being utilized to determine the lipid and fatty acid content and composition of the cells. SDS PAGE is utilized to analyze protein and glycoprotein composition of membranes. Sensitivity of cells to cell-mediated immune attack is measured by ^{51}Cr release. Immunochemical methods, including Sephadex and DEAE chromatography electrophoresis, immunodiffusion and ultracentrifugation are employed to isolate and identify biological macromolecules.

Major Findings: The malignant guinea pig hepatoma cells, line-1 and line-10, are relatively more resistant to killing by antibody plus guinea pig C (GPC) than by antibody plus human C (HuC). Line-10 cells sensitized with anti-Forsman antibody are resistant to killing by GPC whereas line-1 is not. The difference in susceptibility of the two cell lines is not due to the lack of binding of C-fixing antibody and selected C components.

Cells pretreated with certain metabolic inhibitors or chemotherapeutic agents are rendered sensitive to antibody-GPC killing; cells pretreated with selected polypeptide, catecholamine, or steroid hormones are rendered resistant to antibody-HuC killing. The drug and hormone effects are time and concentration dependent and are reversible; the ability of the cells to resist antibody-C killing correlates with their ability to synthesize complex lipids, but not DNA, RNA, protein, or complex carbohydrate.

Metabolic inhibitors that increase the sensitivity of the cells to antibody-C killing interfere with the synthesis and composition of specific lipids in the plasma membrane and intracellular membrane fractions. Drug treated cells recultured in the absence of the drug regained their ability to resist C attack and to synthesize and incorporate lipids in plasma and intracellular membranes.

Line 10 cells rendered resistant to killing by human C following incubation for 1 hour with insulin or hydrocortisone were enhanced in their synthesis of selected lipids into plasma and intracellular membranes. This effect was reversible.

Drug treatment reversibly increased, whereas hormone treatment reversibly decreased the polyunsaturated fatty acid content of the cellular lipids. Hormone treatment also increased the total polarity of cellular fatty acids. The drug and hormone effects on the lipid and fatty acid composition of the cell affect certain molecular relationships in the cells. Drug treatment decreased the cholesterol:phospholipid mole ratio and increases the unsaturated fatty acid content of cellular phospholipids and neutral lipids. Hormone treatment causes the opposite changes. The changes in these molecular relationships could have marked effects on certain physical properties of the cell, especially membrane fluidity, permeability, or thickness. Effects on the physical properties of the cells could explain the drug and hormone effects on the susceptibility of the cells to humoral immune attack.

Analysis of the lipid and fatty acid content of subcellular fractions from the drug- and hormone-treated cells showed that the changes in lipid composition observed in whole cell lipid extracts were a reflection of changes occurring in the plasma membrane as well as in intracellular membranes (i.e., mitochondria, nuclear membrane, endoplasmic reticulum, microsome). Adriamycin treatment decreased the unesterified cholesterol and saturated triglyceride content while increasing the polyunsaturated fatty acid-containing triglycerides and phospholipids in the plasma and intracellular membranes; 5-fluorouracil (a drug that is not effective in increasing the sensitivity of the cells to antibody-C killing) did not have these effects. Treatment of the cells with insulin or hydrocortisone had exactly the opposite effects of those observed in the adriamycin-treated cells. These results suggested that modifications in the chemical composition of intracellular membranes as well as the plasma membrane of nucleated cells may influence certain physical properties of the cell membranes (e.g., fluidity, permeability) and as a result, could affect the outcome of humoral immune attack at the cell surface.

Line-10 tumor cells cultured for 24 hr in lecithin-rich normal human plasma or with synthetic lecithin showed a 5- to 8-fold increase in their lecithin: sphingomyelin mole ratio without being affected in their total lipid content or cholesterol:phospholipid mole ratio. These cells were more sensitive to killing by antibody plus C than untreated controls. Line-10 cells that underwent a homogeneously catalyzed hydrogenation reaction were reduced 6-fold in their content of unsaturated fatty acid compared to controls; the lipid content of these cells was largely unaffected. These cells were more resistant to antibody-C-mediated killing than controls. These modifications in cellular lipid and fatty acid composition could be reversed when the cells were recultured for 24 hr in serum-containing tissue culture medium; the cells regained control levels of susceptibility to antibody-C killing at this time. These results suggested that by manipulating the lipid or fatty acid composition of a tumor cell, either indirectly by changing the lipid composition of the environment in which the cell resides or by directly altering the chemical nature of a cellular lipid constituent, the susceptibility of the cell to immune killing can be modulated.

Studies on the variation and modification of nucleated cells to C-mediated killing were extended to include human lymphoid cell lines, PY and Raji, and the mouse mastocytoma cell, P815. Raji and PY in their lag or stationary phase of growth are relatively more resistant to killing by rabbit ALS or human anti-HLA plus C. The difference in sensitivity observed for the cell

lines was not due to differences in antigen expression as measured by quantitative adsorption test. Cells isolated in G₁ or S phase of the cell cycle were equally sensitive to antibody-C killing indicating the variation in sensitivity of the cells is not directly cell cycle dependent. No correlation was noted between sensitivity to immune attack and net synthesis of DNA, RNA, protein, complex carbohydrate and lipid.

The mouse mastocytoma cell line, designated P815, also demonstrated variation in sensitivity to C-mediated killing. These cells which were relatively more resistant at the log phase of growth expressed a greater amount of antigen compared to sensitive cells. The sensitivity of the line-1, line-10, the human lymphoblastoid and mouse mastocytoma cell lines to killing by C from different species was also determined. Human, rabbit and goat C were generally more effective in killing the guinea pig and human cells. Rat C which was effective in killing line-1 cells sensitized with anti-Forssman or antitumor antibody was only effective in killing line-10 cells sensitized with antitumor antibody and slightly effective in killing human lymphoid cells in stationary phase of growth. With the mouse cell line P815, guinea pig was as effective as human and goat C; rat C was less effective and rabbit C the most effective of the C sources in killing the cells sensitized with the heterologous rabbit anti-P815 antibody. The observation that selected C are effective or more effective with cells sensitized with antibody of one specificity but not another suggests the antibody can influence the effectiveness of C against a particular target.

Analysis of membrane fluid properties were performed on the human and mouse cells at different parts of the cell growth cycle. Fluidity was determined by fluorescence polarization methods on cells labeled with the lipophilic fluorescent probe 1,6-diphenyl hexatriene. No obvious correlation between fluid properties and sensitivity were detected. Membrane fluid properties decreased with age of the culture. Resistant Raji cells cultured for 24 hr at low density (0.5×10^6 cells/ml) in fresh media or media from log or stationary phase cultures became sensitive to C attack; membrane fluidity increased upon culture in fresh medium only. Resistant Raji cells cultured at high density (1.5×10^6 cells/ml) under similar conditions became sensitive only after culture in fresh or log phase media. No detectable change in membrane fluidity was observed. The fluid properties of cells treated with metabolic inhibitors either effective or not effective in rendering cells susceptible to antibody-C killing were generally greater than untreated control cells. These results suggest that the physiological properties of the cells are important in the ability of the cells to resist immune attack.

Lipid synthesis and composition of the mouse and human cells were determined for cells removed from lag, log and stationary phase of growth. Total lipid content did not change significantly. The FFA content was higher in cells removed from stationary phase of growth. The phospholipid content decreased in stationary phase Raji cells compared to lag and log phase. Triglyceride content was less in Raji, P815 and PY cells from stationary phase cultures. No change in CHOL and CHOL-E content was noted. The fatty acid composition studies suggested that cells at the times they were sensitive to killing had a higher content of selected unsaturated fatty acids relative to the resistant populations. This latter observation confirms those of the hepatoma cell

lines, line-1 and line-10, that the unsaturated fatty acid content is increased in the cells at the times the cells are sensitive to immune attack.

Further analysis of lipid synthesis of the human and mouse cells were performed using acetate and glycerol in addition to fatty acids. Cells incubated in fresh media for 1 hr show a decrease in the incorporation of acetate and glycerol. In resistant Raji and P815 cells cultured in fresh media incorporation of unsaturated fatty acids was higher than that of saturated fatty acids. This appears to occur at the times the cells become sensitive to C killing. Although changes in total lipid composition do not occur changes in specific lipids (particularly for fatty acids, triglycerides, phosphatidylcholine and phosphatidylethanolamine) were noted. These results show that changes in lipid synthesis can occur very rapidly in cells and that different precursors of cellular macromolecules are used preferentially at one time but not another.

Analysis of lipid plasmalogen and prostaglandin synthesis (PGE_1 and PGF_1) were performed in sensitive and resistant P815 and Raji cells. No differences were noted in total phospholipid plasmalogens of the resistant and sensitive cells. However, there is suggestive evidence that neutral lipid plasmalogens may be higher in sensitive cells. Preliminary studies on the cellular and extracellular concentration of PGE_1 and PGF_1 indicate no major differences between cells or media for resistant and sensitive populations.

Initial studies with P815 cells indicate that cells for stationary phase of cultures are relatively more resistant than log phase cells to cytotoxic T lymphocyte. This effect was dependent upon the conditions of the test. When experiments were carried out in fresh medium no differences were noted between log or stationary phase cells. Differences were only seen when the experiment was carried out in appropriate conditioned medium (i.e. target cells and attacker cells suspended in cell free medium from stationary phase cultures). No correlation could be seen between resistance to CTL and lipid and fatty acid composition of the cells. The results suggest that factors in addition to lipid composition may influence the sensitivity of cells to cellular immune mechanisms.

Significance to Biomedical Research and the Program of the Institute:

Modification of a tumor cell by metabolic inhibitors, hormones, chemotherapeutic agents and anti-lipidemic agents, or through chemical or physical manipulation of the cell's macromolecular composition furnishes a tool to study the interaction of tumor cells with the immune defense mechanisms of the host. The study of the response by tumor cells to humoral or cellular immune attack through modifications in various cellular metabolic pathways provides information regarding the mechanism of defense or cytomembrane repair processes in these cells. Modification of these processes may lead to cells that are more vulnerable to immune attack mechanisms.

Proposed Course of Project: Radioisotope incorporation studies will be continued as probes to determine the cellular functions that are modified by treatment with drugs or hormones. The chemical attributes of normal cells, tumor cells and treated tumor cells will be studied. Work will continue on the effect of enzymes, hormones and inhibitors of macromolecular synthesis on the metabolic function of the tumor cells. Analysis of membrane-associated and intracellular

macromolecules in these cells will be pursued. Further quantitative chemical analysis of the lipid and fatty acid composition of cells that are susceptible or resistant to immune killing will be made.

The effect of antibody and C on nucleated cells will be further analyzed. This will include studies on the binding and utilization of C components during the cytotoxic reaction. The effects of the stages in the growth cycle of the cell and the physical and biochemical events that occur during formation and transformation of T* will also be studied. T* is an intermediate in the killing of cells by antibody plus C and contains all the components of C required for cell killing to occur.

Studies will be initiated to determine if the human and mouse cells also show a cyclic variation in sensitivity to cell-mediated killing.

Publications

Ohanian, S.H., Yamazaki, M., et al: Cell Growth-dependent Variation in the Sensitivity of Human and Mouse Tumor Cells to Complement-mediated Killing. Cancer Research 43, 491-495, 1983.

Schlager, S.I. and Ohanian, S.H.: Role of membrane lipids in the immunological killing of tumor cells. I. Target cell lipids. Lipids, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB08552-17 LIB
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 Complement Fixation and Action		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) PI: T. Borsos Chief, Humoral Immunity Section LIB NCI		
COOPERATING UNITS (if any) Laboratory of Clinical Investigation, NIAID		
LAB/BRANCH Laboratory of Immunobiology		
SECTION Humoral Immunity Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.0	PROFESSIONAL: 2.0	OTHER: 1.0
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) This is a long-range project investigating the mechanism of complement fixation and action. In particular the interaction of antibody-antigen complexes with the first component of complement and the result of this interaction on the other components are investigated. The relation between antibody action and complement activation is also explored. Finally, the significance of complement in the humoral immune defense mechanism is studied.		

OTHER: R. Ejzemberg
A. Circolo

Guest Worker
Visiting Fellow

LIB NCI
LIB NCI

Project Description

Objectives: To develop new methods and to use available complement (C) fixation tests based on the fixation and transfer of the first component of C (C1) and cytotoxic tests for the analysis of antigen-antibody reactions, in particular in the search for cancer specific antigens.

Methods Employed: The model for studying cytotoxic reactions mediated by antibody and C consists of sheep erythrocytes, hemolytic antibody and C. Purification procedures for antibodies and the C components include: preparative (large-scale) gel filtration, ion exchange chromatography and preparative free electrophoresis. Other techniques used include precipitin and immunoelectrophoretic analysis, analytical, zonal and preparative ultracentrifugation and other immuno- and physico-chemical methods. Development and application of quantitative analytical methods which utilize radiolabeled tracer molecules are being emphasized.

Major Findings: Last year it was reported that during the activation of the classical complement pathway at cell surfaces no C4 was bound to cell bound IgM antibodies; this was in contrast to IgG initiated complement activation where C4 binds to the IgG molecule. In the present study the binding of C3 by IgM anti-methotrexate antibodies was studied. MTX was coupled to the cell surface covalently. C3 was bound to the cells by the classical reaction sequence i.e. via C1, C4 and C2. After binding C3 to the cells, specifically bound IgM was eluted from the cell surface by soluble MTX. By determining the amount of IgM eluted, the number of C3 molecules eluted and remaining on the cells and by reattaching the eluted IgM to fresh cells it was found that no C3 was eluted from the cells, and no C3 transferred from cell to cell. The data conclusively demonstrated that IgM was not capable of binding C3. From these and last years observations the important principle was derived that in the classical pathway lysis of red cells, the whole sequence of C binding occurs at a site distant from the IgM molecule that initiated the sequence.

Regarding activation of C1 by Igs studies continued to elucidate the mechanism of binding vs. activation of C1 by Ig. Rabbit anti-MTX IgG antibody was used to study the effect of MTX density on sheep red cells on the lysis of cells by complement. Under conditions where variation in cell surface hapten density had no effect on binding IgG, the number of C1 (or its active form, C1) bound by the IgG was independent of hapten density. The ability of the C1 binding IgG complex to activate C1, however, decreased with decreasing density of the hapten. The decreased ability to activate bound C1 was paralleled by decreased ability to activate the hemolytic sequence of whole C. The results were interpreted to mean that binding of C1 was the results of aggregation ("doublet formation") by IgG while activation of the bound C1 depended on changes induced in the IgG molecule by straddling hapten molecules at varying distances.

The mechanism whereby C4 binds to the cell surface and acquires the ability of interacting with C2 was also investigated. C4 is a glycoprotein with a MW of 180,000. It has three polypeptide chains held together by disulfide bonds.

Fluid phase C₄ is easily reduced and the three chains can be separated by physico-chemical means. Several years ago members of this laboratory reported that cell bound C₄ was highly resistant to the action of 2-mercaptoethanol (MSH). The present study was designed to explore the nature of the resistance of the bound C₄ and to compare chain structure with activity in the bound C₄. It was confirmed that the activity of cell bound C₄ was not destroyed by MSH; chain analysis showed that MSH removed none of the chains of C₄. By the use of a more active reducing agent, dithiothreitol (DTT), it was shown that this agent was capable of destroying all measurable bound C₄ activity and that the loss of activity was accompanied by loss of 70% of the gamma chain. Neither of the other two chains was affected by the DTT. The data imply heterogeneity of bound C₄ and that the gamma chain is important in the interaction of C₄ with C₂.

Significance to Biomedical Research and the Program of the Institute: C fixation is one of the most widely used diagnostic tools. The development and successful application of a very sensitive C fixation test, the ClFT test, opened up new possibilities in determining antigen-antibody reactions on cell surfaces. Furthermore, cytotoxic reactions due to antibody and C are prime examples of body defense mechanisms. Fundamental research into the nature and mechanism of C fixation and action will contribute greatly to the development of diagnostic tools and to the understanding of the mechanism of immune body defenses.

Proposed Course of Project: This is a long-range project, and little change is expected in the scope of the work during the next few years. The ultimate goals of this project are the development of better diagnostic tools and the elucidation of molecular events associated with the action of C and antibodies. It is hoped that as a result of our program of inquiry into the basic problem of the interaction of antibodies, antigens and components of C, tools will be developed that are of practical significance in the search for cancer antigens and furnish a better understanding of the destruction of cells by antibodies and complement.

Publications

- Borsos, T., and Circolo, A.: Effect of cell surface hapten density and immunoglobulin class on complement lysis and agglutination of cells. Proceedings of the Joint Congress of the ISH and ISBT. Akadémiai Kiadó. Budapest. 1982, in press.
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OTHER: Enrica Alteri Visiting Fellow LIB NCI

Major Findings: It was found previously that human blood monocytes comprise 2 subpopulations: one migrates to the chemoattractant, fMet-Leu-Phe, and has saturable binding sites for this peptide; the other does not migrate, and exhibits little peptide binding. To determine if expression of binding sites was a function of monocyte maturation, human subjects were depleted of blood monocytes by leukapheresis so that the circulation was repopulated by monocytes released from the bone marrow. Pre- and postleukapheresis monocytes were then compared for fMet-Leu-[³H]Phe binding, superoxide generation and chemotactic responses. No significant differences in peptide binding curves were found, suggesting that receptor expression was stable over the maturational span represented by these two groups of cells. This supports the hypothesis that there are two distinct lineages of monocytes with respect to expression of receptors for fMet-Leu-Phe. Despite comparable chemotaxin receptor expression in pre- and post-leukapheresis monocytes, the number of chemotactically responsive cells immediately post-leukapheresis was half the control. This was a transient state; monocyte responses were normal 3 hours after termination of leukapheresis, suggesting that they rapidly become functionally mature. Studies are in progress to determine if this apparent maturation process occurs in vitro.

Human blood monocytes, when stimulated with phorbol myristate acetate (PMA), produced large quantities of superoxide anion at a rate that was constant for at least 15 minutes. Monocytes exposed to fMet-Leu-Phe also produced superoxide, but production lasted 5 minutes at the most. The peptide treated monocytes were refractory to further stimulation with fMet-Leu-Phe, but responded normally to PMA. Although this finding was consistent with specific deactivation of monocytes by fMet-Leu-Phe, a soluble inhibitor was found in the medium of fMet-Leu-Phe treated cells. Medium transferred to fresh monocytes failed to induce superoxide release and rendered the cells unresponsive to fresh fMet-Leu-Phe, but did not prevent their response to PMA.

Macrophage stimulating protein (MSP) is a mammalian serum protein discovered by this laboratory that renders mouse peritoneal macrophages responsive to chemoattractants. It would be of great interest to determine if this protein plays a regulatory role in monocyte/macrophage function. An ELISA assay for human MSP has been developed with a polyclonal rabbit anti-MSP. Such an assay should be suitable for surveying serum MSP concentrations in a search for MSP deficiency and for cellular studies to find the source of production. Large-scale purification of MSP has been completed for generation of monoclonal antibody that will be used as an additional ELISA reagent to assure specificity of the assay.

Previous studies on basophil chemotaxis from this laboratory were made with human mononuclear cell fractions obtained by centrifugation of blood on Ficoll-Hypaque. Basophils comprised 1-2% of the total cells, and the possible effects of these other cells on basophil responses could not be evaluated. A simple, one step purification of human blood basophils has been developed, based on centrifugation on discontinuous Percoll gradients. The majority of

the basophils were recovered in a narrow band. The mean percentage of basophils in this band was 19%, with a range of 5 to 53% - depending on the initial basophil count of the donor. Monocytes and neutrophils were present in very small numbers; the majority of accompanying cells were small lymphocytes. This preparation will be of great interest for chemotaxis studies, since most of the other migrating cell types (neutrophils and monocytes) have been removed.

Significance to the Program of the Institute: analysis of subpopulations of leukocytes and the factors that affect their function may throw light on interactions between tumor and host.

Proposed Course of Project: further characterization of monocyte subpopulations and monocyte maturation. Development of ELISA assay for MSP with monoclonal antibody, and study of human MSP in serum and tissues. Studies of purified human basophils.

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Leonard, E.J. and Skeel, A.: A high molecular weight chemoattractant generated from C5 by ultracentrifugation of mouse serum without activation of complement. Molecular Immunology, in press.

Alteri, E. and Leonard, E.J.: N-formylmethionyl-leucyl-[³H]phenylalanine binding, superoxide release and chemotactic responses of human blood monocytes that repopulate the circulation during leukapheresis. Blood, in press.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CB08576-11 LIB

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.)

Immunological Mechanisms of Tumor Rejection

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

(Name, title, laboratory, and institute affiliation)

PI: M. Meltzer Senior Surgeon LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Immunobiology

SECTION

Immunopathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

B

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

This project is on the interaction of tumor cells and host defense cells. Current studies are on mechanisms of macrophage activation and the impairment of activation in certain strains of mice.

OTHER:	Edward J. Leonard	Chief, Immunopathology Section	LIB	NCI
	Massimo Occhionero	Visiting Fellow	LIB	NCI

Major Findings: Further physicochemical studies were done on the factor or factors in antigen or mitogen stimulated murine spleen cell culture supernatants that induce macrophage mediated tumor cytotoxicity. Although activity was eluted from Sephadex G-100 columns in a region corresponding to a MW of 55,000, rechromatography of the first and second halves of the eluted peak resulted in 2 separate peaks, corresponding in location to the first and second halves of the original peak. This suggests that the original peak comprises 2 activation factors of slightly different MW. In contrast, by electrofocusing there was a single isoelectric point of pH 5.8, with no sign of charge heterogeneity. Denaturation studies showed rapid loss of activity at 60°C, stability over a pH range of 5 to 10, loss of activity at pH 4.0, and loss of 2/3 of the activity in 6M urea. The number of criteria by which this lymphokine has now been characterized should suffice to distinguish it from other lymphokines evaluated by similar methods.

Culture fluids from a phorbol myristate acetate stimulated E1-4 thymoma cell line were found to activate mouse macrophages to become nonspecifically tumoricidal. This activity was compared with that obtained from antigen stimulated mouse spleen cells. By four criteria, functional activity from the two sources was comparable: (1) Macrophage activation could be separated temporally into two steps, priming and triggering. (2) Macrophages activated by the fluids exhibited peak cytotoxic activity within 5-9 hrs; no cytotoxic activity was demonstrable if addition of target cells was delayed 14 hrs. (3) Mouse peritoneal exudate macrophages, but not resident macrophages, became cytotoxic after a 5 hr incubation with culture fluids. (4) Macrophages from certain strains of mice were incapable of activation; the same pattern of strain unresponsiveness was observed with both E1-4 and spleen cell culture fluids. Thus it is likely that the two E1-4 products and the mouse spleen cell lymphokine activate the same cytotoxic mechanism.

Significance to Biomedical Research and the Program of the Institute: Analysis of the effector immune events leading to tumor rejection may provide a rational basis for manipulation of host responses or tumor cell in an effort to eliminate or prevent progression of the tumor.

Proposed Course of Project: this project is being discontinued, at least temporarily, because of the departure of the principal investigator, Dr. Monte Meltzer.

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SUMMARY REPORT
LABORATORY OF TUMOR IMMUNOLOGY AND BIOLOGY
October 1, 1982 to September 30, 1983

The Laboratory of Tumor Immunology and Biology conducts research to identify immunologic markers specific for, or associated with, various carcinomas, with the ultimate aim of employing these toward the diagnosis, prognosis and treatment of human cancer. Thus, the Laboratory carries out research in the generation and characterization of monoclonal antibodies to tumor associated determinants. Conjugate monoclonal antibodies are also constructed to aid in the diagnosis, localization and potentially the elimination of tumor cells. The Laboratory is also involved in research to develop immunoassays that will aid in the characterization of human carcinoma cell populations, and in the diagnosis or prognosis of human carcinomas. A major focus of the Laboratory also involves investigating the association between specific genetic elements and tumorigenesis, employing techniques of molecular biology. Research is also conducted on those factors involved in the differentiation and antigenic modulation of human tumor populations.

The Laboratory of Tumor Immunology and Biology is organized in four Sections:

- Experimental Oncology Section
- Oncogenetics Section
- Biochemistry of Oncogenes Section
- Cellular and Molecular Physiology Section.

Experimental Oncology Section (Dr. Jeffrey Schlom, Chief)

The Experimental Oncology Section conducts research in the generation and characterization of monoclonal antibodies that are reactive with mammalian carcinoma cells. Recent research has identified and characterized specific proteins that are associated with the neoplastic state, with emphasis on human carcinoma systems. Immunoassays have been developed that may aid in the characterization of human carcinoma cell populations and potentially in the diagnosis or prognosis of human carcinomas. The Section has also investigated the use of antibody conjugates that may aid in the detection or therapy of carcinoma lesions. Experiments are being conducted to determine those factors which influence the antigenic phenotype and state of differentiation of human carcinoma cell populations.

Mice were immunized with membrane enriched fractions of metastatic human mammary carcinoma lesions; splenic lymphocytes were fused with non-Ig secretor myeloma cells to generate subsequent hybridoma cultures synthesizing thirteen monoclonal antibodies reactive with human mammary carcinoma cells, two which react with carcinoembryonic antigen. The monoclonals could be placed into six major groups based on (a) reactivity in solid phase RIAs with extracts of metastatic lesions of mammary carcinomas, and (b) binding to the surface of live mammary tumor cells in culture using live cell RIAs and fluorescent activated cell sorter analyses. Some of the monoclonals demonstrated a "pancarcinoma" activity, reacting with the surface of some non-breast carcinomas, but none of the eleven monoclonals reacted with the cell surface of melanomas, sarcomas, various hematopoietic malignancies, and numerous apparently normal cell lines. The immunoperoxidase technique was

used on fixed tissue sections to determine the extent of reactivity of the different monoclonals with various types of primary mammary tumors and with metastatic lesions in lymph nodes and at distal sites. These antibodies react with approximately 85 percent of human mammary and colon carcinomas.

Monoclonal antibodies generated to human mammary tumor metastases were tested for reactivity to novel and known tumor associated antigens. The monoclonals were used to immunoprecipitate antigens from a radiolabeled breast tumor metastasis extract. Monoclonal antibody B72.3 immunoprecipitated a high molecular weight polypeptide complex of approximately 220,000d. B6.2 and four other antibodies immunoprecipitated a 90,000d polypeptide. The four other antibodies cross-react in RIA with monoclonal B6.2 but differ in their ability to compete with the binding of B6.2. Two antibodies, B1.1 and F5.5, were shown to differentially react with carcinoembryonic antigen. The high molecular weight complex identified by monoclonal B72.3 has been preparatively purified, using molecular sieving and antibody affinity chromatography, without loss of immunoreactivity. Studies are in progress to develop radioimmunoassays with several of the monoclonals described. Radioimmunoassays for monoclonal antibody B72.3 have been established and optimized, with a sensitivity in the nanogram range.

Antigenic variation was observed in the expression of specific tumor associated antigens within individual human mammary tumor masses using monoclonal antibodies. This variation was demonstrated by both the pattern and cellular localization of reactivity with a given antibody. This diversity was also observed in human mammary tumor cell lines grown in vivo and in vitro. Analyses of DNA content and cell surface binding of monoclonal antibodies during logarithmic growth phase, and density-dependent arrest, demonstrated that 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 despite prolonged exposure to antibody. Antigenic drift was observed with continued passage of mammary tumor cell lines; consistent with this finding, the "same" mammary tumor cell line obtained from different sources exhibited distinct antigenic phenotypes. Single-cell clones derived from the MCF-7 mammary tumor cell line exhibited at least four distinct antigenic phenotypes; a change in cell surface phenotype of some of the clones was seen during subsequent passage. 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. Purified IgG, F(ab')₂ fragments, and Fab' fragments of monoclonal antibody B6.2 have been generated. The IgG and its fragments were radiolabeled with I-125 without loss of immunoreactivity and were injected into athymic mice bearing human mammary tumor transplants. The radiolabeled antibody localized in the tumor within 24 hours with tumor to tissue ratios rising over a 96 hour period. The F(ab')₂ was better than the IgG and gave tumor to liver and spleen ratios of 15 to 20:1, and tumor to muscle and brain ratios of 50 to 110:1. No localization was observed in mice bearing human melanomas, or with radiolabeled normal murine IgG in human mammary tumor bearing mice. The ability of the radiolabeled antibody to localize in mammary tumors was sufficient to give high quality gamma scans of tumor bearing mice. Monoclonal antibody B6.2 has also been radiolabeled with I-131 and I-123 with similar specificities and localization indices. Several monoclonal antibodies including B72.3 are now being labeled with other radioactive isotopes to test for appropriateness for clinical studies.

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 with the immunoperoxidase method using tissue sections of the primary tumor from the fusion, as well as tissue sections from other primary tumors. One human IgM monoclonal antibody, derived from a human-mouse fusion was used to discriminate between mammary carcinoma cells (from 55 of 59 patients) 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 have also been developed that secrete human monoclonal antibodies; these human Ig's are currently being assayed for immunoreactivity to human breast carcinomas.

The Section has also been concerned with the role and mechanisms by which hormones, growth factors, particularly epidermal growth factor (EGF) and the EGF related transforming growth factors (TGF's) and tumor-promoting phorbol esters regulate cellular proliferation, the production of various extracellular matrix proteins (i.e. collagen and laminin) and the expression of specific tumor-associated cell surface antigens to which various murine monoclonal antibodies have been generated. A variety of cell culture systems are being utilized to address these questions. Specifically, a human epidermoid carcinoma cell line (A431) and a human mammary carcinoma cell line (MCF-7) are being used since both of these lines are sensitive to the biological effects of EGF and tumor promoters (TPA) and both synthesize TGF's. It has been demonstrated that EGF and/or TPA stimulate a 2 to 3-fold increase in the production of type IV (base-membrane) collagen and laminin within 2 hours after exposure of A431 cells to these agents. This response is preceded by a 2- to 3-fold increase in the activity of a phospholipid-dependent protein kinase (protein kinase C) 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 type IV collagen and laminin. 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, namely a reduced serum requirement for growth and a loss of anchorage-dependent growth. TGF's have been isolated from several human breast tumor samples, a transplantable human mammary tumor and in the conditioned medium from MCF-7 cells. The TGF's from these human sources is present in two species, Mr 10 to 12,000 and 6,000 and has a pI of 4.9. Experiments are in progress to purify this activity to a point where murine monoclonal antibodies might be generated against this material. With such a reagent(s), experiments will be conducted to screen for this activity in other human mammary tumors and to study the regulation of its production in MCF-7 cells by other growth factors and hormones. MCF-7 cells are also being utilized to determine whether various hormones and growth factors might enhance the expression of some of the cell surface, tumor-associated antigens.

Oncogenetics Section (Dr. Robert Callahan, Chief)

The Oncogenetics Section is studying the role of infectious mouse mammary tumor virus (MMTV) and genetically transmitted MMTV proviral DNA in the etiology of mammary neoplasia. Their efforts have focused on two general areas: 1) MMTV related sequences on human cellular DNA and 2) genetic studies with inbred

and feral breeding colonies of mice. Low stringency blot hybridization conditions have been used to isolate several recombinant clones of MMTV related human cellular DNA. The major region of homology is at the 3' end of the MMTV pol gene. Earlier works had shown that using these hybridization conditions the MMTV and mammalian type C proviral genomes do not hybridize. We have now found that low stringency hybridization conditions permit stable hybrids to form between the pol gene sequences of infectious type A (M432), 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. In addition, 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. The SMRV LTR hybridizes with restriction fragments containing repeated sequences which are 8.0 kbp apart. The gag and pol related sequences lie between the LTR related sequences. The organization of these sequences in human cellular DNA is consistent with that of a genetically transmitted proviral genome. 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. This family of retroviral genes are present in 20 to 30 copies per cell. Analysis of human-hamster somatic cell hybrids suggests that the retroviral genes are located on four chromosomes. Comparison of restricted cellular DNA from several normal and primary breast tissues has revealed no detectable amplification or variation in pattern of fragments containing related sequences. One exception is the breast tumor tissue culture cell line MCF-7 which contains at least two new related restriction fragments. These sequences appear to be significantly diverged from the cloned human retroviral genome.

In earlier works pedigreed breeding colony of feral mice (CZII) have been 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 by genetically introducing individual MMTV proviral genomes from Balb/c and C3H/ Hef mice into the CZII genetic background. These mice are currently being bred and monitored for the development of mammary tumors. To facilitate this program we have identified, in collaboration with Dr. C. Kozac, the chromosomal locations of the Balb/c and C3H/Hef proviral genomes using mouse-hamster somatic cell hybrids. The Balb/c MMTV proviral genomes are linked to chromosomes 12, 16, and 18. The MMTV related sequences on chromosomes 12 and 18 represent complete proviral genomes whereas the sequences on chromosome 16 correspond only to the MMTV LTR and leader sequences. The expression of the Balb/c MMTV proviral genes was determined in normal and various tumor tissues. Low levels of MMTV genomic and env in RNA was detected in several normal tissues. High levels of a novel 1.6 and 1.2 kbp MMTV RNA was detected respectively in lactating mammary gland and salivary gland. Each of these RNA species hybridized only with the LTR portion of the viral genome. High levels of the 1.6 kbp RNA were also detected in all pristine induced plasma cell tumors. Some of these tumors, as well as carcinogen induced mammary hyperplastic alveolar nodules (HAN) and HAN derived mammary tumors also expressed high levels of genomic and nv MMTV RNA.

Biochemistry of Oncogenes Section (Dr. Robert Bassin, Chief)

The Biochemistry of Oncogenes Section has focused a considerable part of its program on studying the mechanism of transformation by retroviruses and retroviruses-related oncogenes. Two experimental systems have been developed for

this effort: 1) Flat (revertant) derivatives of Ki-MuSV-transformed cells have been isolated which are resistant to transformation by certain oncogenes and not by others. 2) A hormone-dependent ras transformation system originally described by Hager et al. has been adopted to serum-free conditions. Previously, we reported the isolation and initial characterization of flat (revertant) cell lines following mutagenesis of Ki-MuSV-transformed NIH/3T3 cells. These revertant cells have previously been shown to contain 2 copies of the v-Ki-ras gene, elevated levels of p 21 and rescuable sarcoma virus, but they have lost many of the properties associated with transformation including tumor formation in nude mice, and growth in soft agar. Revertants also exhibit a flat cellular morphology in monolayer cultures and cease dividing at low saturation densities.

The ability of oncogenes from various sources to retransform the revertant cell lines has been studied. These experiments were carried out either by super-infection of revertants with appropriate retroviruses or by somatic cell hybridization of the revertants to cells nonproductively transformed by oncogenes from a variety of sources. The revertants were almost completely resistant to retransformation by the Ki-MuSV oncogene v-Ki-ras and to the structurally related oncogenes v-Ha-ras, v-bas and c-Ha-ras (human). The revertants also appear to be resistant to v-fes and v-src, oncogenes not structurally related to the ras gene.

Other viral oncogenes, v-fms, v-mos, and v-sis are not suppressed in the revertant cell lines in the same kinds of cell hybridization and super-infection experiments. It seems likely 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 that certain Ki-Mu SV-transformed cells are more sensitive to ouabain than are NIH/3T3 control cells or the revertant sublines described above indicated that changes in ion transport might be associated with cell transformation. Studies using $^{86}\text{Rb}^+$ and inhibitors of ion transport such as bumetanide and ouabain showed that K^+ transport is altered in transformed cells, perhaps as a result of a more general change in the cell membrane structure. Transfection studies designed to show whether or not ouabain resistance is causally related to the suppression of transformation in the revertants are in progress.

A molecular chimera consisting of the v-Ha-ras gene joined to the LTR of mouse mammary tumor virus is being developed for use both as a novel conditional lethal system for studies on ras-related transformation and as a model for studies on the molecular biology of glucocorticoids. Following adaptation of NIH/3T3 cells transfected with this chimera to a serum-free cell culture system it is possible to measure changes in p21 expression, SGF production, and cell morphology within a short time after turn-on and turn-off of the v-ras gene with glucocorticoids. This system provides a means of assessing the specificity of those changes associated with the transformed state.

Several experimental systems which control growth and differentiation of cultured cells have also been investigated. The role of hormones and cyclic nucleotides in mediating cell growth and differentiation was studied in the DIF-5 cell line. DIF-5 cells constitute a new subline of the F-9 teratocarcinoma cell line which

is apparently blocked in its ability to differentiate completely in the presence of retinoic acid. This DIF-5 cell line is able to grow in the absence of either serum or hormonal supplements and is of potential value in studies on growth regulation.

The F-9 teratocarcinoma system has also been of value in studies on calcium-dependent phosphokinase. Differentiation of the teratocarcinoma cell line F-9 following treatment with retinoic acid is accompanied by an increase in cytosolic kinase activity. On the other hand, treatment of a differentiated cell line, PYS, with phorbol ester results in a shift of hormone activity from the cytosol to the plasma membrane. Studies on the role of calmodulin in the growth and differentiation of cells have been facilitated by application of hydrophobic interaction chromatography for purification of this protein.

Cellular and Molecular Physiology Section (Dr. Herbert Cooper, Chief)

The Cellular and Molecular Physiology Section's work has progressed in three main areas: 1) Studies of the unique posttranslational modification, hypusine formation, and its possible role in regulation of protein synthesis. 2) Studies of the biochemical mechanisms by which the tumor promoting phorbol ester, PMA, affects cell growth and differentiation. 3) Studies of molecular mechanisms in the function and genetic polymorphism of HLA histocompatibility antigens.

In the first area a unique posttranslational modification has been described in which a portion of the polyamine, spermidine, is covalently joined to a lysine in a polypeptide chain. This event affects only one protein, in all animal cells. We have shown that this protein is a translation initiation factor, eIF-4D, which has been previously purified from rabbit reticulocytes. Hypusine formation is virtually absent in quiescent lymphocytes and is very low in other growth-arrested cells, but rises in proportion to the rate of protein synthesis when these cells are activated. Since the unmodified protein is synthesized in quiescent cells, we have proposed 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. It has recently been found that eIF-4D is one of the major non-hemoglobin proteins of the erythrocyte, allowing purification of the factor in large quantities. The function, if any, of this factor in erythrocytes, which do not synthesize proteins, is obscure, and is presently under investigation.

In the second area the effects of 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. We have shown that this sequence is accompanied by the induction of synthesis of a set of proteins which are characteristic of mature monocytes. These proteins thus provide a marker for monocytic differentiation.

During the initial response of HL-60 to PMA, there is a rapid and dramatic increase in phosphorylation of a 17 kD cytosolic protein (ppl7) involving an unusual alkali-resistant serine phosphoester bond. This induction does not occur with phorbol ester analogues lacking tumor promoting activity. Induction of phosphorylation of ppl7 by PMA is also found in other cells which show

different differentiation responses to the agent. This indicates 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. The importance of the early induction of phosphorylation of pp17 for the later differentiation events is suggested by the finding that trifluoroperazine, which inhibites induction of pp17 phosphorylation, also prevents the effects of PMA on growth and differentiation of HL-60 cells.

Studies are in progress to characterize the protein kinase involved in this phosphorylation. At present we know that it is not a tyrosine kinase and that its activation does not require an influx of extracellular calcium. Kinetic studies showed that the phosphate on pp17 is in a state of rapid turnover, suggesting a cyclical function for this protein in its phosphorylated and non-phosphorylated states.

In the third area the synthesis and turnover of HLA-A,B,C molecules in human peripheral lymphocytes has been studied. We showed that these proteins are among the most rapidly turned over of all cell proteins; in the plasma membrane, they are the single most rapidly turned over species of protein. Following growth stimulation of lymphocytes, HLA turnover diminishes and synthesis increases, resulting in net accumulation of HLA molecules in the plasma membrane. These events are 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, we have proposed that rapid turnover of HLA molecules is a biochemical concomitant of this process.

Utilizing modified technique for resolving the molecular species of HLA proteins on 2-dimensional electrophoretic gels, highly characteristic maps of the HLA proteins of single individuals have been produced. Using this technique, we have studied 5 subjects whose HLA-A,B antigens were serologically indistinguishable, but who showd immunological reactions to one another's cells which was traced to specific HLA antigens. In every case, we detected alteration in molecular charge of specific HLA proteins when such responses were present. When immunological tests detected no antigen differences, we found no polymorphism of molecular charge. These studies suggest that amino acid substitutions involving charged amino acids may be an important mechanism in the generation of HLA polymorphism which provokes immunological recognition. Charged amino acids may be a significant factor in the biochemical mechanism by which HLA molecules engage in immunological recognition phenomena.

Other Professional Personnel:

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Project DescriptionObjectives:

To generate and characterize monoclonal antibodies that are reactive with human mammary tumor associated antigens. These monoclonal antibodies may prove useful in the diagnosis, prognosis, monitoring of therapy, and eventual therapy of human carcinomas, as well as in the study of the biology of human carcinoma cell populations.

Methods Employed:

Hybridoma technology was used to generate monoclonal antibodies against human breast tumor metastases. The antibodies were screened in a solid phase radio-immunoassay against extracts of mammary tumor metastases to various organs and corresponding normal tissue. The reactivity of the antibodies to cell surfaces was examined using a live cell RIA and by fluorescent activated cell sorting. The reactivity of these antibodies to tissue sections of primary tumors and metastases to the lymph nodes and distal areas as well as normal tissue were examined using the immunoperoxidase technique.

Major Findings:

I. Generation and characterization of monoclonal antibodies. Mice were immunized with membrane-enriched fractions of human metastatic mammary carcinoma cells (designated Met 1 and Met 2) from either of two involved livers from two different patients. Spleen cells of immunized mice were fused with NS-1 mouse myeloma cells to generate 4,250 primary hybridoma cultures. Supernatant fluids from these cultures were screened in solid-phase RIAs for the presence of immunoglobulin reactive with extracts of metastatic mammary tumor cells from the involved livers, and not reactive with similar extracts of apparently normal liver; 370 cultures synthesized immunoglobulin reactive only with the metastatic carcinoma cell extracts. After passage, double cloning of these cultures by endpoint dilution, and further testing, the monoclonal antibodies synthesized by 11 hybridoma cell lines were chosen for further study. The isotypes were first determined: 10 were IgGs of various subclasses and one was an IgM.

The primary screen for the monoclonal antibodies was a solid-phase RIA employing cell extracts of two breast tumor metastases (Met 1 and Met 2) and apparently normal human liver. The 11 monoclonal antibodies could immediately be divided into three major groups on the basis of their differential reactivity to Met 1 vs. Met 2. All 11 antibodies were negative when tested against similar extracts from normal human liver, the A204 rhabdomyosarcoma cell line, the HBL100 cell

line derived from cultures of human milk, the Mm5mt/c₁ mouse mammary tumor cell line, the C3H10T_{1/2} mouse fibroblast cell line, the CrFK feline kidney cell line, disrupted mouse mammary tumor virus and mouse leukemia virus, purified carcino-embryonic antigen (CEA), and ferritin. Two monoclonal antibodies were used as controls in all these studies: (1) W6/32, an anti-human histocompatibility antigen, and (2) B139, which was generated in these studies, and which showed reactivity to all human cells tested.

Binding to cell surface. To further define the reactivities of the 11 monoclonal antibodies, and to determine if they bind cell surface antigens, each antibody was tested for binding to live cells in culture. Test cells included three mammary carcinoma cell lines (BT-20, MCF-7, and ZR-75-1). Nine monoclonals, grouped together on the basis of their binding to both metastatic cell extracts, could now be separated into three further groups on the basis of their differential binding to the surface of live cells in culture. Monoclonals B6.2, B14.2, B39.1, F64.5, F25.2, B84.1, and B38.1 all bound the three mammary carcinoma lines. Monoclonal B38.1 also bound to the A549 lung carcinoma line, the A431 vulva epidermoid carcinoma line, and the KB oral epidermoid carcinoma line. This antibody, however, did not bind to the sarcoma and melanoma cell lines tested. Monoclonals B50.4, B50.1, and B72.3 constituted a third grouping in that they reacted only with the surface of the MCF-7 cell line. None of the 11 monoclonal antibodies bound to the surface of any of the cell lines tested derived from apparently normal human tissues. Control monoclonal B139, however, did bind to all of these cells.

Fluorescent activated cell sorter analyses. Monoclonal antibodies B6.2 and B38.1 were further analyzed for surface binding using fluorescent activated cell sorter analyses to a panel of human cell lines. These studies were carried out in collaboration with Dr. D. Kufe, Sidney Farber Cancer Center. Antibody B6.2 was reactive with five of six breast carcinoma lines but was unreactive with most other carcinomas. Antibody B38.1, on the other hand, was strongly reactive with all breast carcinoma lines, and was also reactive with many non-breast carcinoma lines. The breast tumor line HS0578T falls into a rare histologic grouping and at one point has been histologically designated a "carcinosarcoma;" it is unreactive with both antibody B6.2 and B38.1. Cell lines derived from melanomas, sarcomas and lymphoid tumors were uniformly unreactive with these antibodies. There was complete agreement in assay results when the same cell lines were tested in live cell RIA and by cell sorter analysis.

A variety of tissues obtained directly from biopsy were also evaluated for reactivity via fluorescence activated cell sorter analyses. Breast carcinoma cells were examined from patients with malignant pleural effusions; three patterns of reactivity were noted. Two pleural effusions demonstrated moderate reactivity with both B6.2 and B38.1. One pleural effusion demonstrated strong reactivity with antibody B6.2 but was unreactive with antibody B38.1; another pleural effusion showed the reciprocal reactivity.

Binding to tumors. To further define the range of reactivity of each of the 11 monoclonal antibodies, the immunoperoxidase technique on tissue sections was employed. All the monoclonals reacted with primary mammary carcinoma cells

(both infiltrating ductal and lobular), but the percentage of tumors that were reactive varied from approximately 50% to 85% for the different monoclonals. In many of the positive primary and metastatic mammary carcinomas, not all tumor cells stained. A high degree of selective reactivity with mammary tumor cells, and not with apparently normal mammary epithelium, stroma, blood vessels, or lymphocytes in the breast was observed with all 11 monoclonal antibodies. The staining patterns of mammary carcinoma cells varied among the different monoclonals. For example, monoclonal B50.4 was reactive with mammary carcinoma cells, displaying a dense focal staining. Monoclonal B6.2, on the other hand, reacts with alternate sections of the same mammary carcinoma, with a more diffuse cytoplasmic pattern. Frozen sections of primary mammary tumors were also tested with some of the monoclonals and were positive. Several of the monoclonals also showed reactivity to selected non-breast carcinomas, such as carcinoma of the colon, thus demonstrating a "pancarcinoma" reactivity.

Experiments were carried out to determine if the 11 monoclonal antibodies could detect mammary carcinoma cell populations at distal sites, i.e., in metastases. Because the monoclonals were all generated by using metastatic mammary carcinoma cells as antigen, it was not unexpected that the monoclonals all reacted, but with different degrees, to various metastases. Several of the monoclonals reacted with metastatic mammary carcinoma cells in lymph nodes of 85 percent of patients, but did not react with uninvolved nodes. None of the monoclonals reacted with normal lymphocytes or stroma from any involved or uninvolved nodes.

Differential binding to human mammary and non-mammary tumors of monoclonal antibodies reactive with carcinoembryonic antigen. The presence of high plasma levels of CEA has been reported to be an indicator of the possible presence of metastatic disease in patients with cancers of the digestive system, breast, lung, as well as other sites. Using assays based on antibodies to colonic CEA, elevated plasma levels of CEA have been reported in 38-79% of patients with mammary carcinomas. There have been several reports, however, indicating that "CEA" is a heterogeneous family of glycoproteins, some of which demonstrate cross-reactivity with each other, as well as with so-called "CEA-related" proteins. One issue that has not yet been clearly resolved is the possibility that a given tumor cell type may produce, or maintain on the cell surface, a CEA that is only partially related to CEAs associated with other malignancies. Monoclonal antibodies should be a valuable reagent toward resolving this point. To date, monoclonal antibodies have been generated by several other groups using CEA from colon carcinomas as the immunogen. In the studies reported here, monoclonal antibodies were generated to membrane enriched fractions of human mammary carcinoma metastases and screened for reactivity with purified CEA. The differential binding properties of two of these antibodies (B1.1 and F5.5) to CEA as well as to breast and non-breast tumors was investigated. Monoclonal B1.1 is an IgG_{2a}, while F5.5 is an IgG₁.

Monoclonals B1.1 and F5.5 were tested for binding to live cells in culture to define their range of reactivities, and to ascertain if they bind to antigenic determinants that are present on the cell surface. Both monoclonals bound to the same three established human mammary carcinoma cell lines and to two colon carcinoma cell lines, but not to a lung carcinoma or vulva carcinoma cell line or to 13 normal human cell lines. The two monoclonals could be distinguished,

however, by their differential reactivity to the surface of certain cell lines. B1.1 bound to three of four melanoma cell lines tested, while F5.5 did not bind to any of the four. Similar differential reactivity with B1.1 was also observed with late passages (greater than passage 80) of the A204 rhabdomyosarcoma cell line, while F5.5 did not bind A204 cells at any passage level.

Monoclonal antibodies B1.1 and F5.5 were compared for range of reactivity with two commercially available monoclonal antibodies prepared against colonic CEA. Monoclonal F5.5 could be distinguished from the three others in that it exhibited a greater binding to mammary cell lines as compared to both purified colon carcinoma CEA, and cell lines derived from colon carcinomas.

To further identify the range of reactivities of monoclonals B1.1 and F5.5 with human mammary carcinomas, the immunoperoxidase technique was used on formalin-fixed tumor sections. Both B1.1 and F5.5 reacted positively with malignant mammary tumor cells in primary lesions and with metastatic cells in lymph nodes and at distal sites. Postive staining was observed with three colon carcinomas and three lung carcinomas tested. Monoclonals F5.5 and B1.1 reacted positively with 55 and 66 percent, respectively, of the mammary carcinomas tested. The positive mammary tumors included infiltrating ductal, in situ, and medullary carcinomas.

II. Monoclonal antibody B72.3 defines the distribution of a novel tumor associated antigen in human mammary carcinoma cell populations. Of the thirteen monoclonals described above, B72.3 (an IgG₁) displayed the most restricted range of reactivity for human mammary tumor versus normal cells. Monoclonal B72.3 was used at various concentrations in immunoperoxidase assays of tissue sections to determine the effect of antibody dose on the staining intensity and the percent of tumor cells stained. Since one cannot titrate antigen in the fixed tissue section, an antibody dilution experiment would give an indication of the relative titer of reactive antigen within a given tissue. A range of antibody concentrations, varying from 0.02 μ g to 10 μ g of purified immunoglobulin (per 200 μ l) per tissue section, was used on each of four mammary carcinomas from different patients. The results demonstrate that: (a) different mammary tumors may vary in the amount of the antigen detected by B72.3, (b) a given mammary tumor may contain tumor cell populations which vary in antigen density, and (c) some mammary tumors may score positive or negative depending on the dose of antibody employed.

To further characterize the range of reactivity of B72.3, the immunoperoxidase technique was used to test a variety of malignant, benign and normal mammary tissues. Using 4 μ g of monoclonal per slide, the percent of positive primary breast tumors was 46% (19/41); 62% (13/21) of the metastatic lesions scored positive. Several histologic types of primary mammary tumors scored positive; these were infiltrating duct, infiltrating lobular and comedo carcinomas. Many of the in situ elements present in the above lesions also stained. None of the six medullary carcinomas tested were positive. Approximately two thirds of the tumors that showed a positive reactivity demonstrated a cell associated membrane and/or diffuse cytoplasmic staining, while approximately five percent showed discrete focal staining of the cytoplasm; approximately one-fourth of the reactive tumors showed an apical or marginal staining patterns. To date, not

enough tumors have been tested to define any correlations between type of staining pattern and such parameters as tumor type or histologic grade. Metastatic breast carcinoma lesions that were positive were in axillary lymph nodes, and at the distal sites of skin, liver, lung, pleura and mesentery. Fifteen benign breast lesions were also tested; these included fibrocystic disease, fibroadenomas and sclerosing adenosis. Two specimens showed positive staining: one case of fibrocystic disease where a few cells in some ducts were faintly positive, and a case of intraductal papillomatosis and sclerosing adenosis with the majority of cells staining strongly. Other primary tumors were also tested for reactivity, including four colon carcinomas, two benign colon tumors, four lung carcinomas, two kidney carcinomas, one prostate carcinoma, one bladder carcinoma, one melanoma, two lymphomas and two sarcomas. Of the above, four colon carcinomas and two lung carcinomas were positive. Monoclonal B72.3 was also tested against normal breast tissue and normal lactating breast from non-cancer patients and showed no reactivity. Occasionally, the histiocytes or the polymorphonuclear leukocytes present in the stroma surrounding breast tumor cells showed positive cytoplasmic staining; this may be due to the reaction of B72.3 with antigen shed by tumor cells and phagocytized by the reactive cells. A variety of non-breast cells and tissues were tested and were negative; these included two uteruses, two livers, two spleens, three lungs, two bone marrows, five colons, one stomach, one salivary gland, five lymph nodes and one kidney.

III. Differential reactivity of monoclonal antibodies with human colon adenocarcinomas and adenomas. Monoclonal antibodies have been generated using membrane enriched extracts of human metastatic mammary carcinoma lesions, some of which demonstrated binding to the surface of human colon carcinoma cell lines. An analysis of the reactivity of three of these monoclonal antibodies was undertaken with fixed tissue sections of human colon adenocarcinomas and adenomas. The three monoclonals employed were B72.3, which is reactive with a 220,000d-400,000d high molecular weight glycoprotein complex; B6.2, reactive with a 90,000d glycoprotein, and B1.1, which is reactive with the 180,000d glycoprotein CEA. B1.1 was least selective in its reactivity to colon carcinoma versus adenoma lesions. Using 10 µg/ml of purified B1.1 IgG per slide, 94% (15 of 16) of carcinomas and 83% (15 of 18) of adenomas showed reactivity. Monoclonal B72.3 demonstrated the most selective reactivity for carcinomas. Eighty-two percent (14 of 17) of carcinomas were positive while none of 18 adenomas examined showed reactivity with greater than a few percent of adenoma cells positive. When a low concentration of purified B72.3 immunoglobulin was used per slide, 8 of 16 carcinomas and none of 46 adenomas or normal colon epithelium scored positive. Monoclonal B72.3 also reacted with cells in areas of "atypia" within adenomas. The reactivity of monoclonal B6.2 was intermediate as compared to B1.1 and B72.3 in its selectivity of reactivity for carcinoma cells. A heterogeneity in the populations of tumor cells showing reactivity with the 3 monoclonals was observed within many of the tumor masses. Both colon adenocarcinomas and adenomas can now be placed in several distinct groups based on their expression of antigens reactive with the three monoclonal antibodies employed.

Significance to Biomedical Research and the Program of the Institute:

These studies have led to the generation of a series of monoclonal antibodies that are reactive with approximately 85% of human mammary and colon carcinomas examined. These antibodies may eventually prove useful in the diagnosis, prognosis, and treatment of human mammary neoplasias, as well as in the study of the basic biology of human mammary tumor cell populations.

Proposed Course of Research:

We plan to further characterize the immunologic and biologic properties of the monoclonal antibodies described, and to generate and characterize new monoclonal antibodies to human colon and mammary tumor associated antigens. Specifically:

Definition of reactivities: To date, we have investigated the surface binding reactivities of each of the monoclonal antibodies described above to over 30 human cell lines; over 50 human carcinomas and over a dozen normal organs have been tested using the immunoperoxidase technique and solid phase RIAs. However, there are several areas of investigation concerning the biological distribution of the antigens detected that will be thoroughly investigated. These include: (a) reactivity with "benign" tumor lesions; (b) correlation of antibody binding (antigen presentation) with biologic behavior such as prognosis and response to therapy.

Generation of new monoclonal antibodies: Not all primary and metastatic mammary and colon tumor masses contain the antigens that are detected by the monoclonal antibodies we have generated to date. Furthermore, in some cases (such as with monoclonal B72.3) we have only one monoclonal, of a given isotype and to a single determinant, to a novel tumor associated antigen. We plan to obtain new monoclonals to different determinants of this antigen, and of differing isotypes and affinities, to be utilized to further define the structure and function of this molecule, and for sandwich radioimmunoassays. We also plan to use as immunogen those primary and metastatic masses that do not react with any of the monoclonals characterized to date, in an attempt to obtain a battery of monoclonal antibodies that are reactive with all primary and metastatic mammary tumor masses.

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Other Professional Personnel:

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Project DescriptionObjectives:

To radiolabel monoclonal antibodies reactive with human mammary tumor associated antigens without loss of immunoreactivity, and to use them to first localize human tumors in athymic mice. To determine what form of the antibody should be used for the radiolocalization studies, and if successful in model systems, to localize tumors in lymph nodes (via lymphangiography) and at distal sites in breast cancer patients.

Methods Employed:

Monoclonal antibodies were purified from ascitic fluids of mice by ion exchange chromatography and molecular sieving. Pepsin was then used to generate F(ab')₂ and Fab' fragments. The IgG and its fragments were radiolabeled with ¹²⁵I and assayed for immunoreactivity in solid phase radioimmunoassays. The radiolabeled antibodies were injected IV into athymic mice bearing human tumors and in vivo distribution of the label was determined by analyzing the tissues for radioactivity and by gamma scanning of the mice.

Major Findings:

(a) Labeling of antibody B6.2 IgG and fragments. Monoclonal B6.2 was purified by salt precipitation and ion exchange chromatography. Some of the purified IgG was used to generate F(ab')₂ and Fab' fragments by pepsin digestion. The fragments were purified by molecular sieving and retained all their immunoreactivity when compared on a molar basis to the intact IgG. The IgG and its fragments were labeled with ¹²⁵I using a variety of techniques, including lactoperoxidase, chloramine T and iodogen. The iodogen method gave the highest yield of labeled antibody without loss of immunoreactivity. The binding of the ¹²⁵I labeled IgG, F(ab')₂, and Fab' was better to the extracts from a breast tumor metastases than to extracts of the MCF-7 breast tumor cell line. No binding was observed to extracts from normal human liver, lymphoid cells, or a rhabdomyosarcoma. The labeled antibody was shown to bind to the surface of MCF-7 cells and retained the same specificity as the unlabeled antibody. More than 70% of the antibody remained immunoreactive after labeling. B6.2 IgG and fragments have also been radiolabeled with isotopes that are more useful for clinical use; namely ¹²³I, ¹³¹I and ¹¹¹In.

(b) Tumor distribution studies.

Athymic mice were trocared with pieces of a transplantable human mammary tumor (Clouser). After approximately 10-20 days, the tumors grew to detectable nodules. The growth rate of the tumors varied as did the final size obtained (0.5 to 2.5 cm diameter). Athymic mice were also injected subcutaneously with cells from a human breast tumor cell line (MCF-7) and a human melanoma cell line (A375). The mice injected with MCF-7 cells required subcutaneous implants of estrogen for the tumors to progress.

Mice were injected intravenously in the tail vein with approximately 0.1 μg of radiolabeled IgG or fragments, sacrificed at various times and the tissues assayed. The number of counts per mg of each tissue was determined and compared to that observed in the tumor of the same mouse.

Athymic mice bearing the Clouser human mammary tumor were first injected with B6.2 IgG labeled with ^{125}I to a specific activity of approximately 15 μCi per μg . The ratio of cpm/mg in the tumor versus various tissues rose over a 4 day period and then fell at 7 days. The tumor to tissue ratio was greater than 10:1 in the liver, spleen and kidney at day 4. Ratios of the counts in the tumor to that found in the brain and muscle were greater than 50:1 and as high as 110:1. Lower tumor to tissue ratios were obtained with blood and lungs (with its large blood pool). The rate of clearance of the labeled IgG from the blood was slightly faster than that published by other investigators, possibly because of the difference in isotopes. Similar results were obtained with B6.2 IgG radiolabeled with ^{123}I , ^{131}I and ^{111}In .

When the athymic mice bearing Clouser tumors were injected with ^{125}I -F(ab')₂ fragments of B6.2, higher tumor to tissue ratios were obtained. The highest tumor to tissue ratios of the organs tested were obtained with liver and spleen with ratios of 15 to 20:1 at 96 hours. The tumor to tissue ratios were somewhat lower with blood and lungs, but were higher than those obtained using IgG. This is probably due to the faster clearance of the F(ab')₂ fragments as compared to the IgG. The tumor to kidney ratio was relatively low and was due to the clearance of Fab' fragments which are either generated from the F(ab')₂ in vivo by the breakage of the cross linking disulfide bonds or are present as minor contaminants (less than 1%) of the starting material. Athymic mice bearing Clouser mammary tumors were also injected with ^{125}I -labeled B6.2 Fab'. The clearance rate of the Fab' fragment was considerably faster than the larger F(ab')₂ fragment and the intact IgG. Good tumor to tissue ratios were obtained within 8 hours, but the fast clearance rate resulted in a large amount of the labeled Fab' being found in the kidney and bladder.

Athymic mice bearing human melanomas were used as controls for non-specific binding of the labeled antibody. No preferential localization of the monoclonal antibody was observed in the tumor, in fact, counts per mg in the melanoma were lower than that found in many organs. Similar negative results were found when either normal murine IgG, F(ab')₂ or IgG₁ (the same isotype as B6.2) from a murine myeloma were inoculated into athymic mice with Clouser mammary tumor or melanoma transplants.

There was a variability in the tumor to blood ratio observed between different athymic mice bearing the Clouser mammary tumor. At 96 hours the ratios observed varied between 4 and 14:1. The variation may be due to either differences observed in tumor growth rate or antigenic differences among tumors (even though they arose from the same inoculum). The variability in tumor to blood ratios between mice may also be due to differences in blood supply to the tumors and thus in the ability of the labeled antibody to bind to the tumor. The mice bearing tumors derived from MCF-7 cells and BT-20 cells gave positive localization of the label, but at lower levels than that obtained with Clouser tumors. This result was not unexpected in that monoclonal B6.2 has consistently bound better to human tumor biops material than to tissue culture cells.

(c) Scanning of athymic mice bearing human tumors.

Studies were undertaken with the Diagnostic Radiology Departments of the NCI and Dana Farber Cancer Institute to determine whether the localization of the radio-labeled antibody in the tumor was sufficient to detect using a gamma camera. Athymic mice bearing the Clouser mammary tumor or the A375 melanoma were injected IV with approximately 30 μ Ci of B6.2 IgG. The mice were scanned and then sacrificed at 24 hour intervals. The B6.2 IgG was easily detected in the Clouser tumor at 24 hours with a small amount of activity visible in the blood pool. The tumor remained strongly positive over the 4 day period with the background activity decreasing to the point where it was barely detectable at 96 hours. No localization was observed in the mice bearing the human melanoma which is unreactive with B6.2. Mice were also injected with ^{125}I -B6.2 F(ab')₂. The mice cleared the fragments faster than the intact IgG and a significant amount of activity can be observed in the kidneys and bladder at 24 hours, but the tumor is clearly positive for localization of the ^{125}I -B6.2 F(ab')₂ fragments. The activity is cleared from the kidney and bladder by 48 hours and the tumor to background ratio increased over the 4 day period of scanning with little background activity observed at 96 hours. No localization of activity was observed in the mice bearing the A375 melanoma.

Significance to Biomedical Research and the Program of the Institute:

Radiolabeled polyclonal antibodies have previously been used to detect metastases. The use of monoclonal antibodies will give to this technique an added specificity and thus less computer manipulation and general applicability. The ability to assess internal mammary chain involvement may be of benefit in the staging of patients. The ability of the antibody to localize metastatic lesions may also prove useful as an adjunct in monitoring efficacy of therapy.

Proposed Course of Research:

F(ab')₂ fragments of monoclonal antibody B6.2 labeled with ^{125}I will be used to determine the minimum size tumor that can be detected by gamma camera scanning. The distribution of the antibody in the tumor will be examined by autoradiography of tumor sections of scanned mice. The efficacy of other radiolabeled monoclonal antibodies made against human breast tumor metastases, such as B72.3 for radio-immunolocalization will be studied.

While ^{125}I -labeled monoclonal antibodies work well in the murine model systems, ^{125}I is not applicable in human studies because of its low energy. Other radioactive iodines will be further studied to determine their effect on the antibody, specific activity obtainable and efficiency of localization in mice. Other isotopes such as ^{111}In are currently being used in nuclear medicine. The feasibility of binding these isotopes to monoclonal antibodies, i.e., maintaining the stability of the label-antibody bond and immunoreactivity, will be further examined. The feasibility of binding isotopes with therapeutic potential will also be studied.

We plan to use radioactively labeled monoclonal antibody B6.2 to localize human mammary tumors in patients with advanced disease. Iodinated antibody will be used in initial studies. The human experiments to detect distal metastases, and lymph node lesions via lymphangiography, will be performed in collaboration with the

Diagnostic Radiology Departments of the Dana Farber Cancer Institute and the NCI.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

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Z01CB05233-02 LTIB
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Z01CP05233-01 LCMB

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.)

Identification and Purification of Mammary Tumor Associated Antigens

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

(Name, title, laboratory, and institute affiliation)

David Colcher, Microbiologist, Lab. of Tumor Immunology and Biology, DCBD, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.3

PROFESSIONAL:

1.3

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Monoclonal antibodies to human mammary tumor metastases were tested for reactivity to novel and known tumor associated antigens. The monoclonals were used to immunoprecipitate antigens from a radiolabeled breast tumor metastasis extract. Monoclonal antibody B72.3 immunoprecipitated a high molecular weight polypeptide complex of approximately 220,000d. B6.2 and four other antibodies immunoprecipitated a 90,000d polypeptide. The four other antibodies cross-react in RIA for monoclonal B6.2 but differ in their ability to compete with the binding of B6.2. Two antibodies, B1.1 and F5.5, were shown to differentially react with carcinoembryonic antigen. The high molecular weight complex identified by monoclonal B72.3 has been preparatively purified, using molecular sieving and antibody affinity chromatography, without loss of immunoreactivity. Studies are in progress to develop radioimmunoassays with several of the monoclonals described. Radioimmunoassays for monoclonal antibody B72.3 have been established and optimized, with a sensitivity in the nanogram range.

Other Professional Personnel:

Andrew Paterson	Visiting Fellow	LTIB, NCI
Jeffrey Schlom	Chief, Lab. of Tumor Immunology & Biology	NCI

Project DescriptionObjectives:

To identify and characterize tumor associated antigens reactive with monoclonal antibodies. To determine if these monoclonal antibodies bind to antigens distinct from known tumor associated antigens such as CEA, ferritin, T and M-N blood group antigens, and determine how many distinct tumor associated antigens are detected by these monoclonal antibodies. To preparatively purify these tumor associated antigens to aid in the development of radioimmunoassays (RIAs) for their detection in human tissues and biological fluids. The RIAs may eventually prove useful in the diagnosis, prognosis, detection of recurrence, and monitoring efficacy of treatment of human mammary cancer.

Methods Employed:

Presence of tumor associated antigens, reactive with monoclonal antibodies, in primary tumor metastases and cell lines was determined by solid phase RIA. The molecular weight of these antigens was determined by immunoprecipitation and SDS polyacrylamide gel electrophoresis and western blotting technique. Purification of the antigens was performed by molecular sieving and antibody affinity column chromatography. A competitive binding assay was used to determine whether monoclonal antibodies reacting with the same antigen reacted also with the same binding site.

Major Findings:

Selection of an antigen source for the identification of tumor associated antigens. As a first step to the identification of tumor associated antigens reactive with the monoclonal antibodies described, the most immunoreactive antigen source for each antibody was determined. Monoclonal antibodies were first screened for reactivity with several previously well characterized tumor associated antigens, including carcinoembryonic antigen (CEA, derived from colon carcinoma), ferritin (derived from liver), acid placental ferritin, T antigen and M-N blood group antigens. Strong positive reactivity to CEA was observed only with monoclonal antibodies B1.1 and F5.5. No reactivity was observed to the other antigens with any of the monoclonal antibodies tested. As a next step in the identification of the best antigen source, monoclonal antibodies were screened by solid phase RIA for reactivity with a variety of mammary tumor and colon tumor extracts, including primary and metastatic tumors and established cell lines. Monoclonals B1.1 and B6.2 reacted similarly with tissue extracts and extracts of the cell lines. Antibody B72.3, however, showed very strong reactivity with some human mammary and colon tumor extracts, but reacted poorly with mammary tumor cell lines. B72.3 did show some weak reactivity to extracts of a colon carcinoma cell line and strong reactivity to tumors in athymic mice, derived from this cell line. Two breast tumor metastases to the liver were chosen as prime sources for antigen identification and purification on the

basis of their broad immunoreactivity to all the monoclonal antibodies and the quantity of tumor tissue available.

Characterization of monoclonal antibodies to CEA. Antibodies B1.1 and F5.5 were shown to bind in a solid phase RIA to CEA purified from a colon carcinoma patient. Cross reactivities have been reported, however, between determinants on CEA and an antigen expressed in normal spleen and on granulocytes termed normal cross-reaction antigen (NCA). Both monoclonals B1.1 and F5.5 were shown to react with some splenic extracts as well as some granulocyte preparations. Purified immunoglobulin preparations of monoclonals B1.1 and F5.5 were titered by serial dilution for binding to CEA preparations purified from five different patients with colon cancer. Monoclonal F5.5 reacted similarly with all five CEA preparations, whereas B1.1 bound differentially to the various preparations. The differences in binding of the antibodies to the different CEA preparations further demonstrate the heterogeneity of the CEA family of glycoproteins.

Immunoprecipitation of tumor associated antigens. Immunoprecipitation studies were initiated to determine the molecular weights of the tumor associated antigens reactive with the monoclonal antibodies described. Purified CEA was iodinated and used as antigen source for the binding of B1.1 and F5.5. SDS-PAGE of the immunoprecipitates shows that the polypeptide precipitated by both monoclonal antibodies is a heterogeneous protein with an average molecular weight of 180,000d. An extract of a breast tumor metastasis to the liver was used as the antigen source for the other monoclonal antibodies described. The reactivity of B1.1 and F5.5 to this breast tumor metastasis was also tested to determine if a polypeptide similar in molecular weight to CEA would be precipitated. Initial attempts to identify the various reactive antigens in radioiodinated extracts of the metastasis were unsuccessful. Experiments were undertaken to increase the relative antigen concentration by partial purification of the extract. The metastatic liver extract was detergent-disrupted and separated using molecular sieving on Ultrogel AcA34. The column fractions were assayed for reactivity with monoclonals B1.1, B6.2, and B72.3 by solid phase RIA. The appropriate immunoreactive fractions were then pooled and labeled with ^{125}I . SDS-PAGE analyses of the immunoprecipitates showed that B72.3 immunoprecipitated a complex of four bands with estimated molecular weights of approximately 220,000, 250,000, 285,000, and 340,000. B1.1 immunoprecipitated a heterogeneous component with an average estimated molecular weight of 180,000. B6.2 immunoprecipitated a 90,000d component as did several other monoclonal antibodies.

Analysis of cell extracts by "western" blotting. Extracts of a breast tumor metastasis to the liver, normal liver, the MCF-7 breast tumor cell line and the LS174T colon tumor cell line and tumors derived from it were disrupted and run on an SDS-polyacrylamide gel. The polypeptides were electrophoretically transferred to nitrocellulose filters, and the filters were incubated with IgG from B1.1, B6.2 or B72.3. The filters were washed, and the remaining antibodies were detected with rabbit anti-murine IgG and ^{125}I -Protein A. B72.3 bound to a high-molecular weight complex of approximately 220,000d in the extracts from the metastasis and the colon tumor. B1.1 bound to a 180,000d polypeptide and B6.2 bound to a 90,000d polypeptide in extracts of the breast tumor metastasis, colon tumor and the MCF-7 cell line. These data demonstrate

that the immunoreactivity of the antigenic determinants are not destroyed by SDS and mercaptoethanol and that molecular weights of the polypeptides in the crude extracts are consistent with those obtained by the immunoprecipitations from semipurified extracts as described above.

Characterization of monoclonal antibodies to the 90,000d antigen. Five of the monoclonal antibodies, including B6.2, were reactive with an antigen of approximately 90,000d. To determine whether these antibodies reacted with the same determinants, a competitive binding assay was established. Purified monoclonal antibody B6.2 was labeled with ^{125}I . Increasing amounts of unlabeled monoclonal antibodies were added to a breast tumor extract followed by the addition of ^{125}I -labeled B6.2 IgG. As little as 10 ng of B6.2 IgG was able to inhibit the binding of the labeled antibody by greater than 90%. Similar inhibition was observed with B84.1 IgG. Various degrees of competition were also observed with other antibodies (B39.1, B14.2, B50.4, F25.2, B84.1). The ability of some of the other monoclonal antibodies to compete for the binding of B6.2 to the breast tumor metastasis extract indicates that these antibodies react to the same antigen.

Purification of the 200,000d high molecular weight complex. Monoclonal antibody B72.3 has been shown to have highly selective reactivity to tumor versus normal tissues. We thus attempted to purify the antigen reactive with B72.3 first, so that further immunological and biochemical characterization could be made. An extract of a breast tumor metastasis to the liver, which contained the highest immunoreactivity with B72.3, was used as the starting material for purification of the 200,000d high molecular weight complex. Following detergent disruption and high speed centrifugation, the supernatant was subjected to molecular sieving using Ultrogel AcA34. Immunoreactive fractions were then passed through a B72.3 antibody affinity column and eluted with 3 M KSCN. Radiolabeled aliquots from the various purification steps were analyzed by SDS-PAGE. Only minimal radioactivity in the high molecular weight range was seen in gel patterns of the AcA34 pool, whereas the affinity column eluant demonstrated the four distinct bands of the 220,000d complex. ^{125}I -labeled B72.3 affinity purified antigen was tested for immunoreactivity by solid phase RIA. Approximately 70% of the purified ^{125}I -labeled antigen was bound in B72.3 antibody excess. The identical method of purification was used with a normal human liver extract as the starting tissue. At no step within the purification scheme was any reactivity with B72.3 detected.

Radioimmunoassay for tumor associated antigens. Soluble antigens which react with the monoclonal antibodies B1.1, B6.2 and B72.3, could be detected and quantitated with a competitive solid phase radioimmunoassay. Samples containing the test or standard antigen were incubated with one of the monoclonal antibodies followed by an incubation of this mixture in microtiter wells that were coated with a target tumor antigen. Free antibody was immobilized via the target antigen and was detected in a third incubation step with ^{125}I -labeled goat anti-mouse IgG antibody. Assay conditions, in which the target antigen coupling procedure, reagent concentrations, buffers and incubation temperature and times were investigated extensively for B6.2 and B72.3 antibodies. Sensitivity was maximized when the extract of breast tumor metastasis to the liver used as the target antigen, was dried onto the microtiter plates at 5 $\mu\text{g}/50 \mu\text{l}$ PBS/well. Decreasing B72.3 antibody concentration from 200 ng to 2 ng/ml

resulted in increased sensitivity. A concentration of 10 ng/ml of this antibody was subsequently used resulting in a sensitivity of 1-2 µg/ml of the tumor extract and approximately 1-2 ng/ml of the antigen bound by monoclonal antibody B72.3.

Significance to Biomedical Research and the Program of the Institute.

The identification and characterization of mammary tumor associated antigens will enable us to compare these antigens with tumor associated antigens from other sources. The purification of these antigens will also aid in the establishment of radioimmunoassays for their detection in human tissues and/or fluids. These assays may prove useful in the diagnosis and prognosis of patients with mammary cancer, in the detection of recurrence of the disease, and in the monitoring the efficacy of various therapeutic regimens. Sequencing of the purified antigens and comparison to other polypeptides may aid in the determination of the biological activity and significance of the tumor associated antigens. The sequence of the tumor associated antigen will (a) enable the synthesis of portions of the peptide that may be useful in subsequent RIAs and (b) enable the synthesis of a cDNA copy that will be useful in studies to clone the genes coding for the peptide.

Proposed Course of Research:

The antigens detected by antibodies B1.1 (180,000d, CEA), B6.2 (90,000d) and B72.3 (220,000d complex) will be purified from the most available source of antigen using gel filtration and antibody affinity chromatography. If these antigens lose immunoreactivity after elution from the affinity column, an alternative purification method will be employed. Ion-exchange chromatography will be performed followed by separation based on the isoelectric point, using either preparative isoelectrofocusing in a granular flat bed or chromatofocusing.

A collaboration has been established to sequence the polypeptides, once sufficient quantities can be purified. The sequencing will be performed using either unlabeled antigens purified from breast tumor metastases or ³H-amino acid labeled polypeptides isolated from cell lines. The sequencing studies will enable us to compare the tumor associated antigens identified by the monoclonal antibodies to antigens that have been previously sequenced. Also, a synthetic peptide could be generated that may be useful in developing radioimmunoassays for the antigens.

Radioimmunoassays for the detection of tumor associated antigens and the antibodies reactive with them will be established. The various RIAs described above will be used for the assay of blood and other biological fluids, and for examination of the biological distribution of the tumor associated antigens in various tissues. Studies will be undertaken to examine sera of breast cancer and colon cancer patients obtained before and during their course of therapy.

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Other Professional Personnel:

John Greiner	Staff Fellow	LTIB, NCI
Jeffrey Schlom	Chief, Lab. of Tumor Immunology and Biology	NCI

Project DescriptionObjectives:

To define the antigenic heterogeneity that may exist within a given human mammary tumor mass. To determine parameters which mediate the expression of various antigenic phenotypes within mammary tumor cell populations. To develop appropriate model systems to study antigenic heterogeneity in vitro and in vivo.

Methods Employed:

The expression of human mammary tumor associated antigens recognized by monoclonal antibodies is determined using (1) the immunoperoxidase method on sections of human mammary tumors and on mammary tumor cell lines; (2) fluorescent activated cell sorter analyses of tumor and normal cell lines; and (3) the live cell radio-immunoassay which determines the binding of the antibody to the surface of cells in culture.

Major Findings:

I. Differential expression of tumor-associated antigens within a given mammary tumor mass. Antigenic variation was observed in the expression of tumor associated antigens within a given mammary tumor. One pattern of antigenic reactivity observed repeatedly, using the immunoperoxidase method and tissue sections, was that one area of a mammary tumor contained tumor associated antigens reactive with a particular monoclonal antibody; while another area of the same mammary tumor was not reactive with the identical antibody. Another type of antigenic heterogeneity was observed among cells in a given area of a tumor mass. This type of antigenic diversity, termed "patchwork," is demonstrated by the presence of tumor cells expressing a specific tumor associated antigen directly adjacent to tumor cells negative for the same antigen. Patterns of reactivity with a specific monoclonal antibody were also observed to vary within a given mammary tumor mass. For example, monoclonal antibody B72.3 reacted with the 220,000d tumor associated antigen present in the cytoplasm of cells in one part of the tumor mass, and on the luminal edge of differentiated structures in a different part of the same tumor mass.

II. Heterogeneity of tumor associated antigen expression within human mammary tumor cell lines. In an attempt to elucidate the phenomenon of heterogeneity of antigenic phenotypes within a human mammary tumor mass, model systems were examined. Human mammary tumor cell lines, transplanted in nude mice, also demonstrate antigenic heterogeneity. To determine if this phenomenon also exists in human mammary tumor cell lines grown in vitro, MCF-7 cells were tested for the presence of tumor associated antigens using the cytoSpin/immunoperoxidase method. The MCF-7 cell line contained various subpopulations of cells as defined by variability in expression of the tumor associated antigen reactive with

antibody B6.2. Positive MCF-7 cells were seen adjacent to cells which scored negative. In addition, variability was observed with respect to the quantity of the 90,000d tumor associated antigen detected among the population of cells which were positive, as demonstrated by differences in the intensity of the stain. A similar heterogeneity, with fewer cells (less than 10%) scoring positive, was also observed using monoclonal B72.3.

III. Parameters affecting expression of tumor associated antigens in vitro.

(a) Fluorescent activated cell sorter (FACS) analyses. Monoclonal antibodies B6.2 and B38.1 were analyzed via FACS for surface binding to a panel of human cell lines. Both antibodies were reactive with breast carcinoma lines BT-20 and MCF-7. A non-reactive monoclonal antibody (anti-human IgM) of the identical IgG-1 isotope showed no binding. The fluorescence pattern with B38.1 was consistently more intense than that seen with B6.2. In contrast to the breast carcinoma cell lines, a cell line derived from prostrate carcinoma (PC-3) showed strong reactivity with B38.1 but was unreactive with B6.2. Both antibodies were unreactive with WI-38 embryonic lung, CEM lymphoblasts, and normal bone marrow.

(b) Relationship of cellular growth patterns to expression of antigenic phenotypes. The cell surface binding of monoclonal antibody B6.2 was monitored on MCF-7 mammary carcinoma cells during logarithmic growth phase and at density-dependent arrest. Antibody binding was monitored each day for seven consecutive days after passage. One day after passage, B6.2 was strongly reactive with the MCF-7 cells. Over the following seven days, the reactivity of the monoclonal decreased progressively and was lowest at density dependent arrest. Passage of these cells on day 7 resulted in the reappearance of the antigen within 24 hours. One explanation for these results would be a cell cycle related expression of the antigen. Similar results were observed with monoclonal B38.1.

To investigate the relationship of antigen expression to cell cycle phase, day 7 cells at density-dependent arrest were passed and monitored 24 hours later for both DNA content and cell surface antigen expression via FACS. DNA staining by Hoechst dye demonstrated that the majority of cells were in the G_0/G_1 phase. The remainder of cells were distributed evenly throughout S-phase and G_2/M phase. G_0/G_1 cells express background fluorescence. In contrast, monoclonal antibody B38.1 was most reactive with cells in S-phase. This S-phase differential binding was also observed using monoclonal antibody B6.2.

(c) Effect of prolonged exposure of MCF-7 cells to antibody on expression of cell surface tumor associated antigens.

In light of the phenomena of "capping," "internalization," and "shedding" of lymphocyte surface antigens after exposure to monoclonal antibodies, studies were undertaken to determine the effect of prolonged exposure of MCF-7 cells to monoclonal antibodies B6.2 and B38.1 on cell surface expression of the reactive antigens. MCF-7 cells were incubated in the presence of monoclonals B6.2 or B38.1 for two hours, washed extensively and then refed with growth medium. Cells were then incubated for an additional 22 hours and analyzed following the addition of G/M FITC. The fluorescence profile was again similar to that achieved during a 2 hour or 24 hour antibody incubation. The membrane expression of the reactive

antigens therefore appears to be stable, despite continued exposure to antibody.

IV. Antigenic drift of mammary tumor cell populations. Studies were undertaken to determine if any drift in antigenic phenotypes could be observed in human mammary tumor cell lines over an extended period of time. The BT-20 cell line, obtained at passage 288 from the Breast Cancer Task Force, was serially passaged and assayed at each passage level during S-phase of the growth cycle. A cell surface HLA antigen detected by monoclonal antibody W6/32, was present at all passage levels, as was the tumor associated antigen detected by monoclonal antibody B38.1. The 90,000d antigen detected by monoclonal antibody B6.2 was expressed on the BT-20 cell surface up to passage 319, but was not evident after this passage level. Similarly, monoclonal B14.2 reacted with BT-20 cells only up to passage 317. This phenomenon was observed in three separate experiments at approximately the same passage levels, using cells thawed from a BT-20 culture viably frozen at passage level 302. Antigenic drift was also observed with the antibodies to the cell surface. MCF-7 cell lines were obtained from four sources and were examined for surface expression of several tumor associated antigens. Karyotype profiles of the four cell lines were examined (W. Peterson, Child Research Center, Detroit) and were identical and characteristic of the MCF-7 cell line. A single LDH band, characteristic of only a few breast tumor cell lines including MCF-7, was also supportive evidence that these cell lines were indeed MCF-7. Using a live cell solid phase RIA, which detects the reactivity of antigens at the cell surface, immunologic profiles of the four MCF-7 cell lines were determined. Monoclonal antibody B139, which recognizes an antigen on the surface of all human cells reacted with all four cell lines. However, when other monoclonal antibodies were tested, several different antigenic phenotypes emerge. The MCF-7 cell line obtained from the Breast Cancer Task Force contained all the tumor associated antigens assayed for, while the MCF-7 (GC) cell line expressed none. Similar results were also observed with BT-20 cell lines obtained from different sources.

V. Antigenic Heterogeneity of Tumor Associated Antigens (TAAs) of MCF-7 clones. To further understand the nature of antigenic heterogeneity of human mammary tumor cell populations, MCF-7 cells were cloned by end-point dilution. Ten different clones were obtained and assayed, using the live cell RIA, for cell surface TAAs. The parent MCF-7 culture reacts most strongly with monoclonal antibody B1.1 and least with monoclonal B72.3. Clone 6F1 appears to have a similar antigenic phenotype to that of the parent. At least three additional major phenotypes were observed among the other clones. Clone 10B5, for example, does not express the TAAs reactive with monoclonals B1.1, B6.2 and B72.3 although it does contain HLA and human antigens detected by monoclonals W6/32 and B139, respectively. A third phenotype, in which four-fold less expression of TAA as compared with the parent was consistently observed, was demonstrated by clone 5H7. Clone 5A9 showed a weak reaction with monoclonals B1.1 and B6.2 and a stronger reaction with monoclonal B72.3; this phenotype is the opposite of that seen with the parent cell line. Variation of expression of cell surface TAAs among the clones was corroborated using the immunoperoxidase/cytospin method. Each of the clones contains several subpopulations of cells as demonstrated by differences in expression of TAAs reactive with monoclonals B1.1, B6.2, and B72.3.

To determine the stability of the cell surface phenotype of the MCF-7 clones,

each cell line was monitored through a four-month period and assayed during log phase at approximately every other passage. While some of the MCF-7 clones maintained a stable antigenic phenotype during this time period, other clones underwent a dramatic change in their cell surface antigen expression. For example, clone 10B5 was negative through passage 6 for antigens reactive with monoclonal antibodies B1.1, B6.2 and B72.3. The antigens detected by monoclonals B6.2 and B1.1 became increasingly expressed on the cell surface from passages 9 to 15. Change in antigenic phenotype appeared to be independent of growth rate of the cloned cell lines.

Significance to Biomedical Research and the Program of the Institute:

Heterogeneity of cells within a given tumor mass, including antigenicity, response to drugs, and presence of cell surface receptors has been observed. The differences among the cell populations present a problem in the development and optimization of immunodiagnostic and therapeutic procedures for breast cancer. Knowledge about the nature of this antigenic heterogeneity may help to predict or control the expression of a specific antigenic phenotype. The studies described here have enabled us to demonstrate this antigenic heterogeneity within human mammary tumor populations both in vivo and in vitro. An examination of the effect of cell cycle, cellular growth patterns, and continued cell passage has enabled us to determine some of the parameters associated with a change in phenotype. Clones of the MCF-7 cell line exhibiting distinct antigenic phenotypes provide excellent model systems to study the nature of antigenic diversity. Moreover, preliminary studies relating the effect of retinoic acid or progesterone on the expression of specific antigens indicate that the potential exists to regulate or amplify the tumor antigen level on the surface of cells. These studies may therefore ultimately aid in both the detection and therapy of breast cancer.

Proposed Course of Research:

Future studies will involve the use of the cloned MCF-7 mammary tumor cell populations for the delineation of the nature of antigenic diversity. Several parameters of antigenic heterogeneity will be examined with cloned cell populations exhibiting distinct antigenic phenotypes, including the relationship between the expression of a specific antigenic phenotype and such parameters as morphology, tumorigenicity, drug susceptibility, estrogen receptors, and growth rate.

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Other Professional Personnel:

Patricia Hand	Chemist	LTIB, NCI
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Project DescriptionObjectives:

To identify chemotherapeutic and other compounds that have immunomodulatory activities which increase the presentation of specific antigens on the cell surface of human tumor cells. To determine whether recombinant forms of human interferons can increase the expression of genes that code for tumor associated as well as histocompatibility antigens on the surface of human breast and colon cells. To develop appropriate model systems to further evaluate the relationship between interferon and other immunoregulatory compounds and the modulation of cell surface antigen expression recognized by the binding of monoclonal antibodies.

Methods Employed:

The expression of human breast and colon tumors associated and normal histocompatibility antigens which bind specific monoclonal antibodies was determined by (1) live cell radioimmunoassay to quantitatively assess the relative amount of monoclonal binding to the cell surface, (2) an immunoperoxidase method using human breast and colon cell lines prepared after treatment with the appropriate monoclonal antibody, (3) fluorescent activated cell sorting and (4) in vivo localization of a tagged monoclonal antibody.

Major Findings:

- I. Enhanced expression of surface antigens after treatment of human tumor cells with interferon

The administration of recombinant leukocyte A interferon (IFN) to human breast (MCF-7) or colon (WIDR) carcinoma cells increases the gene expression of both tumor associated and major histocompatibility antigens. The expression of tumor associated antigens on the surface of MCF-7 cells recognized by monoclonal antibodies B72.3, B1.1 and B6.2 increased followed the addition of IFN. Similarly, the binding of a monoclonal (W6/32) to the histocompatibility antigen, HLA-A, B, C, rose following IFN treatment. The binding of these monoclonals with the exception of B72.3, to the surface of the human colon carcinoma cell, WIDR, was also increased by IFN. WIDR cells do not express the gene encoding for the surface antigen, B72.3. Another monoclonal, B139, which binds to a determinant found on all human cells, whether normal or tumor is unaltered in the MCF-7 cells which are treated with IFN. However, B139 binding to the surface of WIDR cells rose following IFN administration. Therefore, IFN can differentially increase the expression of tumor associated, histocompatibility and other surface antigens on human breast and colon carcinoma cells. Furthermore, IFN treatment cannot initiate the appearance of a gene product (B72.3) in those cells (WIDR) not expressing a constitutive level of the surface antigen.

II. Comparison of IFN effect on various clones of MCF-7

Ten cloned cell lines from the parent MCF-7 cell have been characterized as to their individual antigenic phenotypes. These phenotypes vary widely from those that bind monoclonal antibodies B1.1, B72.3, B139 and W6/32 similar to that observed in the parental MCF-7 cells, to those which have substantially different antigenic heterogeneity. The enhancement of tumor associated and histocompatibility antigens in response to addition of leukocyte IFN also is dramatically different among the MCF-7 clones. IFN treatment had no effect on the binding of any monoclonals to the cell surface of three cloned lines. In two other cloned MCF-7 cell lines, IFN increased the tumor associated antigens, B1.1 and B72.3, but the expression of the normal cell surface antigen constituents recognized by B139 and W6/32 remain unchanged. In another cloned MCF-7 line, IFN treatment increase the normal cell surface antigens but had no effect on the tumor associated antigens which bind monoclonals B1.1 and B72.3. In all, six different types of response to leukocyte IFN was observed in the MCF-7 clones again indicating the tumor cell heterogeneity within this breast carcinoma cell line.

III. Analysis of the effects of IFN on antigen expression in MCF-7 and WIDR cells by immunoperoxidase

The effects of IFN on the expression of tumor associated and normal cell surface antigens observed using the live cell RIA were confirmed with the cyto-spin and immunoperoxidase techniques. Parental MCF-7 cells were treated for 24 h with IFN. Following treatment the cells were incubated in the presence of saturating amounts of B1.1, B72.3, B139 and W6/32 and later fixed with methanol. Staining by the immunoperoxidase technique revealed that IFN treatment resulted in a significantly higher percentage of the cells which stained positive for the respective monoclonal and a concomittant appearance of cells which express a higher intensity of stain. Similar results were also demonstrated with the human colon carcinoma cell line, WIDR. Therefore, the enhanced cell surface antigen expression following IFN treatment seems to be a result of the expression of the surface antigens in more cells of the population and more antigens being expressive on certain cells within that same population.

Significance of Biomedical Research and the Program of the Institute:

The development of monoclonal antibodies to cell surface antigens on human tumor cells presents an efficient, specific mechanism for the delivery of a spectrum of reagents to the tumor site. The studies described within have shown that interferon which can increase the expression of normal histocompatibility antigens can also enhance the presentation of tumor associated antigens at the cell surface. These findings indicate that interferon and possibly other chemotherapeutic-like compounds can regulate the expression of these gene products in tumor cells as well as in normal cells. Furthermore, the varied responsiveness of the different cloned human tumor cell lines to the immunoregulatory (i.e., antigen enhancement) activities of IFN is a further characterization of the heterogeneity of human tumor cells. In any case, the ability to amplify the presentation of tumor associated antigens on the surface of human tumor cells with biological modifiers, such as interferon, has substantial relevance to the

detection and therapy of primary and metastatic human cancers with therapeutically-tagged monoclonal antibodies.

Proposed Course of Research:

Future studies will complete the analysis of the different recombinant forms of IFN on their ability to enhance tumor associated antigen expression on the surface of human breast and colon cells. A further examination of the tumor cell heterogeneity will attempt to correlate antigenic phenotype, responsiveness to IFN, tumorigenicity and IFN-mediated growth inhibition in the different cloned tumor cells. Moreover, the efficacy of IFN enhancement of other tumor associated and histocompatibility antigens on human tumor cells will be evaluated. Upon completion of the IFN study other agents which can differentiate human tumor cells and possibly elicit a concomitant increase in the expression of tumor associated antigens will be examined.

Publications:

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Horan Hand, P., Colcher, D., Wunderlich, D., Nuti, M., Teramoto, Y.A., Kufe, D. and Schlom, J.: Rational basis for the diagnostic, prognostic, and therapeutic utility of monoclonal antibodies in the management of human breast cancer. In Chabner, B.A. (Ed.): Rational Basis for Chemotherapy, Proceedings of the UCLA Symposium. New York, Alan Liss, Inc., 1983, vol. 1, pp. 315-358.

Kufe, D.W., Nadler, L., Sargent, L., Shapiro, H., Hand, P., Austin, F., Colcher, D. and Schlom, J.: Biological behavior of human breast carcinoma-associated antigens expressed during cellular proliferation. Cancer Res. 43: 851-857, 1983.

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Other Professional Personnel:

Patricia Horan Hand	Chemist	LTIB, NCI
Jeffrey Schlom	Chief, Lab. of Tumor Immunology & Biology	NCI

Project DescriptionObjectives:

To generate and characterize monoclonal antibodies to the major structural proteins of murine mammary tumor viruses (MMTVs) in order to: (a) delineate type, group, and interspecies determinants among MMTVs derived from various strains of laboratory mice (M. musculus) and species of the genus Mus; (b) determine if viral coded antigenic determinants are expressed in naturally occurring neoplasms (emphasizing mammary neoplasias) of feral populations of species other than M. musculus; and (c) to determine the degree, if any, of antigenic heterogeneity that exists among naturally occurring mammary neoplasms of the genus Mus, between different mammary tumors of a given species, and within a given tumor mass.

Methods Employed:

Hybridoma technology was employed to generate monoclonal antibodies to MTV gene products. These antibodies were screened by solid phase radioimmunoassays using purified MMTVs from various strains of mice, purified polypeptides from these viruses and membranes of tissue culture cells. The specificity of the monoclonal antibodies were further tested using immunoprecipitation of radiolabeled MTV viral polypeptides. Murine mammary tumors from various species of the genus Mus were tested for reactivity with the monoclonal antibodies using the immunoperoxidase technique.

Major Findings:

I. Specificity of monoclonal antibodies. Mice and rats were immunized with disrupted MMTVs from M. musculus (MMTV(C3H)), M. cervicolor (MC-MTV), and M. cookii (MCo-MTV). Spleen cells from immunized mice were fused with the NS-1 murine non-immunoglobulin secreting myeloma cell line and standard hybridoma technology was employed to obtain double cloned cultures synthesizing monoclonal antibodies. All the monoclonal antibodies bound selectively to disrupted MMTV. Reactivities were further examined for binding to membrane-enriched extracts of cells producing MMTV(C3H). All the monoclonal antibodies bound to membranes from both a C3H mammary tumor cell line and feline cells (CrFK) producing MMTV(C3H). No binding was observed, however, to membranes of C3H fibroblasts or to the uninfected feline cell line. These findings provide evidence that the antigenic determinants recognized by these antibodies are viral mediated and most probably viral coded.

The monoclonal antibodies that were generated against the type B retroviruses from M. cervicolor and M. cookii were tested for reactivity to retroviruses from a variety of species. The monoclonal antibodies M1.1 (prepared from animals immunized with MC-MTV) and M3.1 (prepared from animals immunized against MCo-MTV) both react to MTV's from M. cervicolor milk and from M. musculus produced by either murine or feline cells. None of the monoclonal antibodies tested bound

to murine leukemia viruses from M. musculus, the M432 retrovirus from M. cervicolor, or a variety of retroviruses of different species.

The major MMTV(C3H) polypeptides, gp52, gp36, and p28, were purified using ion-exchange chromatography and molecular sieving. Monoclonal antibodies were then tested in solid-phase RIA's against each purified polypeptide. Antibodies were identified in this manner as reactive with either the MMTV major external glycoproteins, gp52 or gp36, or with the major internal polypeptide p28. The polypeptide specificity of the monoclonal antibodies was further identified by immunoprecipitation of disrupted iodinated MMTV.

II. Diversity of MMTV gene products. The monoclonal antibodies were tested for their ability to distinguish various MMTVs from several strains of mice. Many of the viruses were also propagated in feline cells to ensure that the reactivities observed were directed against viral-coded determinants. All the MMTVs could be easily distinguished. In most cases, the source of the MMTV could be identified using only one monoclonal antibody. The only virus that required more than one monoclonal antibody to distinguish it from the others was the MMTV(BALB/c); that could be accomplished, however, using antibodies M1.1 and M2.1. Several monoclonal antibodies have also been generated that react to all the MMTVs tested.

III. Reactivity with mammary tumors of the genus Mus. The immunoperoxidase technique was utilized to determine if the monoclonal antibodies could be used to detect antigenic determinants in tissue sections of primary and transplanted murine mammary tumors. Two major types of staining patterns were observed: apical, in which the immune reaction was concentrated at the periphery of lumens of acini and focal, in which the immune reaction was detected as discrete intracytoplasmic foci. Two phenomena became apparent when using monoclonal antibodies to monitor expression of a distinct antigenic determinant in tumor cells. The first was that a given antigenic determinant can be expressed in a different manner in two different mammary tumors. The second was the heterogeneity in staining within a given mammary tumor; i.e., most mammary tumors tested from a variety of M. musculus strains and Mus species presented some areas that were positive and some that were negative for expression of a given determinant.

The immunoperoxidase technique was also used to further define interspecies reactivities of certain monoclonal antibodies. Monoclonal antibody M3.1 reacted significantly with sections of primary mammary tumors of M. musculus (C3H), M. cookii, and M. cervicolor. This antibody did not, however, react with sections of lactating mammary glands of C57BL mice, a strain devoid of MTV antigen expression in its milk. The immunoperoxidase method has also proved to be extremely useful in the detection of tumor-associated antigens by monoclonal antibodies in segments of tumors too small to be used in other immunological assays.

Significance to Biomedical Research and the Program of the Institute:

These studies involve the generation and characterization of a repertoire of monoclonal antibodies to the major structural proteins of MMTVs. These antibodies have demonstrated the diversity of defined viral gene products in various mouse strains, and provide defined immunologic reagents to investigate

the most widely studied model of naturally occurring mammary neoplasia and preneoplasia. The use of the immunoperoxidase technique with monoclonal antibodies has revealed the heterogeneity of antigen expression in mammary tumors, i.e., that a single antigenic determinant may be expressed in a different manner not only among different mammary tumors but in different areas of the same mammary tumor. The monoclonal antibodies directed against various interspecies determinants of MTV gene products now also make possible an evaluation of the association between the expression of specific viral gene products and mammary tumorigenesis in feral populations of species other than *M. musculus*. These monoclonal antibodies may prove quite useful in the study of anti-idiotypic responses to antibodies directed against tumor associated antigens in the immunologic control of neoplasia. They may also serve as reagents in an excellent model for studies involving the radioimmunodetection and antibody mediated therapy (either alone, or with conjugated drugs or isotopes) of naturally occurring mammary neoplasia, preneoplasia, and/or micrometastatic lesions.

Proposed Course of Research:

These antibodies are being employed to help determine the degree of viral gene expression in naturally occurring neoplasias of feral populations of several species of the genus *Mus*. Several collaborations with other laboratories are currently in progress. Several of the anti MMTV gp 36 monoclonal antibodies will be injected into BALB/c mice to synthesize anti-idiotypic monoclonal antibodies, i.e., to determine what specific region of the anti-MMTV monoclonal is the anti-idiotypic directed. These antibodies are being used to further define reactivities of MMTV-related antigens in primary cultures of human breast tumors. These antibodies have been, and will continue to be, distributed to numerous other laboratories. We believe that this model system will eventually be extremely valuable in both diagnosis and therapy studies of a well defined naturally occurring carcinoma, and in the detection and therapy studies of both preneoplastic and micro-metastatic lesions.

Publications:

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Project DescriptionObjectives:

To characterize human monoclonal antibodies generated by the fusions of human lymphocytes from axillary lymph nodes of mastectomy patients with murine and human myeloma cells. In addition, to characterize nonhuman primate monoclonal antibodies generated by the fusions of lymphocytes from chimpanzees and old world monkeys immunized with membrane enriched extracts of human mammary cancer. The human and nonhuman primate monoclonal antibodies may ultimately be useful in the in situ diagnosis and/or therapy of human mammary cancer.

Methods Employed:

Human-mouse, human-human, nonhuman primate-mouse, and nonhuman primate-human hybridomas are generated using some modifications of standard fusion and hybridoma techniques. Human and nonhuman primate immunoglobulin production by the hybridomas is detected using specific solid phase and immunobead competition radioimmunoassays. The immunoreactivities of the human and non-human primate monoclonal antibodies are elucidated by employing solid phase radioimmunoassays and the immunoperoxidase technique.

Major Findings:I. Human monoclonal antibodies.

(A) Generation and characterization of human-mouse hybridoma cultures. In our first series of experiments, 1460 microtiter wells have been seeded with fusion products of mouse NS-1 myeloma cells and lymphocytes from 16 patients. Of these, 301 human-mouse hybridoma cultures have been successfully propagated. All of the patients had infiltrating duct or lobular carcinoma. The percentage of wells planted that yielded viable hybridoma cultures varied among patients; the reasons for this variability are known at present. Replicating human-mouse hybridoma cultures were obtained from lymph node segments of 81% of patients.

Hybridoma cultures were first tested for synthesis of human IgG or IgM 14-28 days after fusion. Of the 301 replicating hybridoma cultures, 17% synthesized either human IgG or human IgM. The duration of human Ig synthesis ranged from 14 to greater than 300 days. The level of human Ig production, as measured by liquid immunobead competition and solid phase radioimmunoassays, were comparable to those detected in our laboratory with both mouse-mouse and mouse-rat hybridomas. Many of the cloned human-mouse hybridoma cultures were unstable for Ig production. For example, only 7 of 21 primary clones of hybridomas from patient MB, shown to be positive for Ig production on day 71, were still positive when assayed on day 94. Upon recloning of the three clones highest for Ig synthesis, however, 126 of 129 secondary clones remained positive for human Ig synthesis for greater than the 300 day observation period.

(B) Characterization of human Ig's. Several assays were used to determine if the Ig's secreted by the human-mouse hybridoma cultures were indeed human and not murine. These included: (a) immunobead liquid competition radioimmunoassays,

(b) solid phase linker radioimmunoassays, and (c) Ouchterlony double-diffusion. The immunobead and solid-phase radioimmunoassays could also be used to quantitate human or IgM synthesis. Mouse Ig's did not react in these assays. Two additional anti-murine Ig assays were used to determine if the human-mouse hybridoma supernatant fluids displayed any evidence of murine Ig's; both assays gave negative results. Several of the human-mouse hybridoma supernatant fluids were analyzed by SDS polyacrylamide gel electrophoresis and found to possess complete Ig (i.e., both heavy and light chains). Monoclonal MBE6 also demonstrated a sedimentation coefficient of approximately 19 S, corresponding to pentameric IgM.

(C) Immunoreactivity of human monoclonal antibodies. To define the immunologic reactivities of the human Ig's secreted by human-mouse hybridoma cultures, supernatant fluids were analyzed on tissue sections of human mammary tumors using the immunoperoxidase technique. Supernatant fluids from hybridoma cultures derived from five different patients showed differential reactivity with mammary carcinoma cells in sections of their own mammary tumors. One monoclonal antibody MBE6, from doubled-cloned cultures from the fusion of lymphocytes from patient MB, was chosen for further study. Monoclonal MBE6 was first tested by immunoperoxidase for reactivity with tissue sections of the primary breast tumor mass (infiltrating duct carcinoma) of patient MB, and demonstrated marked cytoplasmic staining and percentage of cells stained were observed in various areas of the tumor. Antibody MBE6 could distinguish clearly between malignant mammary cells and normal mammary epithelium or stromal cells of the same breast. MBE6 was then tested for its ability to detect metastatic mammary carcinoma cells in lymph nodes of patient MB (13) of 16 lymph nodes were histologically positive for tumor); it could be clearly distinguished between metastatic breast cells and adjacent lymphocytes. MBE6 was also tested for its ability to bind to primary and metastatic mammary carcinoma cells of patients other than MB. MBE6 bound to primary breast tumors from 55 of 59 patients and also detected metastatic breast lesions, in lymph nodes and at distal sites, from 20 of 20 patients. Preliminary studies also indicate a cross-reactivity between MBE6 and cells of selected non-breast carcinomas, such as bronchial alveolar carcinoma and medullary carcinoma of the thyroid. MBE6 did not stain smooth muscle, arteries, veins, and nerve bundles of various breasts and did not react with normal colon, thyroid, lung, or cartilage.

(D) Generation and characterization of Human-Human Hybridoma Cultures. Fusions between human lymphocytes from axillary lymph nodes of breast cancer patients with two human lymphoblastoid cell lines, VC-729-6 and LICR-LON-HMY₂, have been performed. To date 1,963 microtiter wells have been seeded with the fusion products from 8 patients. Human-Human Hybridomas have been noted in approximately 300 of these cultures, and 181 have been shown to be synthesizing human immunoglobins. These human Ig's are currently being tested, via the immunoperoxidase method and solid phase radioimmunoassays, for reactivity to human mammary carcinomas.

Lymphocytes from these same 8 patients have also been fused with the murine myeloma cell line NS-1. Comparison of parameters such as fusion efficiency, human immunoglobulin production, and stability of human immunoglobulin

production will be made between the human-human and human-mouse fusions.

II. Nonhuman primate (NHP) monoclonal antibodies.

We have developed solid phase RIAs for the detection of NHP Ig's. Two Patas and two Cynomologous (cyno) monkeys (both Old World) were immunized with membrane enriched fractions from either a human breast metastatic lesion or the MCF-7 mammary carcinoma cell line. Pre-and post-immunization bleeds showed that the monkeys were making Ig's which are preferentially reactive with the immunogen. Using standard hybridoma technology. Lymphocytes from lymph node biopsies (draining immunization sites) were fused with murine NS-1 myeloma cells. Preliminary results indicate that some of the NHP-hybridoma cultures are synthesizing immunoglobulins that are preferentially reactive (in initial screens) with the immunogen.

Significance to Biomedical Research and the Program of the Institute:

The development of a human or NHP monoclonal antibody that is preferentially reactive with human mammary carcinoma cells would have an obvious advantage in in situ diagnosis (gamma scanning for distal metastasis or in lymphangiography), and/or ultimately in antibody mediated therapy of human mammary cancer. In particular, the generation of Fab or Fab'₂ fragments of human or NHP monoclonals in these studies would minimize any host anti-allotype response. It should be pointed out that a recent study from the NIH laboratory of Dr. Fauci has utilized our protocols and succeeded in the development of a human monoclonal antibody to KLH (keyhole limpet hemocyanin) following fusion of human lymphocytes with murine NS-1 myeloma cells.

Proposed Course of Research:

We plan to continue to fuse lymphocytes from lymph nodes of mastectomy patients with the murine and human myeloma lines. We also plan to alter conditions of fusion in an attempt to enhance efficiency of immunoglobulin production. Parameters such as ratios of lymphocytes to myeloma cells, and in vitro immunization with sterile purified antigen preparations will also be investigated.

Publications:

Teramoto, Y.A., Mariani, R., wunderlich, D. and Schlom, J.: The immuno-histochemical reactivity of a human monoclonal antibody with tissue sections of human mammary tumors. Cancer 50: 241-249, 1982.

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Jeffrey Schlom	Chief, Lab. of Tumor Immunology & Biology	NCI

Project DescriptionObjectives:

To determine whether various hormones, growth factors or vitamins might enhance or modulate the expression of cell surface, tumor-associated antigens on human mammary carcinoma cell lines using monoclonal antibodies which were generated against surface antigens on human breast tumor cells. In addition, to correlate the expression of these antigens on human mammary carcinoma cells with other phenotypic markers including: 1. production of transforming growth factors (TGF's); 2. epidermal growth factor (EGF) transferrin and estrogen receptor status; 3. type IV collagen, keratin and laminin production; 4. growth rate and 5. tumorigenicity in nude mice.

Methods Employed:

Three monoclonal antibodies will be utilized to monitor the expression of cell surface antigens. These include B72.3, B6.2 and B139 which recognize respectively the following antigens: 220,000 Mr; 90,000 Mr and HLA-related proteins. Moreover, monoclonal antibodies against the human transferrin receptor (antibody B3/25) and against a melanoma-associated antigen, p94 (Mr, 200,000), using antibody (MAA) will be simultaneously screened for detecting these antigens on human mammary carcinoma cells. Expression and quantitation of these antigens will be accomplished using a live cell radioimmunoassay (LCRIA) and by indirect immunofluorescence. These studies will be conducted on a well characterized human mammary carcinoma cell line (MCF-7) and several clones derived from this line in vitro in a serum-free, hormone-defined medium.

Major Findings:

MCF-7 cells can be propagated in a serum-free medium consisting of Improved Eagle's Minimal Essential Medium (IMEM)-zinc option supplemented with EGF (10 ng/ml), insulin (10 µg/ml), transferrin (10 µg/ml) and Pedersen fetuin (1 mg/ml), human α_2 -macroglobulin (5 µg/ml) or fibronectin (0.5 to 1.0 µg/well) on type I interstitial collagen (0.5 µg/well) or with Holme's α_1 -glycoprotein (200 ng/well) on type IV basement membrane collagen (0.5 µg/well). Cell growth on either type I or type IV collagen under these culture conditions is comparable to that in medium containing 10% fetal calf serum. Recent experiments indicate that the expression of B72.3, B6.2 and B139 is comparable whether the cells were grown in the presence or absence of serum. Likewise, the level of antigen expression was similar for any one antigen whether the cells were maintained on type I or type IV collagen. MCF-7 cells were found to express transferrin receptors which could be detected with monoclonal antibody B3/25. The level of transferrin receptors was higher (approximately 2 to 3-fold) in rapidly proliferating cultures than in more confluent, slow growing cultures. MCF-7 cells also express a high level of the melanoma-associated antigen, p94. Expression of

these antigens is also related to the passage level of the cultures underscoring the phenomenon of antigenic drift. At passage level 165, B72.3 and B6.2 are expressed at low level compared to passage level 129 cells. Moreover, at passage level 165, MCF-7 cells lost their ability to attach and spread on type I or type IV collagen-coated dishes in serum-free medium suggesting that changes in membrane composition may be occurring at this stage. Various clones derived from the MCF-7 the MCF-7 cell line have also been assayed for antigen expression, EGF receptors and TGF production. For example, clones 2B3 and 2A8 produce 3 to 4-fold more TGF than the parental MCF-7 cell line while 2A8 and 2E5 possess approximately 10% of the concentration of EGF receptors found on the parental line. These results suggest that the parental MCF-7 cell line represents a heterogeneous population of cells with respect to a variety of phenotypes exemplifying the concept of tumor cell heterogeneity.

Significance to Biomedical Research and the Program of the Institute:

The purpose of the study is to assess various agents (i.e. hormones and vitamins) in their ability to modulate the expression of tumor-associated cell surface antigens on human mammary carcinoma cells using specific monoclonal antibodies. The ultimate aim of the project should any one of these hormones or vitamins enhance antigen expression in vitro will be to determine whether they are efficacious in vivo with MCF-7 cells in nude mice in the detection of tumor foci by radioimaging.

Proposed Course of Research:

Experiments are in progress to study the effects of the following compounds either alone or in combination on the expression of antigens detected by B3/25, MAA, B72.3, B6.2 and B139: sex steroids (estrogen, progesterone, testosterone); hormones (glucocorticoids, thyroxine, insulin, vasopressin, calcitonin, prolactin) and vitamins (E, α -tocopherol, D, calciferol, C, ascorbic acid and A, retinoic acid). Moreover, changes in antigen expression in either the parental MCF-7 cell line or in clones derived from this line will be correlated with changes in type IV collagen, laminin and keratin production, EGF and estrogen receptors and TGF production to ascertain whether these agents have a selective effect on any one phenotypic marker being expressed by human mammary carcinoma cells.

Other Professional Personnel:

Atul Sahai	Visiting Fellow	LTIB, NCI
Mounanandham Panneerselvam	Visiting Fellow	LTIB, NCI
Nili Feuerstein	Visiting Fellow	LTIB, NCI
Herbert Cooper	Chief, Cellular & Molecular Physiology Section, LTIB, NCI	

Project DescriptionObjectives:

To study the effect of epidermal growth factor (EGF) and the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA) on the growth and synthesis of extracellular matrix proteins (i.e. collagen, laminin and fibronectin) in normal and neoplastic cells and to relate these changes to the level of a calcium and phospholipid-dependent protein kinase (protein kinase C). Moreover, to determine the endogenous, cellular substrates for protein kinase C and how their synthesis and phosphorylation is modified by EGF and/or TPA.

Methods Employed:

Extracellular matrix proteins are identified biochemically and immunologically from conditioned medium and cell extracts by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis and immunoblot assay. Collagen (type I, III and IV), laminin and fibronectin are quantitated by immunoprecipitation and enzyme-linked immune-substrate assay (ELISA). Analysis of protein kinase C activity in vitro is determined by the ability of crude cytoplasmic cellular fractions to phosphorylate endogenous proteins from gamma-(³²P) ATP in the presence of diolein (DG) and phosphatidylinositol (PI). Phosphorylated products are identified by their migration after two-dimensional gel electrophoresis (isoelectric focusing followed by SDS-gel electrophoresis).

Major Findings:

I. Effect of EGF and/or TPA on growth and phosphatidylinositol metabolism in A431 human epidermoid carcinoma cells

The human epidermoid carcinoma cell line, A431 contains a high concentration of membrane receptors for EGF, approximately 2 to 3x10⁶ receptors per cell. EGF as well as TPA induce several rapid biochemical changes in these cells and inhibit their growth following a 24 to 48 hour exposure. For these reasons, A431 cells have been used as a model system in which to study the mechanism(s) of action of EGF and more recently TPA since TPA shares a number of biological responses which are common to EGF. We initially demonstrated that TPA like EGF inhibits the growth of A431 cells. Moreover, TPA synergistically enhances the growth inhibition produced by EGF. To delineate the mechanism(s) by which these agents may regulate cell proliferation, we studied the effect of EGF and/or TPA on the metabolism of phosphatidylinositol (PI) and diacylglycerol (diolein, DG). This approach was taken because several other hormones and growth factors have been demonstrated to enhance PI turnover and to transiently increase the production of DG prior to affecting cell proliferation. Moreover, the activity of a soluble, cyclic-nucleotide independent, phospholipid-

dependent protein kinase C has been demonstrated to be modulated by DG and PI and to fluctuate during enhanced cell growth. EGF induced a rapid yet transient increase in the production of DG (60 to 70% increase) within 10 minutes in A431 cells. This response was followed by a two to three-fold increase in the synthesis of PI after 30 to 60 minutes. TPA also produced a transient increase in DG production but had no effect on PI metabolism. In the presence of EGF, TPA attenuated the increase in PI production produced by EGF.

II. Effect of EGF and/or TPA on protein kinase C in A431 epidermoid carcinoma cells.

The accumulation of DG, derived from the turnover of PI, has been suggested to control the activity of protein kinase C. Since both EGF and TPA were demonstrated to enhance DG production with the latter, EGF also stimulating the synthesis of PI, we investigated the effects of these agents on protein kinase C activity in A431 cells. Furthermore, by SDS-PAGE and two-dimensional gel electrophoresis we have attempted to identify which proteins are being phosphorylated *in vivo* and *in vitro* following exposure of A431 cells to EGF and/or TPA. Protein kinase C was detected in the crude soluble extracts of A431 cells. The enzyme required calcium, PI and DG for maximal activation. Protein kinase C phosphorylated endogenous cytosolic proteins. EGF treatment of A431 cells resulted in a two to three-fold increase in the level of protein kinase C activity detected in crude cytosol extracts within 30 to 60 minutes. TPA had no effect on protein kinase C activity. However, TPA in concert with EGF attenuated the EGF-induced increase in protein kinase C activity. A431 cells were labelled with (^{32}P) orthophosphate and treated with EGF and/or TPA for 1 hour. Soluble, cytosol phosphorylated proteins were then analyzed by two-dimensional gel electrophoresis. EGF enhanced the phosphorylation of three proteins having Mr's of 32 KD, 39 KD and 81 KD, while TPA enhanced the phosphorylation of a 21 KD soluble protein. Addition of TPA in the presence of EGF blocked the EGF-enhanced phosphorylation of the 32 KD protein, but a new protein of 90 KD was now found to be phosphorylated. Cell-free cytosol preparations from EGF-treated cells when assayed for protein kinase C in the presence of PI and DG were found to phosphorylate a 32 KD protein analogous to that observed using intact cells.

III. Effect of EGF and/or TPA on collagen, laminin and fibronectin production in A431 epidermoid carcinoma and normal rat kidney (NRK) cells.

Type IV (basement membrane) collagen, type I (interstitial collagen) and laminin were biochemically and immunologically identified in A431 cell extracts by SDS-PAGE, Westernblot and immunoblot analysis. Laminin and type IV collagen, but not type I collagen were found to be secreted by A431 cells. Both the alpha (200 KD) and beta (400 KD) chains of laminin were identified in the medium and cell extracts. Type IV collagen in the medium was identified as the alpha₁ (160 KD) and alpha₂ (140 KD) chains of mature type IV collagen while the cell-associated type IV collagen was found to be secreted by A431 cells. Both the alpha₁ (185 KD; alpha₂, 170 KD). Type I collagen in the cells exhibited an electrophoretic mobility pattern corresponding to the alpha₁ (I), alpha₂ (I), beta₁, 1, beta 1, 2 and gamma chains of calf skin type I collagen. Both the type IV and type I collagenous peptides were sensitive to digestion with protease-free

bacterial collagenase. A431 cells were treated with EGF and/or TPA for various intervals up to 16 hours. Type IV collagen, type I collagen and laminin were quantitated by immunoprecipitation of medium aliquots or cell extracts. EGF or TPA produced a two to three-fold transient increase in type IV collagen and laminin secretion within 2 hours which was accompanied by a transient increase in cell-associated type IV collagen and laminin. Combination of EGF and TPA was more effective than either agent alone in promoting the secretion of type IV collagen and laminin but produced no further enhancement in the production of either protein. In contrast to its effect on type IV collagen production, TPA had no effect on type I collagen production in A431 cells. However, EGF produced a two-fold increase in in type I collagen production whereas EGF plus TPA caused a 3-fold increase in type I collagen production. The effect of EGF and TPA on extracellular matrix production in NRK cells was investigated since these cells are not transformed and synthesize type I, III and IV collagens, laminin and fibronectin. Overall collagen production was stimulated two to three-fold in NRK cells following a 16 hour exposure to either EGF or TPA. Combination of EGF and TPA produced a four to five-fold increase in collagen production. In contrast, EGF and/or TPA treatment of NRK cells produced a reduction in laminin production ranging from 50 to 80%. Although neither EGF nor TPA had any significant effect on fibronectin production, combination of these agents produced a nearly five-fold increase in fibronectin production.

Significance to Biomedical Research and the Program of the Institute:

A variety of growth factors and hormones function as mitogens by regulating the synthesis and/or turnover of various components of the extracellular matrix. Moreover, the mitogenic response to several different growth factors and hormones in a variety of cell types is dependent upon the cells ability to interact with and deposit an appropriate extracellular matrix. Following transformation, cell-substratum interactions become deranged such that cells are now anchorage-independent in their growth. These effects are preceded by changes in extracellular matrix production and the response to exogenous growth factors. The studies presently being conducted should prove to be advantageous in elucidating the mechanism(s) by which a well defined growth factor, EGF and a tumor promoter, TPA affect the production of a pericellular matrix by normal and transformed cells and how these changes in matrix production may be related to or mediated by changes in intracellular phosphorylation reactions.

Proposed Course of Research:

Studies are in progress to identify the proteins which are rapidly phosphorylated in response to EGF and/or TPA; to relate changes in the synthesis or phosphorylation of these proteins to the effect of these agents on the synthesis of specific extracellular matrix proteins and to determine whether transforming growth factors produce analogous responses.

Publications:

Sahai, A., Smith, K.B., Panneerselvam, M. and Salomon, D.S.: Activation of a calcium and phospholipid-dependent protein kinase by epidermal growth factor in A431 cells: attenuation by 12-O-tetradecanoylphorbol-13-acetate. Biochem. Biophys. Res. Commun. 109: 1206-1214, 1982.

Smith, K.B. Losonczy, I., Sahai, A., Panneerselvam, M., Fehnel, P. and Salomon, D.S.: Effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on the growth inhibitory and increased phosphatidylinositol response induced by epidermal growth factor (EGF) in A431 cells. J. Cell. Physiol. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CB09003-01 LTIB
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.)		
Transforming Growth Factors in Tumors and Retrovirus Transformed Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.)		
(Name, title, laboratory, and institute affiliation)		
David S. Salomon, Expert, LTIB, DCBD, NCI		
COOPERATING UNITS (if any)		
W. Kidwell, J. Zwiebel, M. Bano, LPP, DCBD, NCI		
LAB/BRANCH		
Laboratory of Tumor Immunology and Biology		
SECTION		
Experimental Oncology Section		
INSTITUTE AND LOCATION		
NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		A
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>Transforming growth factors (TGF's) are EGF-related peptides which have been isolated from a variety of rodent and human tumors and from the conditioned medium of rodent and human carcinoma cell lines and from retrovirus transformed cells. TGF's are able to reversibly confer upon normal cells certain properties associated with the transformed phenotype namely a reduced serum requirement for growth and a loss of anchorage-dependent growth. TGF's have recently been isolated from rat mammary adenocarcinomas. These TGF's are: 1. able to compete with EGF for receptor binding; 2. induce the anchorage-independent growth of mouse and rat fibroblasts in soft agar; 3. reduce the serum requirement and function as potent mitogens for fibroblasts and normal mammary epithelial cells in monolayer culture and 4. differentially stimulate collagen production in both epithelial and mesenchymal cells. The rodent mammary TGF's have a molecular weight of 68,000 and a pI of 5.9. Comparable, but as yet not fully characterized, activities have been isolated from several human breast tumor samples, a transplantable human mammary tumor and in the conditioned medium of a human mammary carcinoma cell line (MCF-7) and several clones derived from this line. The activity in human breast tumor samples differ from the rat mammary TGF in that this activity has a pI of 4.9. Kirsten transformed mouse 3T3 cells provide a useful system in which to study the production of TGF's and to relate its production to the expression of the viral <u>onc</u> gene protein, p21. Cellular revertants of Kirsten transformed 3T3 cells have been isolated (C1 and F2) which possess 2 genomic copies of the <u>v-kis</u> sequences; express elevated levels of p21 but are no longer transformed or tumorigenic. The revertants, however, like the parental transformed cells lack detectable EGF receptors and secrete into the culture medium TGF as detected by the ability of conditioned medium concentrates to: 1. inhibit EGF binding to 3T3 cells; 2. stimulate the growth of fibroblasts in soft agar as colonies and 3, differentially stimulate the production of collagen. These results suggest that the lesion(s) in revertants are distal to the <u>the</u> expression of p21 or the production of TGF.</p>		

Other Professional Personnel:

Robert Bassin Chief, Biochemistry of Oncogenes Section LTIB, NCI

Project DescriptionObjectives:

To isolate and characterize transforming growth factors (TGF's) from rat and human mammary tumors and to determine their role in normal and neoplastic mammary gland development and their production in human mammary carcinoma cell lines. In addition, to generate and characterize monoclonal antibodies against these mammary tumor-associated TGF's for use in the detection and quantitation of TGF's in human breast tumor samples. Likewise, to study the production of TGF's in Kirsten transformed NIH/3T3 cells with respect to the expression of the viral onc gene protein, p21.

Methods Employed:

TGF's are isolated from acid-ethanol extracts of rat and human mammary tumor samples and from conditioned medium (CM) concentrates obtained from retrovirus transformed and human mammary carcinoma cells (MCF-7). TGF's are being purified by preparative isoelectric focusing followed by high performance liquid ultra-filtration and reverse phase chromatography. Activity of the TGF's is being monitored by the ability of TGF to: 1. inhibit the binding of epidermal growth factor (EGF) to its receptor in a radioreceptor assay (RRA); 2. induce the growth of normal rat kidney (NRK) cells in soft agar and 3. differentially stimulate the production of collagen in NRK cells.

Major Findings:

I. Production of Transforming Growth Factors (TGF's) by Rat Mammary Adenocarcinomas

Dimethylbenzene(α)anthracene (DMBA) and Nitrosomethylurea (NMU)-induced rat mammary adenocarcinomas contain TGF's which biologically resemble the TGF's isolated from a variety of rodent and human tumors and from the CM of rodent and human carcinoma cell lines but which are not physiochemically identical to these activities. DMBA rat mammary tumor cells were found to possess fewer EGF receptors when compared to normal rat mammary epithelial (RME) cells (2.5×10^4 versus 8.4×10^4 receptor sites per cell, respectively) in primary culture. For this reason, CM concentrates or acid-ethanol extracts of the tumors were screened for the presence of TGF-like activities. TGF's were identified in CM concentrates and in tumor extracts. These mammary-associated TGF's were found to be acid stable and heat and protease sensitive. They inhibit the binding of EGF, but not insulin, to mouse 3T3 or embryonal carcinoma cells in a RRA. They confer upon NRK or 3T3 cells the ability to grow in soft agar. They are mitogenic for RME, NRK, 3T3 and chick embryo fibroblasts in monolayer culture. Finally, they cause a 2 to 10-fold differential increase in collagen production in RME, NRK and 3T3 cells. However, these activities fail to stimulate the proliferation of primary cultures of rat DMBA tumor cells or to cause an increase in collagen production in these cells. Following gel filtration, two major molecular weight species of TGF, eluting at 6,000 and 68,000 daltons are present in the acid-ethanol extracts of primary DMBA or NMU-induced rat mammary tumors. The major high molecular weight species (68,000) has a pI of 5.9. In contrast, in transplantable tumors derived from primary DMBA or NMU-tumors little,

or no TGF could be detected. Since the primary carcinogen-induced mammary tumors are well differentiated, hormone-dependent (estrogen) adenocarcinomas while their transplantable counterparts are generally poorly differentiated carcinomas which are hormone-independent, it is possible that TGF production is under hormonal control of the host and present in normal mammary tissue during certain stages of the gland's development. In support of this, TGF can be recovered in acidethanol extracts prepared from estrogen and progesterone-stimulated bovine mammary glands.

II. Production of TGF's by Human Mammary Tumors

TGF can be recovered from several human breast tumor samples, a transplantable human mammary tumor (Clouser) and in CM concentrates from a human mammary carcinoma cell line (MCF-7) and several clones derived from this line. Likewise, milk also contains a substantial quantity of this material. Although these activities from human material have not yet been fully characterized, the activity isolated from human breast tumor samples and human milk apparently differ from the rat mammary tumor TGF's in that the human TGF has a pI of 4.9. The human breast tumor and milk TGF's like the rat mammary tumor TGF's: 1. compete with EGF for binding in a RRA; 2. induce the anchorage-independent growth of NRK cells in soft agar and 3. differentially stimulate the production of collagen in NRK and A431 human epidermoid carcinoma cells. Since human milk appears to be a rich source for this material, has a similar isoelectric focusing profile as that obtained from human breast tumor samples and possesses far less other proteins than the tumor extracts, this material is being utilized to further purify human TGF. Preliminary experiments indicate that the milk-associated TGF can be removed from the bulk of milk caesins by isoelectric precipitation, can be bound to a zinc-agarose affinity column and is sensitive to reduction by dithiothreitol yet heat stable.

III. Relationship of Retrovirus onc Gene Expression and TGF Production in Kirsten Virus Transformed Cells

Moloney, Kirsten, feline, Abelson and Simian retrovirus transformed cells have previously been demonstrated to possess a reduced number of EGF receptors when compared to their nontransformed counterparts. This observation led to the discovery of sarcoma growth factor (SGF), an EGF-related peptide which is now known to be a prototype for the TGF's. Kirsten transformed NIH/3T3 cells provide a useful in vitro model system in which to study the production of SGF and to relate its production to the expression of the viral onc gene protein, p21. Cellular revertants of Kirsten transformed NIH/3T3 cells have been isolated (C11 and F2) which possess 2 genomic copies of the v-kis sequences, express elevated levels of p21 like the Kirsten transformed cells and are no longer transformed or tumorigenic in nude mice. However, these revertants like the parental Kirsten transformed 3T3 cells lack detectable EGF receptors and secrete SGF into the culture medium as monitored by the ability of CM concentrates to: 1. inhibit EGF binding to normal NIH/3T3 cells in a RRA; 2. stimulate the growth of NRK, NIH/3T3 and NR6/3T3 cells in soft agar and 3. differentially stimulate the production of collagen in NRK cells. These results suggest that the lesion(s) in the C11 and F2 revertants with respect to not being transformed must be distal to the expression of p21 and the production of SGF. To more fully delineate the relationship between p21 expression, SGF production and the

acquisition of the transformed phenotype, a cellular chimera (NIH 3T3/433) containing the mouse MMTV-LTR (long terminal repeat) linked to the v-Ha-ras gene is being utilized. This system is unique in that glucocorticoids (i.e. dexamethasone) induce the expression of p21 in 433 cells within 5 hours followed by morphological transformation within 15 to 24 hours. This presumably occurs by steroid activation through a receptor-related pathway of the MMTV-LTR and the subsequent promotion of the v-Ha-ras gene. 433 cells possess EGF receptors at a concentration equivalent to that found on NIH/3T3 cells. In NIH/3T3 cells, dexamethasone ($10^{-8}M$) induces a 2-fold increase in the number of EGF receptors within 5 hours. However, in comparably treated 433 cells, dexamethasone produces an 80% reduction in the number of EGF receptors which can first be detected 10 to 12 hours after steroid administration. Moreover, CM concentrates from steroid-treated 433 cells, but not CM concentrates from untreated cultures are able to stimulate the anchorage-independent growth of NIH/3T3 and NRK cells in soft agar. These results provide evidence for glucocorticoid-induced production of SGF in 433 cells which occurs subsequent to the expression of the p21 onc gene protein.

Significance to Biomedical Research and the Program of the Institute:

The detection, characterization and purification of TGF's from rodent and human mammary adenocarcinomas should prove useful in the possible diagnostic detection and pathological staging of breast tumor samples assuming: 1. that increased production of TGF's is associated with neoplastic cells; 2. that more differentiated tumors (i.e. adenocarcinomas) express relatively more TGF's than their undifferentiated counterparts (i.e. carcinomas) and 3. C-onc gene products may be involved in regulating the production of TGF's. The generation of monoclonal antibodies against mammary-derived TGF's will aid in addressing these questions.

Proposed Course of Research:

Experiments are in progress to further characterize and purify TGF's from human breast tumors to generate monoclonal antibodies against these activities; to study their production in various human mammary carcinoma cell lines with respect to the ability of other mammotropic hormones to modulate their production and to relate the production of TGF's to the expression of v-onc gene proteins (i.e. p21) in Kirsten virus transformed 3T3 cells and in NIH/3T3 cells transfected with other transforming sequences isolated from human carcinomas.

Publications:

Zwiebel, J.A., Davis, M.R., Kohn, E., Salomon, D.S. and Kidwell, W.R.: Anchorage-independent growth-conferring factor production by rat mammary tumor cells. Cancer Res. 42: 5117-5125, 1982.

Bano, M., Zwiebel, J.A., Salomon, D.S. and Kidwell, W.R.: Detection and partial characterization of collagen synthesis stimulating activities in rat mammary adenocarcinomas. J. Biol. Chem. 258: 2729-2735, 1983.

Other professional personnel:

Charles Theillet	Visiting Fellow	LTIB, NCI
Jeffrey Schlom	Chief, Lab. of Tumor Immunology & Biology	NCI

Project DescriptionObjectives:

To characterize at a molecular and biological level mouse mammary tumor virus (MMTV) related endogenous human proviral sequences and assess their role in neoplasia.

Methods Employed:

Retroviral related sequences were detected in restriction endonuclease digested recombinant and cellular DNAs by Southern transfer - blot hybridization technique. Standard techniques were used to obtain recombinant clones of cellular and unintegrated retroviral DNA.

Major Findings:

DNA sequences homology between infectious type A, B, and D retroviral DNA.

In earlier work we have shown that by lowering the stringency of blot hybridization conditions recombinant MMTV proviral DNA hybridizes with a recombinant clone of human cellular DNA but not with recombinant mammalian type C proviral DNA. We have now, in collaboration with I.M. Chiu and S. Tronick, extended this study to include recombinant proviral genomes of infectious type A (M432) and type D (Squirrel Monkey retrovirus, SMRV) retroviruses. In each case we have been able to detect reciprocal DNA sequence homology between the pol genes of the type A, B and D retroviral genomes. With respect to the MMTV genome the sequence homology has been localized to 600 bp at the 3' end of the pol gene. This sequence also contains the major homology region with the human recombinant clones. In addition weak homology could be detected between the gag genes of Type A and Type B proviral genomes. We conclude that the Type A, B and D classes of retroviruses constitute a family within Retroviridae which had a common ancestor. Consistent with this interpretation is the morphological similarity of intracellular particles and the divalent cation requirement for activity of the respective reverse transcriptase enzymes.

Characterization of Recombinant clones of MMTV related human cellular DNA.

We have characterized five recombinant clones in detail by restriction endonuclease mapping and heteroduplex analysis. These studies have led us to the following conclusions. 1) Three of the five clones contain 8 kbp of continuous homology, the other two clones contain a portion of these sequences truncated to lambda phage DNA. 2) The 8 kb homology region is terminated at each end by a 0.6 to 1.0 kbp repeat sequence. These sequences hybridize to restriction fragment of the SMRV proviral genome which contains the LTR. 3) The major region of homology between the human recombinant clones and the type A, B and D retroviral pol genes is a 0.6 kbp restriction fragment which is located within the human 8.0 kbp homo-

logy region. 4) Weak homology between the SMRV gag gene and the 8.0 kbp human homology region could also be detected. The organization of the gag, pol and LTR related sequences within the human recombinant clones is consistent with that of a genetically transmitted proviral genome.

Quantitation and Organization of the retroviral like sequences in human cellular DNA.

We have quantitated the number of the novel retroviral like sequences in restricted human cellular DNA using an enzyme which does not cleave these sequences. The results suggest that there are 20 to 30 copies per cell. Variations in the restriction sites present in each of the human recombinant clones as well as multiple related restriction fragments in cellular DNA suggests that they represent a family of related but nonidentical retroviral genomes. In collaboration with Dr. Croce, human-hamster somatic cell hybrids which are segregating subsets of human chromosomes have been analyzed for the presence of the human retroviral related sequences. The results indicated that these sequences are located on four chromosomes. Comparison of restricted cellular DNA from several normal tissues and primary breast tumors has revealed no detectable amplification or variation in the pattern of fragments containing MMTV related sequences. One exception is the breast tumor tissue culture cell line MCF-7 which contains at least two additional related restriction fragments. These sequences appear to be significantly diverged from the cloned human retroviral like genome.

Significance to Biomedical Research and the Program of the Institute:

The role of genetically transmitted retroviral genomes in the etiology of human neoplasia is at present unknown. The isolation of recombinant clones of human cellular DNA which share sequenced homology with the murine type B MMTV genome and still greater sequence homology with the primate type D SMRV genome raise the possibility that the latter class of retroviruses are primate analogues of MMTV. Attempts to identify components of type B and D retroviral particles by biochemical and immunological techniques in human breast tumors have met with some success. The existence of recombinant clones containing type B and D retroviral related sequences offers the opportunity to determine if a relationship exists between their expression and human mammary neoplasia.

Proposed Course of Research:

Currently our efforts are focused in four areas. (1) Determine the biological activity of the recombinant clones and chimeric recombinant DNA consisting of the putative human LTR related sequences and the Herpes thymidine kinase gene, by transfection of the NIH 3T3 tissue culture cell line (2) Determine whether RNA sequences related to the human recombinant clones are expressed in breast tumors by Northern blot analysis and in situ DNA:RNA hybridization. (3) Determine the sequence of type B and D related regions of the human recombinant clones. The sequences will allow us to prepare synthetic peptides which will be used to raise heterologous antisera and monoclonal antibodies. (4) Isolate recombinant clones of the novel MMTV Pol related restriction fragments in MCF-7 cellular DNA and MMTV env related sequences.

Publications:

Callahan, R., Drohan, W., Tronick, S. and Schlom, J.: Detection and cloning of human DNA sequences related to the mouse mammary tumor virus genome. Proc. Natl. Acad. Sci. USA 79: 5503-5507, 1982.

Other Professional Personnel:

Dr. Chantel Theillet

Visiting Fellow

LTIB, NCI

Project DescriptionObjectives:

To dissect the genetic and molecular interaction between genetically transmitted mouse mammary tumor virus (MMTV) proviral genomes and exogenous carcinogens.

Methods Employed:

Retroviral related sequences were detected in restriction endonuclease digested recombinant and cellular DNAs by the Southern transfer-blot hybridization technique. Recombinant clones containing MMTV related sequences were isolated from libraries of cellular DNA in Charon lambda phage. The pedigreed breeding colonies of feral mice have been described in Callahan et al, Proc. Natl. Acad. Sci. U.S.A. 79, 4113-4117, 1982.

Major Findings:I. Derivation of mouse strains containing a single genetically transmitted MMTV proviral genome

The pedigreed feral CZII mice have been previously shown to lack genetically transmitted MMTV proviral genomes. To assess the role which they play in mammary neoplasia we have begun to genetically introduce single germ line MMTV proviral genomes from Balb/c and C3H/Hef mice into the CZII genetic background. The MMTV proviral genes of feral strain of mice (designed CL) are also being transferred to the CZII genetic background. Earlier work has shown that CL mice contain two copies of the MMTV LTR on separate chromosomes, but none of the MMTV structural genes. These mice are currently being bred and monitored for the development of mammary tumors.

We have also monitored the CZII colony for the development of spontaneous tumors. After one year one spontaneous mammary tumor has been observed in 100 force-bred females. This tumor was a Type A mammary adenocarcinoma. Analysis of restricted tumor cellular DNA confirmed the absence of MMTV related sequences. This preliminary result is consistent with the proposal that MMTV infection is sufficient but not necessary for tumor induction. After six months force-bred female CZII mice treated with the chemical carcinogen dimethylbenzanthracine (DMBA) or infectious MMTV (C3H) have begun to develop mammary tumors (respectively 4/30 and 2/30).

II. Chromosomal location of genetically transmitted MMTV proviral genomes

To facilitate the program of introducing genetically transmitted MMTV proviral genomes into CZII mice we have attempted to identify their chromosomal location. In collaboration with C. Kozac, we have used mouse-hamster somatic cell hybrids to determine the location of the Balb/c MMTV proviral genomes. The results of these studies indicated that the three Balb/c MMTV genomes are located on, respectively

chromosomes 12, 16, and 18. The MMTV related sequences on chromosomes 12 and 18 represent complete proviral genomes where as the sequences on chromosome 16 correspond only to MMTV LTR and leader sequences. The location of the MMTV proviral genome on chromosome 12 was confirmed by Southern blot analysis of the Balb/c congenic strain C4W which carries the chromosome 12 linked FV-4 locus of the wild Japanese mouse, Mus musculus molossinus. M. m. molossinus cellular DNA lacks the 10.5 and 7.7 kbp MMTV related EcoRI fragments as does the Balb/c C4W cellular DNA. The cellular DNA strain does, however, contain the 16 kbp and 8.3 - 6.6 kbp MMTV fragments. C4W mice carry the wild mouse allele of the Pre⁻¹ locus on chromosome 12. However, Hind III and EcoRI restricted Balb/c and C4W cellular DNA show identical patterns of Igh related sequences. Since the gene order of these loci is centromere Igh-Pre-1FV-4, we conclude that the 10.5-7.7 kbp MMTV provirus is located distal to the Igh locus on chromosome 12. The location of the 16 kbp MMTV related EcoRI fragment on chromosome 16 was confirmed using a genetic cross between Balb/c mice and the CL strain of feral M. m. domesticus. The MMTV proviral genomes of CL mice do not correspond to those of Balb/c mice and they also differ at the immunoglobulin lambda chain locus (Igl) on chromosome 16. Analysis of EcoRI digested cellular DNA from F₂ generation mice show linkage between the Balb/c Igl locus and the 16 kbp MMTV related EcoRI fragment. In other experiments one of the CL mouse MMTV proviral LTR sequences was shown to be linked to chromosome 7, 17 centi Morgans from the albino locus.

III. Expression of the Balb/c MMTV proviral sequences in normal tissues and tumors

The location of a MMTV proviral genome near immunoglobulin Igl loci on chromosome 12 is of interest due to the 12, 15 chromosomal translocations found in plasma cell tumors. These translocations result in aberrant tumor immunoglobulin gene rearrangements and altered transcriptional activity of genes located near the chromosomal break points. To determine whether there is increased expression of MMTV proviral loci in these tumors, we have compared the level of RNA expression in normal and tumor tissues. Low levels of MMTV genomic and env mRNA were detected in several normal tissues. High levels of a MMTV related 1.6 kbp RNA was detected in the mammary gland and a 1.2 kbp RNA in salivary gland tissue from lactating females. Each of these RNAs hybridized only with the LTR region of the MMTV genome. High levels of the 1.6 kbp MMTV RNA were also detected in all pristine induced Balb/c plasma cell tumors. In each of these tumors the chromosome 16 Balb/c MMTV related sequences were hypomethylated. This suggests that the 1.6 kbp MMTV RNA is expressed from the aberrant MMTV proviral genome on this chromosome. Some of the plasma cell tumors also expressed high levels of genomic and env MMTV RNA. In addition to these tumors carcinogen-induced mammary hyperplastic alveolar nodules (HAN) and HAN derived mammary tumors also expressed high levels of genomic and env MMTV RNA. Whether this expression reflects some etiological role for these sequences in the induction or maintenance of neoplasia is unknown.

Significance to Biomedical Research and the Program of the Institute:

The development of the CZII pedigreed breeding colony which has a low incidence of spontaneous tumors will allow us to define the role of individual genetically transmitted MMTV proviral genomes in the development spontaneous and chemical

carcinogen induced neoplasias. Previous genetic studies have assigned specific MMTV proviruses to chromosomes 1, 4, 6, 7, and 18 in different mouse strains. The genetic mapping of 2 BALB proviral genomes to chromosomes 12 and 16 provides additional evidence that MMTV proviruses are located at nonallelic sites and on different chromosomes in the genomes of inbred mice. More importantly, the known chromosomal assignments for these MMTV loci makes it possible to extensively explore the role of these proviruses in the etiology of neoplasia. The existence of MMTV proviral genomes containing only the MMTV LTR sequences will allow us to test directly the role of this subviral genetic element in the development of tumors and their effect on the expression of adjacent cellular genes. Finally mice lacking genetically transmitted MMTV proviral genome will allow us to unambiguously identify and define the role of insertions of exogenous MMTV proviral genes into preferred domains of the cellular genome on the development of mammary tumors.

Proposed Course of Research:

We are currently continuing the breeding program to genetically fix the Balb/c, C3H/ Hef, and CL MMTV proviral genomes into the genetic background of the CZII mice. During this program we are also monitoring the mice for the development of mammary tumors. Genetic crosses have been initiated to confirm the chromosome 18 linked MMTV proviral genome in Balb/c mice. This MMTV proviral genome has been found in all inbred strains of mice which have been tested. The chromosomal location of the unique C3H/Hef and CL MMTV proviral genome are also being determined by appropriate genetic crosses and with the aid of somatic cell hybrids. During the next year we will determine whether there are preferred integration sites in mammary tumors in MMTV (C3H) infected CZII mice and in the high incidence strain of M. cervicolor popaeus. The latter species has a high incidence of mammary tumors which histologically appear to be different than those of M. musculus.

Publications:

Callahan, R., Drohan, W., Gallahan, D. D'Hoostelaere, L. and Potter, M.: Novel class of mouse mammary tumor virus related DNA sequences found in all species of Mus, including mice lacking the virus proviral genome. Proc. Natl. Acad. Sci. USA 79: 4113-4117, 1982.

Callahan, R.: Expression of mouse mammary tumor virus genes in Balb/c plasma cell tumors. In F. Melchers, M. Potter and M. Weigert (eds.): Mechanisms of B cell Neoplasia Workshop at the Basel Institute for Immunology, Roche, Basle, Switzerland, 1983, pp. 92-98.

Other Professional Personnel:

Makoto Noda	Visiting Fellow	LTIB, NCI
David Salomon	Expert	LTIB, NCI

Project DescriptionObjectives:

- 1) The ultimate objective of these studies is the elucidation of the functional role of oncogenes, especially the v-ras gene, in cell transformation and tumor formation. A near-term goal involves the identification of those cellular constituents with which the ras gene interacts and an analysis at the molecular level of their role in cell metabolism. A secondary objective, which does not require a total understanding of the mechanism of transformation by the ras gene, consists of a comparative analysis of different onc genes, including those associated with human tumors, according to their functional characteristics.
- 2) The objective of these studies is the biological refinement of a hormone-dependent transformation system (originally developed by Gordon Hager) so that it can be used both for the molecular analysis of the mechanism of glucocorticoid control of RNA transcription and also as a conditional lethal system with which to study cell transformation.

Methods Employed:

- 1) In addition to the standard tissue culture methods employed by cell biology laboratories, the following biological techniques were used in these studies: mutagenesis of cells followed by selection in a variety of media including those supplemented with ouabain, thioguanine or BUdr; selection of flat revertant cell lines following mutagenesis of transformed cells; hybridization of various cell lines in the presence of polyethylene glycol followed by selection for hybrids; analysis of hybrids for transformed phenotype using colony formation in either liquid medium or in agar suspension cultures; transfection of both high molecular weight DNA and cloned DNA samples including co-transfection with 2 independently selectable markers; analysis of the ras-gene product, p21, by Western blotting; characterization of the ras genes present in various cell lines by the Southern gel transfer technique. Studies on monovalent cation transport using ⁸⁶Rb as a tracer and various inhibitors of potassium uptake such as bumethanide and ouabain.
- 2) In addition to many of the above techniques and procedures, these experiments required the use of serum-free medium procedures for growing cells, and analysis of cellular p21 content by immunoprecipitation.

Major Findings:

- 1) Previously, we had shown that flat revertants, which were isolated from Ki-MuSV-transformed NIH/3T3 cells, contained functional v-ras genes which

were capable of transforming fresh cells following rescue and infection or transfection. Since these revertants appeared to resist transformation by the v-ras gene, we initiated a large number of cell hybridization experiments to determine the ability of these revertants to suppress transformation in cells transformed by ras itself, by ras-related oncogenes, and by oncogenes unrelated to ras. Briefly, each of the 2 revertants which were used in this study were able to suppress transformation by v-Ki-ras and by all oncogenes related to it structurally, including v-Ha-ras, v-bas, and c-Ha-ras 1 (human). Many oncogenes unrelated to ras structurally were not suppressive in the cell hybridization studies. These included v-fms, v-sis, and v-mos. Still another group of oncogenes, which include v-fes and v-src, are structurally related to ras but were suppressed in hybrids with the 2 revertant cell lines. Thus, oncogenes may exhibit functional similarities and diversities which are not readily apparent from their structural relationships.

Potassium transport studies in the presence of specific inhibitors such as bumethanide and ouabain indicated that certain Ki-MuSV-transformed cell lines were altered in their monovalent cation fluxes. This alteration could be demonstrated by $^{86}\text{Rb}^+$ flux experiments or by colony formation in cell culture experiments. The significance of this finding must await additional experimental results. Experiments have been initiated with the aim of transfecting ouabain resistance from revertant cells into transformed cells. These experiments should help to define the role of monovalent cation transport in transformation and reversion mediated by ras.

2) Adaptation of cells transfected with the MMTV LTR - ras gene chimeric DNA to serum free culture systems was refined during the past year. Under these conditions addition of glucocorticoids results in a complete morphological change of the cells within 3 days, and loss of the transformed phenotype is complete by 2-3 days following removal of the hormone. The kinetics of p21 synthesis following addition and removal of dexamethasone were studied by electrophoretic analysis of immunoprecipitates. Following addition of 2.5×10^{-6} M dexamethasone, p21 synthesis can first be detected by 3 hours and reaches a maximum by 6 hours. Following removal of the hormone, p21 synthesis ceases 1-2 hours later. Also, the minimum amount of dexamethasone required for cell transformation in this system was approximately 1×10^{-9} M.

Significance to Biomedical Research and the Program of the Institute:

1) It has been suggested that some human tumors are associated with activation of cellular homologues of retroviral onc genes. The revertant cell lines isolated in this study were shown to be specifically resistant to the transformation mediated by a group of certain onc genes. Thus, one should be able to address which type of onc gene is responsible for, at least a part of the malignant phenotype expressed by a given tumor by fusing our revertant cells to either the tumor cells or NIH/3T3 cells transformed with the tumor DNA via transfection and examining the growth properties of resulting hybrids. Besides such diagnostic applications, biochemical characterization of the cellular revertants will be valuable in delineating the mechanism of transformation

produced by ras gene expression and other related onc genes. The findings that at least, some of the ras-transformed cell lines are highly sensitive to ouabain and that the flat revertants exhibit altered responses to ouabain treatment may suggest involvement of a membrane component, possibly ion transport systems, in the transformation process.

2) The hormone-dependent transformation system developed in this study will be useful to elucidate mechanisms of action of glucocorticoid hormones under defined, serum-free conditions and to study mechanism(s) of transformation. Practically, transformed phenotyped mediated by p21 will provide selectable markers for cellular responses to glucocorticoids whereas glucocorticoids can be utilized to induce transformation.

Proposed Course of Research:

1) To establish functional similarities among oncogenes responsible for the malignancy of naturally occurring tumors, cell hybridization studies will be extended to human tumor cells and/or NIH/3T3 cells transformed with either cloned or uncloned human oncogenes. To address the question why the cellular revertants are resistant to transformation by a group of structurally unrelated onc genes, we will determine the nature of these onc gene products being expressed within the revertants and cell hybrids. Studies on the role of cellular ion-transport system in transformation will be continued.

2) Using transformation as a marker, we will attempt to isolate and characterize cellular mutants which cannot respond to the addition or removal of glucocorticoid hormones. These mutants may represent either mutations in MMTV-LTR-ras sequences or those in cellular genes involved in hormonal reactions (e.g. receptors). We will also use this system to study the kinetics of appearance and disappearance of transformation associated parameters after the addition or removal of glucocorticoids, respectively. By so doing, one should be able to establish the sequence of events in the transformation process. Comparative studies of this sort using two independent conditional mutant systems, that is the hormone-dependent transformation and ts-MusV transformation, will provide strong evidence for specific changes responsible for the acquisition of malignant phenotypes which are associated with expression of the ras gene.

Publications:

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.

Bassin, R.H., Noda, M., and Selinger, Z.: Cellular revertants isolated from Kirsten sarcoma virus-transformed cells are resistant to transformation by specific oncogens. Proc. XX Congresso Nazionale Soc. Italiana Microbiol. (In Press).

Other Professional Personnel:

Robert Bassin Chief, Biochemistry of Oncogenes Section LTIB, NCI

Project DescriptionObjectives

Although a large body of detailed information concerning the structure of viral and cellular onc genes has accumulated in the past several years, little is known about the mechanism of action of the proteins, which these onc genes specify, in the process of carcinogenesis as well as in normal situations. Our objective is to identify cellular components involved in transformation processes by isolating and characterizing cellular mutants which are resistant to the action of these onc-gene products.

Methods Employed:

Morphologically flat revertants of NIH/3T3 cells originally transformed by 2 copies of Kirsten murine sarcoma virus (Ki-MuSV) genomes were isolated. A selection system for flat (revertant) cells was based on the observation that transformed cells are more sensitive to a Na/K-ATPase inhibitor, ouabain, than are the parental untransformed NIH/3T3 cells. Growth in soft agar was used to test the anchorage dependency of various cell mutants and transformants, while other cell properties were assessed by routine cell culture techniques. Standard Southern and Western blotting techniques were used to detect the Ki-MuSV transforming gene, v-Ki-ras, and its product, p21 protein, respectively. Somatic cell hybridization by polyethylene glycol was employed to investigate the genetic properties of mutant cell lines.

^{86}Rb was used as a tracer for K⁺-flux to test whether alteration in cation transport can be correlated with morphological changes in revertant cells.

Major Findings:

Two clones of flat revertants isolated using above selection techniques have been characterized most extensively. Both revertants contain 2 copies of v-Ki-ras genes without any gross rearrangement, elevated amounts of p21, and rescuable transforming virus. Therefore, these mutants appear to contain alteration(s) in cellular component(s) involved in the expression of the transformed phenotypes.

We tested the ability of these revertants to resist re-transformation with various onc genes by superinfection with sarcoma viruses or by cell fusion with transformed cell lines. As expected, neither mutant could be retransformed by ras-related genes. On the other hand, these revertants were susceptible to transformation by mos, fms and sis. Interestingly, however, the action of fes and src, which are apparently unrelated to one another structurally, seemed to be suppressed in the cell hybrids. Thus, there appeared to be functional relationships and diversities among onc-genes not readily apparent from their structural characteristics.

Since our revertants were obtained by mutagenization followed by ouabain-selection, we conducted two lines of experiments as follows to correlate revertant phenotypes and their decreased sensitivity to ouabain. First, K⁺ ion transport in the revertants as well as normal and Ki-MuSV transformed NIH/3T3 cells was measured in the presence and absence of ouabain: The revertants appeared to possess a unique pattern of ouabain-resistant K⁺ influx. Second, DNAs extracted from these revertants were transfected into fresh Ki-MuSV-transformed cells, and the recipient cells were selected with ouabain: Some flat colonies appeared following transfection with revertant DNA but not with normal NIH/3T3 DNA. These observations suggest that genetic alteration(s) are present in the revertant genome (possibly in the gene(s) coding for membrane component(s)) which can be transferred into fresh cells by DNA-transfection technique.

Significance to Biomedical Research and the Program of the Institute:

It has been suggested that activation of cellular homologues of retroviral oncogenes (proto-oncogenes) may play significant roles in carcinogenesis. From molecular biological evidences, it is now clear that retroviral oncogenes and tumor oncogenes may have been arisen by activation of proto-oncogenes in similar manners (e.g. transcriptional activation by strong promoters and/or point mutations within the genes which render alteration in protein structure). Therefore, it is reasonable to assume that viral oncogenes transform cells by the same pathways as do corresponding tumor oncogenes. This notion was supported by the finding that our revertant cell lines, specifically resistant to the transformation by viral ras and related genes, was also resistant to the transformation by bladder tumor DNA.

Although physiological functions of the proto-oncogene products are still unknown, it is expected that these products are essential in regulations of cell growth and/or cell differentiation. Analysis of the alteration(s) which had occurred in the cellular mutants resistant to the action of specific oncogenes should provide valuable informations on the nature of naturally occurring tumors as well as on the regulation of normal growth and differentiation of the cells.

Proposed Course of Research:

Based on above findings, the following three projects seem to be worth pursuing:

- 1) To test cell lines transformed with other onc genes, including those of human tumors, by cell fusion experiments with the revertants. It should be possible to divide these onc genes on the basis of functional relationships.
- 2) To try to identify membrane components responsible for the behavior of the revertants (among which is their altered response to ouabain). We propose to concentrate on examining membrane components, because it is known that p21 and pp60^{src} exhibit palmitate-binding activities and are located in plasma membrane.
- 3) To identify the gene(s) which has been mutated in the revertant cells using DNA-transfection and molecular cloning techniques.

Publication:

R.H. Bassin, M. Noda and Z. Selinger: Cellular revertants isolated from Kirsten sarcoma virus-transformed cells are resistant to transformation by specific oncogenes. Proc. XXth Congresso Nazionale Soc. Italiana Microbiol. (In Press).

Other Professional Personnel:

Agnes Estival

Guest Worker

LTIB, NCI

Project DescriptionObjectives:

To elucidate the mechanism by which tumor promoters such as phorbol esters modulate Ca^{2+} , phospholipid-dependent protein kinase activity and to determine the role of this kinase in regulating cell growth.

Methods Employed:

Cell culture, standard biochemical analysis of protein kinase activities, protein purification procedures including ion-exchange chromatography and hydrophobic interaction chromatography, SDS-poyacrylamide gel electrophoresis and radiographic analysis.

Major Findings:

Protein kinases have been implicated in the regulation of cell growth and differentiation, as well as in the viral transformation of cells. Since phorbol esters have been shown to modulate the growth and differentiation of numerous cell types. Studies have been devoted to determining phorbol ester effects on protein kinase activities. Previously, it was established that cytosolic calcium, phospholipid-dependent protein kinase (Ca, PL-PK) activity in EL 4 thymoma cells is dramatically decreased following treatment of the cells with phorbol esters. This study has been extended to PYS cells, where exposure to phorbol ester also is found to provoke a rapid (within minutes) loss (approx 90% decrease) in cytosolic Ca, PL-PK activity.

Direct addition of phorbol ester to the reaction mixture in the presence of phosphatidylserine and diacylglycerol has no effect on cytosolic Ca, PL-PK activity. However, direct activation of Ca, PL-PK is noted when phorbol ester is added to a reaction mixture containing no diacylglycerol. It thus appears that phorbol esters are able to replace the PYS enzymes' requirement for unsaturated diacylglycerol.

It was found that concomitant with the loss in cytosolic Ca, PL-PK activity there is a significant increase in the amount of Ca, PL-PK activity associated with the plasma membrane fraction. Quantitation of the amount of Ca, PL-PK activity lost from the cytosol with phorbol ester treatment and the amount gained by the particulate fractions indicates that most of the lost activity can be accounted for now associated with the plasma membrane fraction. Apparently, phorbol esters either provoke the rapid translocation of cytosolic Ca, PL-PK activity to the plasma membrane, or stabilize this kinase in association with the plasma membrane. Indeed, evidence suggests that this protein kinase may itself be a phorbol ester receptor, or at least a component of a receptor complex.

Significance to Biochemical Research and the Program of the Institute:

Phorbol esters are tumor promoters that can amplify the effects of a low dose of a carcinogen, apparently by acting primarily at the cell surface. Since phorbol esters also modulate the growth and differentiation of numerous cell types, they are useful tools also to probe the mechanisms involved in controlling these biological processes. Phosphorylation of membrane proteins is an initial event induced by growth factors, and the intracellular location of certain viral transformation protein kinases is at the plasma membrane. Thus, it is of importance to establish how, and why, protein kinases such as Ca, PL-PK interact with the plasma membrane, and to establish which endogenous membrane substrates are phosphorylated, in response to growth factors, tumor promoters, and viral protein kinases. This will help to elucidate common intermediates in the process of growth control and tumor promotion.

Proposed Course of Research:

Studies will continue to elucidate the role of calcium, phospholipid-dependent protein kinase activity in the regulation of cell growth and differentiation, and to determine if alterations in this kinase activity are required to mediate other effects of phorbol esters. This will include studies to determine the mechanism for the phorbol ester-induced increase in Ca, PL-PK activity associated with the plasma membrane, and to determine if other tumor promoters might cause a similar change in Ca, PL-PK activity. Other studies will be carried out to determine the effects of viral transformation on the activity and subcellular distribution of this kinase. Attempts also will be made to determine specific endogenous membrane proteins phosphorylated in response to phorbol esters and to ascertain the function of such endogenous substrates.

Publications:

Kraft, A.S., Anderson, W.B., Cooper, H.L., and Sando, J.J.: Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL & Thymoma cells. J. Biol. Chem. 257: 13193-13196, 1982.

Kraft, A.S., and Anderson, W.B.: Phorbol Esters increase the amount of Ca^{2+} , phospholipid-dependent protein kinase associated with plasma membrane. Nature 301: 621-623, 1983.

Kraft, A.S., and Anderson, W.B.: Characterization of cytosolic calcium, phospholipid-dependent protein kinase activity in embryonal carcinoma cells: Effect of retinoic acid-induced differentiation of F9 cells to parietal endoderm. J. Biol. Chem. (In press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CB08256-04 LTIB
formerly
Z01CB08256-03 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.)

Role of Hormones & Cyclic Nucleotides in Mediating Cell Growth & Differentiation

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

(Name, title, laboratory, and institute affiliation)

Wayne Anderson, Research Chemist, LTIB, DCBD, NCI

COOPERATING UNITS (if any)

Daniele Evain-Brion, Unite INSERM 188, Paris, France
S. Peter Nissley, Metabolism Branch, NCI, NIH
Anton Jetten, NIEHS, Research Triangle Park, North Carolina

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Biochemistry of Oncogenes Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.6

PROFESSIONAL:

1.2

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Treatment of F9 embryonal carcinoma cells with retinoic acid induces differentiation to an endoderm cell type. A new cell line (DIF 5) derived from F9 cells after prolonged treatment with retinoic acid has been isolated and characterized. DIF 5 cells appear to represent an intermediate in differentiation which is blocked in its ability to complete retinoid-induced differentiation. DIF 5 cells secrete and deposit large amounts of extracellular matrix components (fibronectin, laminin and type IV collagen). These cells also exhibit good cell growth in the complete absence of serum or hormonal supplements. These cells thus offer an excellent system to study the modulation of extracellular matrix formation. In addition DIF 5 cells will be useful to study mechanism involved in growth regulation and the production of embryonic growth factors.

Studies indicate that DIF 5 cells do produce an insulin-like growth factor (IGF). When DIF 5 conditioned growth medium was chromatographed on Sephadex G75 in 1M acetic acid two peaks of activity are observed which compete for specific [¹²⁵I] multiplication stimulating activity (MSA) binding. MSA is the rat homologue of human IGF-II. The high molecular weight fraction (Mr ~ 60K) apparently corresponds to IGF binding protein, while the low molecular weight fraction (Mr ~ 8-10K) apparently is biologically active IGF. These findings suggest that ectopically produced IGF may serve as an early embryonic growth factor.

Other Professional Personnel:

Lalitha Nagarajan	Guest Worker	LTIB, NCI
Robert Bassin	Chief, Biochemistry of Oncogenes Section	LTIB, NCI
Patricia Horan-Hand	Chemist	LTIB, NCI
David Colcher	Microbiologist	LTIB, NCI
Robert Bowen	Medical Staff Fellow	LTIB, NCI

Project DescriptionObjectives:

To better understand how retinoic acid acts to modulate cell growth and differentiation and to determine the role of growth factors and hormones produced and secreted by embryonal carcinoma cells on the growth and differentiation of these cells.

Methods Employed:

Cell culture, standard biochemical analysis of adenylate cyclase and protein kinase activities, hormone binding studies, SDS-polyacrylamide gel electrophoresis and radiographic analysis, immunoprecipitation and radioimmunoassay.

Major Findings:

Teratocarcinoma cells in culture offer an in vitro system to study some early biochemical events involved in embryonic growth and differentiation. Treatment of F9 teratocarcinoma cells with retinoic acid induces extensive differentiation to an endoderm cell type. It has been suggested that endoderm-like cells arising during retinoid treatment of F9 cells are analogous to primitive endoderm cells. Primitive endoderm cells, in turn, may be directed to differentiate into either parietal or visceral endoderm cells by modulating cell substratum interactions or by regulating cyclic AMP levels.

A new cell line (Dif 5) has been derived from F9 cells after prolonged (30 days) treatment with 1 M retinoic acid. This new cell line does not appear to be tumorigenic in syngeneic host strain 129 mice. Dif 5 cells synthesize and deposit an extracellular matrix consisting of several components of Reichert's membrane (fibronectin, laminin, and type IV collagen). These cells also secrete plasminogen activator but they do not synthesize alpha-feto protein. An interesting property of these cells is their ability to grow in the absence of serum or other hormonal supplements. These retinoid-derived cells do possess cell surface receptors for epidermal growth factor (EGF), while specific EGF binding is not observed with either F9 or parietal endoderm cells. Growth arrested Dif 5 cells can be reinitiated to proliferate by the addition of fetal calf serum.

The determined properties of Dif 5 cells fail to meet all the characteristics described for either parietal or visceral endoderm cells. This raises the possibility that Dif 5 cells might represent a primitive endodermal cell type which is intermediate in differentiation to either parietal or visceral endoderm but which lacks the biochemical signal to complete this stage of differentiation. These cells will be useful in studying the modulation of growth and extra

cellular matrix formation during early embryonic development.

Since the Dif 5 cells apparently are able to produce mitogenic factor(s) which may be involved in the promotion of embryonic cell growth, we have attempted to isolate and characterize possible growth promoters present in growth medium conditions by Dif 5 cells do produce both an insulin-like factor (IGF) and IGF binding protein. IGF produced by endoderm cells could act in an autocrine fashion by having effects on the same cell which produced this factor, or it could act as a paracrine system to promote the growth of other nearby cells during early embryonic development.

Significance to Biochemical Research and the Program of the Institute:

Teratocarcinomas are malignant tumors which are characterized by the presence of a cell type known as embryonal carcinoma (EC) cells. EC cells have the capacity, depending upon their environment, either to form tumors or to differentiate into normal cells. Thus, the study of these cells allows the analysis of biochemical events relating to early mammalian development and to neoplasia. Treatments which result in the differentiation of stem cells within a tumor might negate the malignant cell type and result in a benign neoplasm. Further, since EC cells represent a good model for the study of early embryonic development, we can gain a better understanding of the process of normal embryonic cell differentiation and can learn how malignancy alters this process.

Proposed Course of Research:

Studies will continue to determine the possible ectopic production of, and altered responsiveness to, specific hormones and growth factors which may be involved in mediating the growth and differentiation of teratocarcinoma cells. Of interest will be investigations to establish if the ectopic production of calcitonin and/or parathyroid hormone, as well as growth promoters such as IGF, contribute to the maintenance of the malignant state and to the regulation of cell growth and differentiation. This will include studies on the ability of these hormones to alter cyclic AMP levels and calcium flux, and to modulate extracellular matrix production. Investigations also will continue to elucidate the mechanism by which retinoic acid rapidly increases cyclic AMP-dependent protein kinase activity and selectively enhances R_{II} association with the plasma membrane. A radioimmune assay to detect and quantitate the amount of a transformation sensitive P53 protein will be developed. Studies will be carried out to quantitate p53 in various tumors and transformed cells, and to determine the role this protein might play in regulating cell growth and in mediating the onset of the transformed state.

Publications:

Chandrasekaran, K., Mora, P.T., Nagarajan, L., and Anderson, W.B.: The amount of a specific cellular protein (p53) is a correlate of differentiation in embryonal carcinoma cells. J. Cell Physiol. 113: 134-140, 1982.

Nagarajan, L., Jetten, A.M., and Anderson, W.B.: A new differentiated cell line (Dif 5) derived by retinoic acid treatment of F9 teratocarcinoma cells capable of extracellular matrix production and growth in the absence of serum. Exp. Cell Res., (In press).

Nagarajan, L., Jetten, A. M., and Anderson, W.B.: Characterization of a new endodermal cell line (Dif 5) derived from retinoic acid treated F9 teratocarcinoma cells. In L. Silver, G. Martin, and S. Strickland (eds.): Cold Spring Harbor Conferences on Cell Proliferation, Vol. 10, Teratocarcinoma Stem Cells. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (In press).

Anderson, W.B., Nagarajan, L., Jetten, A., Rechler, M.M., and Nissley, S.P.: Production of insulin-like growth factor by Dif 5 endoderm cells: possible early embryonic growth hormone. Cell Biol. Intl. Rpts. (In press).

Other Professional Personnel:

Rayudu Gopalakrishna

Visiting Fellow

LTIB, NCI

Project DescriptionObjectives:

To identify biological activities controlled by calcium which might serve to regulate early biochemical events involved in mediating cell growth and differentiation.

Methods Employed:

Cell culture, biochemical analysis of calmodulin-stimulated enzymatic activities, hydrophobic interaction chromatography, affinity chromatography, SDS-polyacrylamide gel electrophoresis and radiographic analysis.

Major Findings:

Calmodulin (CaM) is a low molecular weight Ca^{2+} - binding protein which serves to mediate numerous Ca^{2+} -regulated enzyme systems and cellular processes. A new method for the determination of calmodulin was established by utilizing Ca^{2+} -dependent hydrophobic interaction with phenyl-Sepharose to rapidly and quantitatively isolate calmodulin from tissues and cells. The use of low Ca^{2+} (100 μM) during sample application to the phenyl-Sepharose column is important to avoid non-specific interaction of proteins to the Sepharose. Further, a monovalent cation (K^+ , Na^+ , or NH_4^+) wash is essential to elute contaminating proteins bound in a Ca^{2+} - dependent manner to the phenyl-Sepharose column. Monovalent cations apparently compete for Ca^{2+} binding to interfering proteins.

Since homogeneous calmodulin preparation's can be isolated with essentially complete recovery utilizing hydrophobic interaction chromatography, calmodulin concentration can readily be determined by assaying directly for protein. If the ability of tissue or cells is limited it is necessary to use either the phosphodiesterase stimulation assay or a radioimmunoassay to determine calmodulin in diluted fractions.

To better understand the nature of the calmodulin binding site on target proteins, we have carried out studies with the calmodulin-dependent cyclic nucleotide phosphodiesterase and the calmodulin inhibitor protein calcineurin. Hydrophobic interaction chromatography was employed to detect and characterize hydrophobic regions on these calmodulin binding proteins. To determine the degree of hydrophobic interactions between the calmodulin binding protein and the phenyl-Sepharose affinity column, various agents and buffer systems were used to elute the bound proteins from the column. The results established that while the calmodulin target proteins do possess hydrophobic regions, the presence of exposed hydrophobic regions on these proteins is not the sole criteria

for determining specific binding to calmodulin. Rather, the isoelectric point of the protein, as well as the ionic environment surrounding the hydrophobic region, apparently play a critical role in modulating such interactions.

The relative degree of affinity for calmodulin (dictated by a combination of hydrophobic and ionic interaction) might play a role in determining the sequential order of activation of these enzymes by the Ca^{2+} -calmodulin complex in response to increased intracellular Ca^{2+} by a given physiological stimulus.

Significance to Biomedical Research and the Program of the Institute:

Numerous studies have implicated calcium and calmodulin as regulators of cell growth and differentiation, and calmodulin levels have been shown to be elevated in a number of transformed cell types. It is thought transformation factors might act by perturbing major cascade regulation systems. The pivotal role of calmodulin as a cellular regulator makes the calmodulin regulatory system an excellent potential target during transformation. Thus, it is of interest to establish the nature of calmodulin interaction with target proteins and to attempt to establish which activities under calmodulin control might be involved in regulating cell growth and differentiation.

Proposed Course of Research:

The newly established quantitation procedure for calmodulin will be used to determine possible alteration in calmodulin levels and subcellular distribution at various periods of the cell cycle and with viral transformation of cells. Also, studies will be carried out to determine if treatment of cells with agents such as retinoic acid and phorbol esters alter the intracellular levels and localization of calmodulin. Other studies will be concerned with establishing the possible role of membrane-associated calmodulin in regulating the secretion of proteins such as hormones and extracellular matrix components.

Publications:

Gopalakrishna, R., and Anderson, W.B.: Calmodulin interacts with cyclic nucleotide phosphodiesterase and calcineurin by binding to a metal ion-independent hydrophobic region on these proteins. J. Biol. Chem. 258: 2405-2409, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01CB09005-01 LTIB
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.)		
Post-translational Hypusine Formation and Control of Protein		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.)		
(Name, title, laboratory, and institute affiliation)		
Herbert L. Cooper, Chief, Cellular & Molecular Physiology Sect., LTIB, DCBD, NCI		
COOPERATING UNITS (if any)		
J. Folk, M.H. Park, Lab. of Protein Chemistry, NIDR; B. Safer, Lab. of Molecular Hematology, NHLBI		
LAB/BRANCH		
Laboratory of Tumor Immunology and Biology		
SECTION		
Cellular and Molecular Physiology Section		
INSTITUTE AND LOCATION		
NCI, NIH, Bethesda, Maryland 20205		
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SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)		
<p>A unique post-translational modification of a single protein in all animal cells has been described for the first time in our laboratories. This modification involves the conversion of a peptide-bonded lysine residue to the unusual amino acid, hypusine by covalent linkage between the epsilon amino terminal of the lysine and a butylamine group derived from the polyamine, spermidine. This is the only specific biochemical reaction yet described for polyamines in intact cells which can be related to a particular biochemical function. The rate of formation of hypusine was found to be proportional to the rate of protein synthesis in human lymphocytes, although the unique protein in which it occurs is synthesized continuously at all levels of protein synthesis. We have shown that this unique protein is the translation initiation factor 4D (eIF-4D). We have purified this protein in large quantities from human erythrocytes, where it is one of the major non-hemoglobin proteins. The role of initiation factor, and therefore in control of protein synthesis is being studied. The function of this protein in erythrocytes, where no protein synthesis occurs, is also under investigation.</p>		

Project DescriptionObjectives:

Studies in the section this year have focussed on the general question of neoplastic human and animal cells. Since proper maintenance of qualitative and quantitative controls on synthesis of proteins is an essential component of physiological control of cell growth and differentiation, knowledge of these mechanisms is essential to an understanding of the basis derangements occurring in neoplasia.

One of the important mechanisms which both regulate protein synthesis and influence the activity of proteins during cellular growth, function and differentiation is posttranslational modification of proteins. Enzymatic modification of proteins by phosphorylation is thought to play an important role in regulation of protein synthesis (by modulating initiation factor 2 activity) and in control of cell growth and differentiation through a great variety of protein kinase reactions. Some of the latter may be critical in the activity of certain tumor producing viruses.

We have recently elucidated another type of posttranslational modification which is chemically highly specific but universally found among animal cells. Accumulating evidence suggests that this posttranslational modification may be a critical control mechanism regulating the increase and decrease in overall translation rate during changes in cell growth and differentiated activity. This modification involves the conversion of a lysine residue in a polypeptide chain to an unusual amino acid, hypusine (N^E-[4-amino-2-hydroxybutyl] lysine).

Methods Employed:

Human peripheral lymphocytes were purified from heparinized whole blood (normal blood donors) by ficoll-hypaque sedimentation and plastic adherence. Erythrocytes were obtained from the same preparations. Cultured lymphoid cell lines were maintained by standard cell culture techniques. Lymphocyte growth was induced by exposure to mitogens (phytohemagglutinin) when indicated. Proteins were radiolabeled in intact cells by incubation with labeled amino acids or with radiolabeled polyamines. Preparation for analysis of proteins were obtained from whole cells and from subcellular fractions prepared by detergent lysis protocols, by nitrogen cavitation and by sonication followed by density sedimentation and ultracentrifugation. Proteins were analyzed by one- and two-dimensional polyacrylamide gel electrophoresis, by ion exchange chromatography and by high performance liquid chromatography. Protein purifications will be described. Proteins were visualized in one-and-two dimensional electrophoretic gels by staining with Coomassie blue or silver, or by radiofluorography for labeled proteins.

Major Findings:

Hypusine formation as a post-translational modification was first demonstrated by us when it was observed that incubation of growing lymphocytes with radio-labeled polyamines (putrescine or spermidine) resulted in acid insoluble labeling of cell protein. Analysis of hydrolyzed protein labeled in this way

showed that viturally all radioactivity was found in an unusual amino acid. Subsequent analysis revealed this amino acid to be hypusine, a component first discovered in body fluids by a Japanese group, who named the material and characterized it, but who did not determine its biological significance.

In further chemical and synthetic studies we found that hypusine was formed by covalent linkage of a butylamino group derived from the polyamine, spermidine, to the E-amino terminus of a peptide-bonded lysine residue. Subsequently the 2-carbon of the added group is hydroxylated.

By two-dimensional electrophoresis of whole cell proteins we found that essentially all this material was confined to a single protein of M_r ca. 18000 and p.I 5.1. In studies of several cell types from a number of species we found that all growing cells, or cells engaged in high rates of protein synthesis formed hypusine in a protein with 2-dimensional mobility identical to that in human lymphocytes. As judged by the relative staining characteristics in 2-dimensional gels, this protein is among the 10% most common proteins of most cells. Purification of small amounts of completely pure protein was accomplished by a preparative 2-dimensional technique. Analysis of tryptic peptides and amino acid analysis of such material showed that only a single lysine residue in this single protein is converted to hypusine. Thus, the enzyme system performing this complex conversion shows a remarkable specificity for a single amino acid site among all cellular proteins.

Cell growth studies show that, while hypusine is formed in growing lymphocytes, essentially none is produced in quiescent (non-growing) lymphocytes which are characterized by very low baseline rates of protein synthesis seen in lymphocytes after exposure to a mitogen. This rise begins within the first few hours of growth stimulation. The unique protein which undergoes hypusine modification, however, is synthesized or turned over continuously in both resting and growing cells, but is only modified in growing ones. Thus, activation of the enzyme system that forms hypusine seems to be an early event in the mitogen-induced growth of lymphocytes. Similar studies with serum-deprived 3T3 fibroblasts showed a marked reduction of hypusine formation associated with cessation of cell growth, indicating the generally of the observation.

Subcellular fractionation studies show that the hypusine-bearing protein is predominantly cytosolic, although small amounts are associated with cytoskeletal components. This distribution, together with its prevalence, its M_r , pI and association with changing rate of protein synthesis, suggested that the hypusine-bearing protein might be one of the known translation initiation factors, eIF-4D, whose described characteristics are similar. By 2-dimensional co-electrophoresis and ion-exchange chromatography of purified rabbit reticulocyte eIF-4D we showed that eIF-4D has M_r and pI identical to that of the hypusine-bearing protein, and contains one hypusine per molecule, thereby proving the identification. They hypusine-bearing protein will henceforth be called eIF-4D.

Since future studies will require large quantities of purified eIF-4D we have sought appropriate sources. It was found that this protein is a prominent component of mature human erythrocytes. Using a purification involving DEAE chromatography, $(NH_4)_2SO_4$ precipitation, and TSK-2000 HPLC separation we have

produced large quantities (mg amounts) of purified eIF-4D from the erythrocytes which are a by-product of our routine lymphocyte preparations. This material contains hypusine, and its presence in erythrocytes in such quantities is currently unexplained. Using this material we have produced sheep antiserum which is currently being affinity purified. Production of a monoclonal antibody is also in progress. Also of great interest is the unmodified form of the protein found in resting lymphocytes. To investigate the role of this material will also require a good source of supply. Since the amounts recoverable from peripheral lymphocytes are comparatively small, alternative sources are being considered.

Significance to Biomedical Research and the Program of the Institute:

Our work has shown that the production of the translation initiation factor eIF-4D involves a unique posttranslational modification whose occurrence parallels the prevailing rate of cell protein synthesis. Since the unmodified form of the protein is apparently produced in quiescent cells, it is possible that the function of eIF-4D as an initiation factor is modulated by conversion of a specific lysine to hypusine in this precursor form. Activation of the hypusine-forming enzyme system as an early event in growth stimulation may govern this modulation, thereby regulating the normal increase in protein synthesis and influencing cell growth. The cessation of hypusine formation during cell growth arrest suggests that this mechanism may be involved in the turn-off phase of cell growth as well. Biochemical control mechanisms at both these stages are of obvious importance in study of neoplastic cell growth.

At a more basic level, these studies will expand the current area of study of mechanisms of regulation of protein synthesis, which are largely focused on eIF-2 and its associated proteins. The discovery of a unique modification of eIF-4D which may modulate its function should attract considerable interest from workers in this field.

In another direction, this work has indentified the only specific biochemical reaction, occuring in vivo and related to particular cellular pocesses, which has yet been described for the ubiquitous polyamines, despite many years of intense study in that area.

Proposed Course of Research:

1. Purification and characterization of both hypusine-modified and unmodified forms of eIF-4D for further studies of biochemical function.
2. Determination in cell-free systems of the roles of the modified and unmodified forms of eIF-4D in protein synthesis. Currently, there is uncertainty as to the precise function of eIF-4D. We particularly wish to distinguish between two possibilities: a) The unmodified form is an inactive precursor which is activated by hypusine formation; b) The unmodified form is a negative regulatory factor which is inactivated by hypusine formation.
3. Characterization of the enzyme system which performs the hypusine modification. Since cell growth may be regulated by the activity of this system, it

is essential to understand how its function is regulated as part of the growth activation and cessation sequence. In particular, it is important to determine how mitogen binding events at the cell membrane cause activation of this enzyme system.

4. Clarification of the role, if any, of the large amount of eIF-4D in erythrocytes. Since these cells do not synthesize protein the presence of this material is puzzling. It is possible that the factor is merely left over from an earlier stage when protein synthesis was active. Alternatively, eIF-4D may perform other functions.
5. An examination of the control of hypusine formation in the context of changing cell growth conditions in malignant as compared to normal cells. It is possible that improper turn-off of protein synthesis occurs in some neoplastic cells because of defective control of the modification of eIF-4D.

Publications:

Park, M.H., Cooper, H.L., and Folk, J.E.: The biosynthesis of protein-bound hypusine (N^E -(4-amino-2-hydroxybutyl) lysine): Lysine as the amino acid precursor and the intermediate role of deoxyhypusine (N^E -(4-aminobutyl) lysine). J. Biol. Chem. 257: 7217-7222, 1982.

Cooper, H.L., Park, M.H., and Folk, J.E.: Post-translational formation of hypusine in a single major protein occurs generally in growing cells and is associated with activation of lymphocyte growth. Cell 29: 791-797, 1982.

Cooper, H.L., Park, M.H., Folk, J.E., Safer, B. and Braverman, R.: Identification of HY^+ , the unique hypusine-containing protein, as translation initiation factor eIF-4D. Proc. Nat Acad. Sci. USA. 80: 1854-1857, 1983.

Park, M.H., Cooper, H.L. and Folk, J.E.: Chromatographic identification of hypusine (N^E -(4-amino-2-hydroxybutyl)lysine and deoxyhypusine (N^E (4-aminobutyl) lysine). Meth. in Enzymol. (In press).

Cooper, H.L., Park, M.H., and Folk, J.E.: Hypusine formation: a unique post-translational modification in translation initiation factor eIF-4D. Methods in Enzymology, (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB09006-01 LTIB
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 Events in Phorbol Ester Effects on Cell Growth and Differentiation		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Nili Feuerstein, Visiting Fellow, LTIB, DCBD, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Tumor Immunology and Biology		
SECTION Cellular and Molecular Physiology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
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SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Treatment of cells of the promyelocytic leukemia line, HL-60, with the phorbol ester, PMA, results in arrest of cell growth and induces terminal differentiation into monocytes. We have shown that in this response PMA initiates a rapid (<30 min.) and dramatic increase in phosphorylation of a 17kD cytosol protein (ppl2). Although the phosphoester bond of this protein was highly alkalai stable, phosphoamino acid analysis revealed that the protein is not phosphorylated at tyrosine residues, but only at phosphoserine. Induction of phosphorylation of ppl7 is intimately associated with the initial events by which PMA interacts with cells, since inactive phorbol esters--which do not bind to cellular binding sites--fail to induce the phosphorylation event. Moreover, increased phosphorylation of ppl7 was also observed during the interaction of PMA with other cell types (U937, A431, and human lymphocytes). This suggests that induction of phosphorylation of ppl7 may be a generalized initial response of cells to PMA treatment, promoting activation of the particular differentiation program of the cell lineage involved. The importance of this induction in activating the sequence of events leading to cellular differentiation is indicated by the failure of PMA to induce differentiation in the presence of trifluorperazine, which inhibits the PMA-induced phosphorylation of ppl7.</p> <p>Kinetic studies showed that the phosphate group in ppl7 is not stable <u>in vivo</u>, but undergoes rapid phosphorylation and dephosphorylation. Induction of phosphorylation of ppl7 is not dependent on influx of extracellular calcium. Further characterization of the protein kinase involved in this reaction is in progress. Studies with methylation inhibitor, 3-deazaadenosine, indicate that the initial response of HL-60 cells to PMA, leading to induction of phosphorylation of ppl7, does not require transmethylation reactions, although such reactions may play a role at a later time during differentiation.</p>		
1013		

Other Professional Personnel:

Herbert L. Cooper Chief, Cellular & Molecular Physiology Sect. LTIB, NCI

Project DescriptionObjectives:

Phorbol esters, in addition to being powerful tumor promoters, have profound effects on cellular processes of growth and differentiation. Striking effects of this type are seen when human leukemic cells are treated with nanomolar concentrations of phorbol-12-myristate-13-acetate (PMA), an extremely active tumor promoting phorbol ester derivative. Such treatment results in prompt arrest of cell growth and induction of terminal differentiation. Evidently, interaction of PMA with the cells sets in motion a series of biochemical events which affect both the control of cell growth and expression of differentiated characteristics. We have used this system as a model for studying these events, concentrating on the induction and phosphorylation of specific proteins.

Methods Employed:

HL-60 promyelocytis leukemia cells were cultured under standard conditions. Radiolabeling, cell fractionation, 2-dimensional gel electrophoresis were as in project 1. Phosphoamino acids were analyzed on acid hydrolysates of specific proteins by two-dimensional thin-layer electrophoresis and chromatography. Cell adherence was studied in Boyden chambers with nucleopore membranes.

Major Findings:

Within 24 hrs of addition of $10^{-7}M$ PMA to undifferentiated HL-60 cells growing rapidly in suspension, cell growth ceases entirely and the cells become strongly adherent to the culture vessel, exhibiting spontaneous motility. The cells remain viable and are highly active. The exhibit phagocytosis and enzymatic studies have shown them to resemble monocytes at this time. Incubation with a radioactive amino acid ($[^3H]$ leucine) and analysis of whole cell proteins by 2-dimensional electrophoresis reveals that this monocytoïd differentiation is accompanied by induction of synthesis of a specific set of proteins, some present in the cytosol, others confined to the crude membrane fraction (m1-m6, m10). Proteins m1-m6 and m10 are also prominently synthesized by mature monocytes isolated from peripheral blood, but are not detected in lymphocytes. This is evidence that PMA treatment indeed activates the biochemical program leading to monocytoïd differentiation, allowing distinction between cells of this lineage and other types. The results demonstrate that 2-dimensional analysis of whole cell, or cell fraction proteins is a powerful method for detecting cell and tissue specificities, once marker proteins are established.

In a study of phosphoproteins formed in HL-60 cells we found that PMA induces a rapid and dramatic increase in phosphorylation of a 17 kD protein (pI: 5.1) pp17) and also an increase in phosphorylation of a 27 kD protein with similar pI (pp27). This effect is maximal within 30 min of addition of PMA. Both of these proteins are cytosolic in location. Coomassie blue stained gels of

cytosol preparations revealed a stained peptide approximately one charge unit more acidic than pp17, with identical M_r . This may be the substrate protein which is phosphorylated after PMA treatment. Derivatives of phorbol ester which are inactive in tumor promotion are also inactive in induction of monocytoid differentiation. We showed that these inactive derivatives also failed to induce phosphorylation of pp17 and pp27, indicating a close relationship between induction of phosphorylation of these proteins and differentiation.

The importance of the early phosphorylation of pp17 to the differentiation response is further indicated by the effect of another drug, trifluoroperazine. This substance inhibits PMA-induced phosphorylation of pp17 and also prevents the monocytoid differentiation of HL-60 in a dose-dependent manner. These observations suggest that induction of phosphorylation of pp17 and pp27 is a rapid consequence of PMA binding which may be essential to subsequent steps in the differentiation pathway.

Recent evidence suggests that activation of tyrosine-specific protein kinases may be involved in biological processes associated with control of cell growth and malignant transformation by oncogenic viruses. Moreover, treatment of certain cells with PMA is reported to induce phenotypic changes similar to those associated with transformation by Rous sarcoma virus or to enhance the expression in transformed cells of the properties associated with the transformed state. Therefore, we characterized the phosphoamino acids in pp17 and pp27. We found that the phosphoester bond of pp17, but not of pp27, was highly stable to alkali treatment. Such stability has been ascribed to phosphotirosine residues in contrast to the more common phosphoserine. However, phosphoamino acid analysis of pp17 recovered from electrophoretic gels showed phosphorylation only of phosphoserine; neither tyrosine nor threonine were phosphorylated.

Other investigators have proposed that methylation of phospholipids is an early event that is stimulated following receptor binding and plays an essential role in transmembrane signal propagation in a variety of systems. Since induction of phosphorylation of pp17 follows shortly after the binding of PMA to its receptor, we questioned whether this induction was dependent on presumptive methylation events connected with PMA binding. A potent inhibitor of methylation, 3-deazaadenosine, had no effect on the induction of phosphorylation of pp17 and pp27, nor did it prevent the arrest of cell proliferation caused by PMA treatment. Thus, the rapid induction of these phosphorylation events occurs independently of any methylation reactions, indicating that the initial transmembrane propagation of signals in this system is not mediated by methylation reactions.

However, development of cell adherence was strongly inhibited by the methylation inhibitor. In addition, 3-DZA caused a moderate inhibition of induction of proteins m1-m6 and a strong inhibition of induction of m10. In contrast, the drug did not inhibit synthesis of m10 in mature monocytes. This indicates that the effect of 3-DZA on these late developing events is on processes involved in the differentiation sequence, but not on the final differentiated function. Thus, methylation reactions may be involved at some point in the differentiation process, but they are not essential for the initial signal propagation.

Studies on the nature of the protein kinase responsible for phosphorylation of ppl7 are in progress. Thus far, we know that it is not a tyrosine kinase. Studies with Ca^{++} chelators suggest that influx of extracellular calcium is not required for the activity of this kinase.

Significance to Biochemical Research and the Program of the Institute:

These studies contribute to the reconstruction of the sequence of biochemical events leading from the initial interaction of undifferentiated cells with a powerful tumor promoter (PMA) to the emergence of a highly differentiated cell type. This approach will provide necessary information on the regulation of cell growth and differentiation at a basic level. Understanding of the biochemical processes involved in reestablishing the differentiated state in a line of malignant, undifferentiated cells will provide important information about the nature of the derangement in the neoplastic condition and suggest possibilities for intervention.

Our results have delineated crucial, early phosphorylation events in the re-establishment of differentiation and cessation of cell growth: phosphorylation of ppl7 and pp27. The control of these events and their significance to differentiation are under study. Our findings place these events in perspective in comparison with other systems. Thus, although oncogenic viruses and certain other ligands are reported to function through activation of tyrosine-specific protein kinases, we have shown that this is not the case for PMA-induced phosphorylation of ppl7 and pp27. Similarly, the intervention of methylation events, claimed to be crucial in propagation of signals resulting from binding of certain ligands, was not involved in transmission of such signals from the PMA-bound receptor to the activation site of the protein kinase responsible for phosphorylating the cytosolic proteins, ppl7 and pp27. Our results suggest that the importance of methylation events may lie in subsequent steps in the differentiation program.

We have shown that 2-dimensional electrophoretic analysis of whole-cell and cell fraction proteins can be a powerful method for determining the cell lineage and state of differentiation of cells once marker proteins have been established. This may have practical utility in characterizing cells as to malignant potential in clinical situations, as well as providing a possible means of assessing therapeutic effects.

Proposed Course of Research:

1. Elucidation of the biochemical mechanisms underlying early phosphorylation of ppl7 and pp27 following PMA treatment in HL-60. Specifically, further studies to characterize the protein kinase involved in these reactions and its mode of regulation. Development of cell-free systems to study this kinase and its substrates.
2. Study of PMA effects in other cell types to determine whether induction of phosphorylation of ppl7 and pp27 is a general response to PMA binding. Investigation of other ligands to determine whether these events are restricted to PMA response or may be a common mechanism in reaction of cells to ligands

which modify growth and/or differentiation.

3. Investigation of the biochemical linkage between early phosphorylation events and other physiological effects induced by PMA; relationship to phospholipid and arachidonate metabolism. Study of the role of phosphorylation events in induction of synthesis of specific proteins (m1-m6, m10). Exploration of modifications of the transcriptional-translational mechanism involved in such inductions.

Publications:

Feuerstein, N. and Cooper H.: Rapid protein phosphorylation induced by phorbol ester in HL-60 cells: unique alkali stable phosphorylation of a 17000 dalton protein detected by two-dimensional electrophoresis. J. Biol. Chem. (In press).

Other Professional Personnel:

Herbert Cooper Chief, Cellular and Molecular Physiology Section LTIB, NCI

Project DescriptionObjectives:

The HLA antigens have important functions in human immune phenomena, particularly in the detection and rejection of cells which are recognized as foreign by virtue of surface alterations related to viral infection and possibly to neoplastic transformation. The class I HLA molecules (HLA-A, B,C) of immunological cell membranes are involved in the presentation of foreign antigens to T lymphocytes in an appropriate context for activation of antigen specific clones of cytotoxic cells. However, the biochemical mechanisms involved in these recognition and activation phenomena are unknown, as are the precise means by which HLA Molecules participate in them. While current immunological theory assigns a clear role to the HLA molecules on antigen presenting cells, a function for these ubiquitous molecules on the responding cells is seldom mentioned. In this project, we have begun to explore the possible role of HLA molecules on responding T lymphocytes and to examine the biochemical mechanisms involved in their function.

Methods Employed:

Preparation of lymphocytes, culture conditions, mitogen treatment, cell fractionation, radiolabeling of proteins and their analysis by two-dimensional polyacrylamide gel electrophoresis was as in project 1. Iodination of plasma proteins was done by the lactoperoxidase method. Immunoselection of HLA antigens and B₂-micro-globulin was done using either monoclonal anti-HLA antibodies, or anti-B₂-Microglobulin rabbit antiserum, combined with Protein-A precipitation. Functional immunological responses were assayed by allogeneic mixed lymphocyte activity (MLR) and antigen-specific cell-mediated lympholysis (CML) performed by our collaborators.

Major Findings:

By combining two-dimensional electrophoretic analysis of whole-cell proteins with analysis of immunoselected HLA and B₂-M, we were able to identify the HLA molecules among the total complement of proteins synthesized and radiolabeled in resting and growing human peripheral lymphocytes. Comparison of Coomassie blue-stained gels with radiofluorographs of the same gels allowed an examination of steady-state versus synthetic parameters of individual proteins. This permitted comparisons of the relative rates of synthesis of HLA and other proteins, and of the effects of growth activation on these relations using samples of whole cell proteins. In this way, kinetic studies of the synthesis and degradation of HLA molecules could be carried out which are only possible with great difficulty, if at all, by standard immunological procedures.

We found that in resting T-lymphocytes, in the absence of any immunological activation, HLA molecules are among the most actively turned over proteins of the cell. Among plasma membrane proteins, HLA molecules are the most actively turned over proteins. This is in contrast to other plasma membrane proteins of the quiescent lymphocyte, which are relatively stable. Thus, resting lymphocytes are actively engaged in synthesizing and replacing surface HLA molecules to a much greater extent than other surface proteins, an activity which must be presumed to be related to the biological function of these cells. When we examined the turnover kinetics of HLA in comparison with other cell proteins, we found that HLA molecules had a much shorter half-life (ca. 8 hrs) than total cell protein (12-16 hrs), confirming the special characteristics of the metabolism of HLA molecules.

Activation of lymphocytes by a non-specific mitogen (phytohemagglutinin) caused an increase in overall protein synthesis, as is well known. Examination of the relative stimulation of various proteins showed that synthesis of HLA was stimulated to a lesser degree than most proteins. However, the half-life of newly synthesized HLA molecules was prolonged, indicating a reduction in rate of turnover. The result was a net accumulation of HLA on the cell surface in activated lymphocytes. This observation provides the biochemical background for the immunological observations which have shown an increase in the expression of HLA molecules on lymphocyte surfaces after activation. Clearly, HLA molecules are subjected to specific biochemical processes which regulate their quantity and metabolic fate in relation to the chemical events at the cell surface which constitute the activation sequence.

Activation of lymphocytes in vivo molecular recognition events in which HLA molecules are essential participants. We have begun to examine the chemical basis of this recognition, and at the same time have explored the nature of HLA polymorphism. By modifying our 2-dimensional electrophoretic technique (altered acrylamide; bisacrylamide ratios; ampholyte selection) we have significantly improved our ability to resolve the complicated electrophoretic pattern which characterizes the group of HLA proteins. Charge heterogeneity was reduced by neuraminidase treatment to eliminate variable degrees of sialylation. As a result of these changes we were enabled to obtain highly resolved 2-dimensional electrophoretic maps of the HLA proteins of specific subjects. These were found to be highly specific and very reproducible, demonstrating a previously unknown small degree of molecular size heterogeneity, as well as wide molecular charge heterogeneity among the various HLA gene products. Using this technique we investigated the sensitivity of the method in detecting genetic variation among the HLA molecules of different donors. We studied a panel of 5 donors whose HLA-A,B antigens were indistinguishable by the usual HLA serological techniques. However, functional tests (see methods) revealed that among the group, some specific antigens were recognized as foreign by other members of the group who carried an antigen which was serologically identical. This indicated a minimal degree of difference between the HLA molecules in question. Analysis by our technique allowed us to detect all of the variants unequivocally, because each showed an alteration in molecular charge detected as a change in isoelectric point.

In addition to providing a sensitive method of analysis of differences in HLA antigens by biochemical rather than immunological parameters, the finding raised the question of whether altered molecular charge was a universal finding among HLA molecules which provoke functional immunological responses but may be indistinguishable serologically. An examination of the available data in the literature revealed the striking fact that among 16 HLA and H2 (the murine equivalent) molecules studied, 14 showed amino acid substitutions which caused altered molecular charge. This was true, even though the average number of amino acid substitutions involved in these mutations was only 1.6. In our data, among the 14 HLA molecules which showed no functional evidence of nonidentity we found no molecules with functionally silent charge alterations. Thus, mutations which produce charge alteration appear always to produce functionally recognized variants. This has led us to propose as a working hypothesis that amino acid substitutions which produce charge alterations are an important mechanism in the generation of functionally significant HLA polymorphism. At the biochemical level, this suggests that the chemical events involved in immunological recognition utilize charged amino acids as important landmarks.

Significance to Biomedical Research and the Program of the Institute:

Our finding that HLA molecules are the major proteins undergoing turnover in resting lymphocyte plasma membranes was completely unknown to immunologists. It points strongly toward a significant biological role for HLA molecules in resting lymphocytes, although current immunological theory provides no such role. We have suggested that this intense biochemical activity may relate to the biological function thought to be performed by resting T-lymphocytes in vivo, namely continuous surveillance for the appearance of non-self antigenic specificities on cell surfaces. Our model to describe this activity proposes that T-lymphocytes utilize their surface HLA molecules as recognition elements which "see" corresponding HLA molecules on the surfaces of antigen presenting cells by transient chemical interactions. In the absence of activation, such interactions are terminated by proteolytic event, resulting in turnover, of the HLA protein. If HLA molecules on the presenting cell are configured by the presence of foreign antigen, the surveillance interaction results in T-cell activation rather than termination. Our studies on the basis of functionally significant HLA diversity suggest that the chemical basis for the participation of HLA molecules in immunologic surveillance may depend strongly on charged amino acids in those regions of the HLA molecule which interact with the detecting mechanism on T lymphocytes. This model has many experimentally testable consequences, which will form a major part of the continuation of this project.

Since neoplasms are often characterized by the appearance of surface antigens which are immunologically foreign to the host, the possibility of mounting an effective immunological defense against the tumor may ultimately be of major therapeutic importance. Thorough understanding of the chemical basis for immunological detection and cytolysis of cells bearing foreign determinants may provide us with clues as to where these normal defense mechanisms may be enhanced or supplemented for therapeutic purposes.

Proposed Course of Research:

1. Exploration of the biochemical basis of the prominent turnover of HLA proteins in resting T lymphocytes. This will involve examining the conditions of cellular interaction which promote or inhibit such turnover, the proteolytic processes involved in turnover, and the mechanisms by which they are regulated. Evidence for HLA interactions with other membrane-bound molecules will be sought, with examination of the effect of charge modification on such interactions.
2. Structural relationships between HLA molecules in plasma membranes and the recently identified putative antigen-specific T-cell receptor will be examined. Our model (see above) predicts that fairly close juxtaposition of HLA sites and T-cell receptor sites will exist.
3. Extensions of our studies on the specific modifications of HLA molecules which are recognized as functional signals in immunological responses. In a collaborative study (Lab. of Immunogenetics, NIAID) we will identify HLA molecules which have minimal structural differences recognized only by functional tests, determine the degree of charge variation present, identify tryptic fragments which bear the variants and determine the amino acid modifications. This will allow us to test our hypothesis that substitution of charged amino acids is a major source of functionally significant HLA diversity.
4. In a related area, we intend to examine the proteins and peptide fragments which are released into the culture fluids of lymphocytes under various conditions of growth. In part, this will be an effort to search for components derived from proteolytic breakdown of HLA molecules, to test various predictions of our hypothesis on the meaning of HLA turnover in resting lymphocytes. In addition, this study will be a more general examination of the types of proteins, particularly those with growth regulatory activity (lymphokines), elaborated by resting and growing lymphocytes in the course of immunological activity.
5. An examination of the synthesis of HLA molecules by neoplastically transformed cells in comparison with normal cells of the same tissue. This question will explore the possibility that failure of immunological rejection of tumor cells may be related to improper expression of histocompatibility antigens necessary for correct antigen recognition.

Publications:

Monos, D. and Cooper, H.L.: Rapid turnover of HLA proteins in quiescent lymphocytes: proposed connection with immunologic surveillance. J. Immunol. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CB00944-21 LTIB

formerly

Z01CB00944-20 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.)

Total Metabolism of Cancer Cachexia

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

(Name, title, laboratory, and institute affiliation)

Seoras D. Morrison, Research Physiologist, LTIB, DCBD, NCI

COOPERATING UNITS (if any)

Jeffrey Norton, Surgical Metabolism Section, Surgery Branch, NCI

LAB/BRANCH

Laboratory of Tumor Immunology and Biology

SECTION

Cellular and Molecular Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

The project is directed toward identifying the causes of the nutritional depletion and general deterioration of the cancerous host, known as cancer cachexia. The object is to find ways of blocking or reversing these systemic effects of cancer so that the cancer patient would become more accessible to anti-cancer therapies. Work on normal and cancerous organisms is in progress on: total energy, water and other material exchanges; physiological and behavioral control of food and water intake; metabolic abnormalities in nitrogen and lipid metabolism; effects of imposed motor activity and of insulin on nitrogen and lipid depletion; discrimination of mass and metabolic effects of tumor; identification of plasma components triggering the abnormal host responses; modifications of composition of IV aliment to minimize tumor growth and maximize host retention.

Project Description:Objectives:

(a) Investigation of the patterns of heat production of rats with concomitant recording of feeding behavior and other activity with a view to finding how the energy expenditure, feeding and general behavior pattern are related to tumor growth, to finding the nature of the loads imposed by a tumor on its host, and to identifying the causes of cancer cachexia. (b) Investigation of relative changes in water and material exchanges in tissue compartments of the rat during imposed and induced changes in food and water intake and the relation of these to tumor induction and growth. (c) Examination of metabolic effects of total parenteral nutrition and chemotherapy in non-tumor-bearing rats and on the cachexia and decline of food intake induced by tumor growth. (d) Identification of functional sites and causes of breakdown of control of food intake during tumor growth. (e) Identification of molecular fragments in plasma inducing anorexia and metabolic change. (f) Development of conceptual models of control of food and water intake and regulation of energy and water exchange and their inter-relationships for normal animals and for the cancer cachectic process.

Methods Employed:

The methods of indirect, total, long-term calorimetry, operant conditional responses, placement of electrolytic lesions in or stimulation of the central nervous system, continuous or programmed infusions into unrestrained animals, and methods of orthodox nutritional studies. Computer methods of numerical analysis of serial records of tumor growth, continuous records of gaseous exchange and of change in feeding and drinking patterns in relation to total energy exchange and tumor growth. Sprague-Dawley, Buffalo and Fischer rats are used in all animal experiments. Most tumor-bearing rats are inoculated with Walker 256 carcinoma or MCA sarcoma, but other transplantable tumors are also used.

Major Findings:

The feeding response of tumor-bearing animals to exogenous insulin demonstrated earlier (Morrison, 1982) is being further examined. There is evidence that this treatment delays irreversible anorexia. Work is under way to define optimum dose and period of treatment and composition of spared carcass tissue.

A method was devised to discriminate the mechanical effect of large mass of transplantable tumors from their biological cachectic effect. The mechanical effect leads to overestimate of cachectic weight loss and underestimate of cachectic asthenia.

Investigation of reduced perception of hunger or premature satiety as immediate mechanisms of cancer hypophagia indicates that premature satiety is the dominant effect.

A project is underway to examine effects on tumor-stimulated gluconeogenesis of limiting glucose availability by supplying all non-protein energy as glycerol

and simultaneously blocking the phospho-enol-pyruvate gluconeogenic pathway. Preliminary results indicate that this does not prevent skeletal muscle nitrogen depletion.

Significance to Biomedical Research and the Program of the Institute:

The findings on control of feeding in tumor growth are throwing light on the nature of cancer cachexia and should be utilizable in the development of effective methods for improving the nutritional condition of cancer patients. The section of the National Cancer Plan that the work most closely approximates is: Objective 6 (Develop the means to cure cancers and to retard the progress of cancers not cured). Approach 4 (Enhance the host's ability to eliminate or prevent further development of Cancer). It is also immediately relevant to the 1974 Amendment to the Cancer Act (Collect ... information respecting nutrition programs for cancer patients and the relationship between nutrition and cancer).

Proposed Course of Research:

Work in energy and water exchange of tumor bearers will be continued along with study on the interaction of metabolic and behavioral responses of normal and cancerous animals, with particular reference to the reasons for the changes in food and water intake and depletion of host tissues that occur during tumor growth. Work is planned on identification of the humoral agents in plasma of tumor-bearers that immediately induce decline of food intake and metabolic abnormalities.

Publications:

Morrison, S.D.: Control of food intake in experimental tumor growth. Cancer Treatment Rpts 65 (Suppl. 5): 9-14, 1981.

Morrison, S.D.: Feeding response of tumor-bearing rats to insulin and insulin withdrawal and the contribution of autonomous tumor drain to cachectic depletion. Cancer Res. 42: 3642-3647, 1982.

Popp, M.B., Morrison, S.D. and Brennan, M.F.: Total parenteral nutrition in a methylcholanthrene-induced rat sarcoma model. Cancer Treatment Rpts. 65(Suppl. 5): 137-143, 1981.

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Morrison, S.D.: Nutrition and longevity. Nutr. Rev., (In press).

Morrison, S.D.: In vivo estimation of size of experimental tumors. J. Natl. Cancer Inst. (In press).

Other Professional Personnel:

Magdi M. Mossoba	Visiting Fellow	LTIB, NCI
Alasdair Carmichael	Visiting Fellow	LTIB, NCI

Project DescriptionObjectives:

The effects of ionizing and ultraviolet radiation on biological macromolecules and their constituents are being investigated. For reproductive death, DNA is the target molecule in viruses and is at least a part of the target molecule in bacteria and mammalian cells. Radiation damage to DNA is produced by the "direct effect" through the formation of radical ions, electrons, excited states and neutral free radicals or by the "indirect effect" where radical species are hydrated electrons, hydrogen atoms and hydroxyl radicals. In the case of radiation damage to the chromosome, radicals formed in the nucleohistone may cause damage to the DNA.

In the chain of events that leads to loss of biological activity, free radicals play an important role. Chemical compounds have been discovered which significantly modify radiation effects. These include: (a) electron affinity sensitizers which act on hypoxic tumor cells, (b) halogenated pyrimidines which are incorporated into DNA and (c) cancer chemotherapy agents of the intercalating or alkylating type which sensitize tumor and normal cells.

Studies of the mechanism of action of radio-sensitizers and radio-protectors are necessary to design improved combinations of chemotherapy and radiation therapy.

An understanding of the mechanisms by which ionizing radiation brings about the loss of biological activity in macromolecules is likely to help in the development of new methods for altering the efficiency of cell killing with possible benefits to radiation therapy.

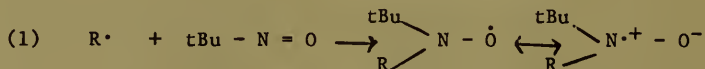
In the last few years it has become apparent that superoxide anion radicals and hydroxyl radicals are found in many biological systems in the absence of either ionizing radiation or UV-photolysis. Recent reports have indicated that radicals are produced in the presence of certain anti-cancer drugs such as bleomycin and adriamycin. The significance of radical reactions is therefore not confined to radiation biology.

Methods Employed:

Nucleic acids, proteins and their constituents were γ -irradiated either in the solid state at 2×10^{-5} torr or in aqueous solutions in a 800-curie Cobalt γ -source. Electron spin resonance studies were carried out with a Varian E-9 Spectrometer connected to a Nicolet Lab 80 computer. For photolysis studies at specific wavelengths, a 1000-watt high pressure Mercury-Xenon arc source and monochromator were employed.

Spin-trapping Method:

In the spin-trapping method, the short-lived free radicals react with a diamagnetic scavenger (the spin-trap) to produce longer-lived radicals (the spin-adduct) which can be conveniently investigated by e.s.r. In our studies, *t*-nitrosobutane (tNB) was employed as the spin-trap, equation (1):



The e.s.r. spectrum of the spin-adduct nitroxide shows a primary triplet splitting due to the ^{14}N nucleus and secondary splittings which usually arise from the magnetic nuclei of the trapped radical $R\cdot$. From the e.s.r. spectra of the nitroxide, the structure of $R\cdot$ can often be identified. For some experiments in which several radicals were spin-trapped simultaneously, the resulting spin-adduct nitroxides were separated by means of a Waters High Pressure Liquid Chromatograph with a C_{18} -micro BONDAPAK column and water-methanol elution gradients. A capillary flow-detector in the e.s.r. cavity was used to indicate the positions of the free radical peaks.

Major Findings:I. Photochemistry of Daunomycin and Adriamycin in Aqueous Solutions

The free radicals generated by photolysis of aqueous aerated solutions of daunomycin and adriamycin at 438 nm were investigated by ESR and spin-trapping. This is of interest in relation to the possible use of these drugs in the photo-radiation therapy of tumors. With the spin trap 4-[(1,1-dimethylethyl)imino]-1-methylpyridinium N-oxide (PYBN) the formation of superoxide anion radicals in aqueous aerated solutions could be established. The absence of hydroxyl radicals was shown by competition studies with formate and ethanol which demonstrated that the DMPO-OH spin-adduct was formed directly from the $\text{DMPO-O}_2^{\cdot-}$ adduct and not from free hydroxyl radicals. The absence of photoionization was indicated by the absence of the deamination radicals from alanine and glycine which are efficiently spin-trapped and identified by 2-methyl-2-nitrosopropane. The absence of singlet oxygen was demonstrated by the method of Lion which utilizes the conversion of 2,2,6,6 tetramethyl-4-piperidone by singlet oxygen to the corresponding nitroxide radical which is ESR - detectable. Fluorescence measurements in aqueous solutions of daunomycin and adriamycin clearly showed quenching of the singlet state by oxygen. The formation of superoxide anion radicals occurs either by the reaction of quinone anion radicals or of quinone singlet or triplet states with oxygen,.

II. Quantitative Aspects of ESR and Spin Trapping with DMPO and POBN in γ -irradiated Aqueous Solutions

The spin traps 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and α -4-pyridyl-N-tert-butyl nitron 1-oxide (POBN) are frequently used to identify hydroxyl radicals and hydrogen atoms, respectively. However, the quantitative aspects of these spin trapping reactions in aqueous solutions containing DMPO and POBN

are not known. The effects of dose, spin trap concentration, pH and of the stability of the spin adducts on the spin trapping efficiency were investigated.

The efficiency of DMPO as the spin trap for hydroxyl radicals in degassed solutions is 35%. Assuming a direct hydrogen atom yield from γ -radiolysis of $G_H = 0.55$, the hydrogen atom spin trapping efficiency of POBN in the degassed solutions is 1.3%. These values were obtained in the plateau region of the plots of radical yields vs. spin trap concentrations and under conditions where the decay of the spin adducts is negligible. Control experiments indicated that there was no reaction between hydrogen peroxide formed by γ -radiolysis and the spin adducts under our conditions. The low spin trapping efficiency of DMPD for OH radicals is explained in terms of hydrogen abstraction reactions which compete with the addition of hydroxyl radicals to the nitron double bond.

III. E.s.r. of spin-trapped radicals in γ -irradiated polycrystalline DL-alanine. A quantitative determination of radical yield. (In collaboration with Yves Lion and Guy Denis at the Institute of Physics, Univ. of Liege, Belgium.)

The quantitative aspects of determining free radicals in polycrystalline amino acids γ -irradiated at room temperature and subsequently dissolved in spin-trap solutions were investigated. The deamination radical in DL-alanine was used for detailed studies and 2-methyl-2-nitrosopropane (MNP) was employed as the spintrap. The spin-trapping efficiency (the number of radicals spin-trapped in solution divided by the number of radicals initially present in the γ -irradiated solid) was found to be in the range 1 to 10 per cent for aqueous solutions depending on the experimental conditions. The effects of dose, particle size, pH, spin-trap concentration, age of spin-trap solution, MNP monomer to dimer ratio and the presence or organic solvents were investigated. Several reactions were found to decrease the spin-trapping efficiency; radicalradical recombination, the competition between the spin-adduct and the spin-trap for radicals and the reaction of radicals with the MNP dimer. The reaction of intact DL-alanine molecules with deamination radicals to produce H-abstraction radicals which are not spin-trapped does not significantly lower the spintrapping efficiency. The results obtained with compounds such as glycine, glycyglycine, L-valine and L-proline suggest that the low spin-trapping efficiency found for DL-alanine may be representative of polycrystalline amino acids.

IV. ESR of Spin Trapped Radicals in γ -Irradiated Aqueous Uracil Solutions. Chromatographic Separation of Radicals. (with K. Makino and M.M. Mossoba)

Air-saturated aqueous uracil solutions were γ -irradiated at room temperature in the presence of 2-methyl-2-nitrosopropane (the spin trap). The resulting radicals were separated subsequently by high-performance liquid chromatography and detected by ESR. With a phosphate buffer (pH 7.0), the spin adduct, $\text{NH-C(=O)-NH-C(=O)-CH(OH)-CH-N(O}\cdot\text{)-}^t\text{Bu}$ (A) was obtained while with a $\text{Na}_2\text{HPO}_4\text{-NaOH}$ buffer (pH 11.0), the spin adduct (B) which has the same chemical structure as (A) but shows a different ESR spectrum from that of (A) was separated. The spin adduct, (A) and (B), were found to be cis-trans isomers

of each other. The assignments for these spin adducts were confirmed by studying the ESR spectra of the spin adducts in H₂O and D₂O at various pH and pD values and also by investigating the ESR spectra obtained from γ -irradiated aqueous solutions containing uracil-5-d₁. In addition to these spin adducts, NH-C(=O)-NH-C(=O)-CH-(N(O \cdot)-^tBu) -CH-OH (C) was obtained. The ESR spectra of (A), (B), and (C) were compared with those obtained from UV-irradiated aqueous dihydrouracil solutions containing MNP and H₂O₂ at various pH values and were found not to be due to H-addition products at the C5 or C6 of uracil. The spin adducts, (A), (B), and (C), are formed from the radicals generated by addition of hydroxyl radicals to the 5,6 double bond of uracil.

V. Pulse Radiolysis Studies of some Aza Analogues of Nucleic Acid Components (with I. Rosenthal and M. Farragi, Dept. of Chemistry, Nuclear Research Center-Negev, Beer-Sheva, Israel)

The technique of pulse radiolysis has been utilized to study the reactions of some aza analogues of nucleic acids components with hydrated electrons and OH radicals. The absorption spectra of the transient free radical adducts which result from these reactions and their decay kinetics were determined. The 5-aza analogues gave similar results to those of pyrimidine bases. The 6-aza analogues also showed similar kinetics, however, transient spectra were different. The presence of the sugar moiety in these aza analogues changed the rate law of the OH adduct transient decay from a second order to first order kinetics.

VI. Long-Range Proton Hyperfine Coupling in Alicyclic Nitroxide Radicals by Resolution Enhanced Electron Paramagnetic Resonance, RE-EPR (with M.M. Mossoba, K. Makino and in collaboration with Ray C. Perkins, Jr. of Varian Associates, Palo Alto, CA)

The free radicals 4-R-2,2,6,6-tetramethyl piperidine-1-oxyl, R = OH (TEMPOL), NH₂ (TEMPAMINE), H₂ (TEMPO) and O (TEMPONE), and 2-R-5,5-dimethyl pyrrolidine-1-oxyl, R = COOH, CH₃, CD₃, OH and OD have been investigated in aqueous solutions at room temperature by RE-EPR. In contrast to in-phase EPR, the present method provides a means for further identification of radicals which is particularly useful for species that are otherwise indistinguishable. The superhyperfine coupling constants of γ and δ nuclei in piperidine derivatives, which were obtained by computer simulation, are in agreement with those calculated from literature ¹H-NMR data observed for the same compounds and indicate that TEMPOL and TEMPAMINE are stable in chair conformations, while TEMPO and TEMPONE are rapidly interconverting between two identical chair and twist conformations, respectively. The data obtained for the pyrrolidine-1-oxyl derivatives containing a carboxy or methyl group at C2 were consistent with slightly puckered rings while the derivative with a hydroxy substituent was found to favor a deformed ring with a pucker at C2.

VII. Chemical Effects of Ultrasound on Aqueous Solutions. Evidence for the Formation of Hydroxyl Radicals and Hydrogen Atoms by Spin Trapping and Electron (with K. Makino, M.M. Mossoba)

The study of the chemical effects of ultrasound on aqueous solutions is of

therapy in medicine. (Br. J. Cancer 45 Suppl. V: Ultrasound, Microwave and Radiofrequency Radiations: The Basis for Their Potential in Cancer Therapy. 1982). The collapsing cavities which may occur during sonolysis lead to chemical damage and sonoluminescence due to the production of high local instantaneous temperatures and pressures. Recently, the formation of free gas bubbles in living mammalian tissue by 0.75 MHz ultrasound irradiation at 680 mWcm⁻² has been demonstrated by G. ter Haar, S. Daniels, K.C. Eastaugh and C.R. Hill (Br. J. Cancer 45 Suppl. V: 151, 1982).

In the present work, conclusive evidence for the formation of $\cdot\text{OH}$ and $\cdot\text{H}$ in the cavitation bubbles produced by ultrasound in argon-saturated aqueous solution is presented. A Bransonic 12 ultrasonic bath with a frequency of 50 kHz was employed. The methods of spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), (4-N-methyl-pyridinium) tert-butyl nitron (PYBN) and 4-pyridyl-1-oxide N-tert-butyl nitron (POBN) combined with ESR were used for the detection of $\cdot\text{OH}$ and $\cdot\text{H}$. With either DMPO or PYBN, the OH- and H-spin adducts were obtained, and with POBN, the H-adduct was observed. These results were confirmed by sonolysis of D₂O solutions containing the same spin traps.

By studying the competition reactions for $\cdot\text{OH}$ and $\cdot\text{H}$ between the spin traps, DMPO and POBN, and $\cdot\text{OH}$ and $\cdot\text{H}$ scavengers [O₂, formate, thiocyanate, benzoate, methanol, ethanol, n-propanol, t-butanol, acetone, 2-methyl-2-nitrosopropane (MNP)], further verification for the formation of $\cdot\text{OH}$ and $\cdot\text{H}$ was obtained. Sonolysis of aqueous solutions containing DMPO and POBN in the presence of air, suppressed formation of H-adducts but not that of OH-DMPO. The unusually large effects of acetone and MNP on the spin adduct yields could be explained either by the scavenging of radicals in the gas phase of the cavitation bubbles or by preventing the collapse of the gas bubbles during cavitation and thus reducing radical formation. From the results of the present work, it was inferred that $\cdot\text{OH}$ and $\cdot\text{H}$ are formed in the cavitation bubbles.

Hydroxyl radicals and hydrogen atoms are also produced in argon-saturated aqueous solutions exposed to ultrasound using a typical clinical dental instrument (frequency 25 kHz).

Significance to Cancer Research and the Program of the Institute:

Studies of the effects of ionizing radiation are of importance in relation to (1) radiation therapy, (2) carcinogenesis, (3) stability of the genetic pool, (4) the suppression of the immune mechanism, and (5) aging. The effects of ionizing radiation on nucleic acids are being studied in order to understand the nature of radiobiological death in normal and tumor cells. The addition of radioprotective and radiosensitizing agents is being investigated so that a therapeutic advantage may be gained. Objective 6, Approach 1.

Proposed Course of Research:

To continue studies on the effects of ionizing radiation on macromolecules of biological importance. The mechanism of radioprotective and radiosensitizing agents and the interaction of radiation and cancer chemotherapy agents will be

investigated. New areas of interest include photosensitized cell killing by porphyrins in relation to photoradiation therapy.

Publications:

Makino, K. and Riesz, P.: E.s.r of spin-trapped radicals in gamma-irradiated polycrystalline amino acids. Chromatographic separation of radicals. Int. J. Radiat. Biol. 41: 615-624, 1982.

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Lion, Y., Denis, G., Mossoba, M.M. and Riesz, P.: E.s.r. of spin-trapped radicals in γ -irradiated polycrystalline DL-alanine. A quantitative determination of radical yield. Int. J. Rad. Biol. 43: 71-83, 1983.

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Makino, K., Mossoba, M.M. and Riesz, P.: ESR and spin trapping study of γ -irradiated aqueous hydantoin solutions. Keto-enol equilibrium and post-radiolysis growth. Rad. Res. (In press).

Makino, K., Mossoba, M.M. and Riesz, P.: Formation of $\cdot\text{OH}$ and $\cdot\text{H}$ in aqueous solutions by ultrasound using clinical equipment. Rad. Res. (In press).

Rosenthal, I., Riesz, and Faraggi, M.: Pulse radiolysis studies of some aza analogues of nucleic acid components. Int. J. Rad. Biol. (In press).

Annual Report of the Laboratory of Cell Biology
National Cancer Institute
October 1, 1982 - September 30, 1983

The following are selected highlights of the research efforts of staff and collaborators of the Laboratory of Cell Biology.

Tumor Specific Antigens (TATA)

TATA, tumor specific rejection antigens have now been isolated from the cytosol of several methylcholanthrene-induced neoplasms. Both Meth A and CI-4 antigens have now been purified to homogeneity using immunoaffinity chromatography following hexylamine agarose, gel filtration and hydroxylapatite chromatography; both have MWs of 75,000. Their individual specificities are retained as determined by tumor rejection assays in syngeneic mice. These two antigens are closely related but are not identical; they differ in their chromatographic behaviour, isoelectric points and amino acid compositions. Both present as single bands on SDS-PAGE.

Recently, two additional 75 Kd proteins from CII-7 and mKSA have been isolated and purified using immunoaffinity chromatography. CII-7 is an MC-induced neoplasm. Both purified 75 Kd materials retain their specificities in tumor rejection assays and, like MethA and CI-4, 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. Apparently 75 Kd, a normal cell constituent, has been activated in mKSA by conversion to malignancy by SV40.

A question raised by our results is whether expression of 75 Kd was widespread or limited to chemically induced neoplasms. Through the use of an anti-75 Kd antiserum, we have identified, in binding and precipitation assays, 75 Kd in all neoplasms no matter what the species or the oncogen. Thus 75 Kds appear to represent a family of closely related proteins. Analyses are in progress of the primary structure of the different 75 Kds.

Assignment of the Gene Coding for Meth A TATA to Chromosome 12

Through analysis of somatic cell hybrids of Meth A microcells and Chinese hamster E36 cells, we have been able to assign the gene for Meth A TATA to the distal region of Chromosome 12, the region coding for immunoglobulin (Ig) heavy chains. Only those microcell hybrids containing X¹² (Chromosome 12 translocated to X) were capable of specifically immunizing against Meth A cell challenge. These same hybrids had been shown previously to completely absorb anti-meth A cytotoxicity from the syngeneic antiserum of DeLeo, thus recognizing Meth A TSSA.

No evidence for linkage of the Meth A coding gene and the H-2 complex was observed. None of the hybrids expressing Meth A antigen had a recognizable Chromosome 17 (that carries H-2). This further supports evidence obtained in this laboratory and by others that Meth A TATA is distinct from H-2 products.

Tumor Antigens of Leukemia Cells

Work continues concerning the biochemical and immunogenic characterization

of leukemia TATAs, specifically of the strong tumor rejection antigen of the T cell leukemia RBL-5. Unlike the unique TATAs of chemically induced and spontaneous neoplasms, the TATA of RBL-5 is group specific -- crossreacting with all FMR leukemias, but not with other leukemias. This TATA has now been purified to apparent homogeneity. It has a MW similar to Meth A, 75 Kd, but is a glycoprotein. As little as 50 ng of this protein provides complete protection against TLD₁₀₀ challenges of RBL-5 cells.

A rabbit antiserum prepared against 75 Kd precipitated a single protein of 175 Kd MW from metabolically labelled RBL-5 cells. Through the use of this antiserum as an immunoabsorbant, it was possible to purify a mixture of 175 Kd and 75 Kd proteins. 175 Kd was immunogenic against RBL-5 and it was found that 75 Kd was a stable breakdown product. The 175 Kd protein was found to be expressed on all neoplasms and diverse normal cells.

As with Meth A, CI-4 and CII-7 proteins, this glycoprotein is being sequenced with the hope of preparing synthetic probes that will allow isolation of the determinant encoding this protein.

Regulation of T Cell-mediated Immunity

Using in vitro assays, principally the ¹²⁵IuDR release assay and FBL-3 leukemic cells, mechanisms for tumor cell-induced suppressions, the development of tumor immunity in tumor bearing hosts and prostaglandin regulation of immune responses have been studied. In syngeneic mixed lymphocyte-tumor cell cultures, it was found that tumor cells triggered a suppressor mechanism mediated through macrophages. Prostaglandins were also found to induce the generation of suppressor T cells. When in vivo assays were used, it was found that adherent macrophages isolated from progressively growing RBL-5 leukemias also suppressed the protective effect of immune T cells; this activity was on the effector phase of the immune response.

When T cells from the site of a progressively growing tumor (leukemia ascites) are assayed in vivo, they fail to provide long lasting protection against tumor challenge due to the presence of suppressor macrophages. However, these selectively grown T cells in medium supplemented IL-2 were able to provide long lasting specific protection. NK cell lines are also now being maintained in medium supplemented with partially purified IL-2.

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). That this latter site was the antigen-Ia interaction site was confirmed by testing immune responses to four different antigen specificities. In each case the same interaction was found; these results suggested that the presenting cell and T cell independently recognize their respective sites. Attempts at restimulation and blocking support the data, indicating specific interactions between antigen and Ia control responses of T cell clones.

Studies of Rat-tropic RNA Tumor Viruses

Attention has been focused upon a rat hepatoma leukemia-helper virus (RHHV)

and a wild rat leukemia-helper virus (WR-RaLV). Both have been isolated in this laboratory. Analyses of the genomic complexity of these endogenous viruses have continued in order to gain insight into the "helper" sequences and their functions especially when recombined with C-src sequences in the genome of a transforming rat virus. DNA sequences have been identified specific for both of the above viruses as 8.0 - 8.8 K bases in length. These viral DNAs were successfully cloned via the pBR322 vector and cultured in E. coli. Genomic maps have been constructed from the various restriction enzyme cleavages of both viruses. Studies continue using heteroduplex analysis of the convergent and divergent DNA sequences of these rat viruses.

The biologic activity of cloned RHHV DNA in restricted subgenomic fragments was assessed by intranuclear microinjection into NRK cells. The 5.8 - 6.2 kb DNA fragment proximal to the 5' terminus of cloned RHHV 8.8 kb DNA was able to rescue a transforming replication-competent pseudotype virus. Other DNA fragments were ineffective.

Effect of Interferon on Murine Retroviruses

The effects of interferon, using mixtures of α and β interferons, occur after the synthesis of viral RNA and structural proteins -- that is in the late stages of virus assembly and maturation. This effect, however, varies among the different classes of retroviruses and it has been observed recently that cloned γ interferon has most of the same properties as the $\alpha\beta$ mixture.

The effects of interferon on a newer class of murine retrovirus, MCF, appear to reveal changes analogous to those seen for Gross and Rauscher viruses. Treatment for 24-48 hours reduced the titer of infectious virus 100-fold but reduced particle production by only 10-fold. Kinetic studies indicated no changes in intracellular processing of viral proteins to mature structural proteins but that there was an accumulation of viral p30 and p12. Viral particles produced from interferon treated cells were nearly devoid of gp70 and had lower amounts of p30. The paucity of gp70 is consistent with the lack of infectivity in these virions.

Trophoblast Cells and Viral Growth

Mouse trophoblast cell lines were established in this laboratory from cultured midterm placenta. These cells had characteristics of trophoblast; including a lack of SSEA-1, presence of cytoskeletal proteins EndoA and EndoB and absence of, or low expression of H-2 and β -2 M. These trophoblast cells were found to be resistant to the growth of wild type polyoma virus; more differentiated cells, however, are permissive for wild polyoma replication. However several mutant viruses (Py Tr) were shown to overcome this restriction, were isolated and found to have genetic changes within a regulatory region of the polyoma virus genome. Mutants growing in embryonal sarcoma cells (EC) differ from these mutants growing in trophoblast cells. Thus viral mutants differ in both phenotype and genome depending upon degree of differentiation of recipient tissues. Nucleotide sequences are being performed upon a series of virus mutants. In preliminary data, a series of deletions have been found and the position of these deletions may have some bearing in determining the phenotype of these mutants.

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Histocompatibility Antigens

Recently, several laboratories have isolated both cDNA and genomic clones encoding the heavy chain of the H-2 antigens. We have previously reported the expression of H-2D^d and H-2L^d genomic clones in L cells after DNA-mediated gene transfer. The H-2 antigens expressed on the mouse L cells are indistinguishable from their normal counterparts. They evoke a strong specific serological response and are able to serve as targets for alloreactive T cells. Since these results, we have started a study of the influence of amino acid sequence on the three dimensional structure and function of the H-2 antigens. The method is oligonucleotide-directed mutagenesis, which involves priming *in vitro* DNA synthesis with a chemically synthesized oligonucleotide that carries at least a single base mismatch with the complementary strain of the "wild type" DNA. Eight different oligonucleotides have been synthesized, corresponding to different parts of the heavy chain of H-2 and all carry at least one base change. After molecular cloning in the M13 vector and expression in the L cells, we hope to increase our understanding of the relationship between primary structure and biological function of H-2 through these mutant proteins.

We have reported previously that the F9 teratocarcinoma cells do not express H-2 antigens. To analyze further this phenomenon, individual clones (designated clone 1 and clone 9) derived from retinoic acid treated F9 stem cells were analyzed for their stage of differentiation, expression of H-2K genes and the degree of methylation of these genes. Clone 9 cells, isolated after four days of treatment with retinoic acid, have characteristics of endoderm cells; whereas, clone 1 cells, isolated after prolonged exposure to the same agent appear similar to fibroblast cells. By several biological criteria, the stages of differentiation of these cells were determined to be in the order F9→clone 9→clone 1. In contrast to F9 cells which do not express the H-2 antigens, clone 9 cells express low levels, and clone 1 cells express high levels of these antigens. In order to determine if methylation plays a role in the regulation of expression of the H-2 genes, we compared the methylation pattern of the H-2K gene(s) in the various cell lines by the use of the methylation-sensitive restriction enzymes, Msp II and Hpa II. Unlike most genes which are highly methylated, it was found that the H-2K genes are poorly methylated in the differentiated F9 stem cells. And whereas differentiation is usually associated with a decrease in the methylation state of most genes, the H-2K genes of clone 9 and clone 1 cells become more highly methylated. Specifically, when compared to F9 cells, clone 9 cells which express low levels of the H-2 antigens have the H-2K genes from one chromosome hypermethylated, whereas clone 1 cells which express high levels of the H-2 product have the

H-2K genes from both chromosomes hypermethylated. 5-Azacytidine inhibition studies have been carried out and the results clearly show that the increased level of methylation is directly responsible for the increase in expression of the H-2K genes.

T Cell Immune Response

The T cell immune response to the carboxyl terminal cyanogen bromide peptide fragment of pigeon cytochrome C has been the subject of numerous studies. Comparison of the sequence and antigenic strength of the naturally occurring peptides did not allow identification of any single amino acid residue which accounted for the T cell immune response. We have studied the immune response to a series of 15 and 16 residue cytochrome C synthetic peptides. In this series, T cell memory was controlled by residue 11(Lys) and 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). In order to confirm that this latter site was the antigen-Ia interaction site, we tested the immune responses to several different antigen specificities (epitopes). A total of 4 epitopes has been tested and in each case the same antigen-Ia interaction was found. These results suggested that the presenting cell and the T cell might independently recognize their respective sites. We therefore attempted to restimulate a primed T cell population by use of a peptide whose T cell and antigen-Ia site had been separated by a 4 residue spacer. This experiment resulted in no significant restimulation. Secondly, we attempted to block the antigen-Ia interaction with a peptide which contained the antigen-Ia site but not the T cell site. No inhibition was found. Thus, our data support the concepts of a specific interaction between the antigen and Ia which controls the responses of several T cell clones.

Tumor Antigens

It has been recognized that neoplastic transformation by chemical carcinogens is accompanied by the expression of tumor specific transplantation antigens(TSTAS). These antigens impart transplantation immunity to the tumor of origin by prior immunization with that tumor or a soluble form of the antigen derived from it. Independently transformed cell lines express different transplantation antigens, even when they are the progeny of a fibroblast clone. Recent work carried out in our laboratory indicates that for methylcholanthrene-induced fibrosarcomas, the TSTAs are derived from a single protein or a family of closely related proteins. These proteins have been called p75. An immediate question raised by our results was whether expression of the p75 family was widespread or limited to the chemically induced fibrosarcomas. Examination of a variety of tumors, both virally and chemically transformed, as well as short term cultures of Con A stimulated spleen cells, indicated that all these cell types contain a serologically cross-reactive protein of the same molecular weight. This protein in chemically transformed cells must acquire individually expressed antigenic determinants which are recognized by a tumor graft rejection assay. Attempts to carry out in vitro assays of T cell immunity have failed to detect a dominant individually expressed determinant. Preliminary results, on the contrary, indicate that p75 is either a mitogen or is able to induce a primary in vitro response. These data raise two basic questions which we are currently addressing. 1) What are the cell types and specificity of the

in vitro priming p75 dependent proliferation? 2) What is the relevance of this phenomenon to tumor specific graft rejection? Another study that we have undertaken is an analysis of the primary structure of the different p75s from the chemically induced fibrosarcomas. This will facilitate the isolation of the nucleic acid clones and give an insight into the molecular organization of the genes involved in the expression of these antigens.

p53 is a cellular-encoded transformation-related protein, synthesized in elevated amounts by many transformed cell lines and primary tumor cells of various species. It is a phospho-protein capable of complexing with several tumor antigens. The fact that it is overproduced in a wide range of tumor cells suggests that the elevated level of p53 is associated with neoplastic transformation. To elucidate the molecular mechanism controlling p53 expression, we have attempted to purify it in a native form using a mouse monoclonal antibody and elution under native conditions. Our results indicate that such a purification method will allow us to obtain purified p53 protein suitable for enzymatic and DNA binding assays.

Protein chemistry and Peptide synthesis.

Progress in recombinant DNA technology and nucleotide sequence analysis has made possible the isolation of genes and the definition of their structure. However, there still remains the problem of characterizing the gene products and their function in vivo. One solution is to synthesize by solid phase methods peptides derived from the predicted protein sequence, attach them to immunogenic protein carrier and inject them into rabbits. Using this approach, we have raised rabbit antibodies to the peptide Glu-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu, which represents a part of middle T antigen of polyoma virus that is considered important in inducing the phenotype of transformed cells. The antibodies react with native as well as denatured middle T antigen. In addition the antibodies precipitate a cellular protein with an apparent molecular weight of 130,000. The relationship of this protein to middle T antigen is being investigated. Another possibility is the use of this antibody to purify middle T in a kinase active form, since such purification cannot be carried out with standard antitumor sera.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05550-14 LCBGY
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.) Oncogenesis by Retroviruses and its Regulation by Cell Differentiation		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) K. S. S. Chang, Medical Officer, LCBGY, NCI		
COOPERATING UNITS (if any) LMM, NIALD Meharry Medical College, Nashville, TN		
LAB/BRANCH Laboratory of Cell Biology		
SECTION Viral Oncogenesis Section		
INSTITUTE AND LOCATION NIH, NCI, Bethesda, MD 20205		
TOTAL MANYEARS: 1.50	PROFESSIONAL: 1.50	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews B		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The long range purpose of this project is to investigate the role of type C retroviruses as an etiologic agent and a vector of genetic information for neoplasms and the use of viral mutants to analyze the mechanism of regulation of gene expression associated with cell differentiation and oncogenesis.</p> <p>The topics of current interest are: 1) molecular cloning and characterization of viral DNA derived from amphotropic virus strains isolated in this laboratory, 2) characterization and nuclotide sequencing of polyoma virus mutants that can replicate in embryonal carcinoma and trophoblast cells, 3) role of methylation affecting the expression of type C retroviruses and oncogenes in embryonal carcinoma and trophoblast cells, 4) <u>in vitro</u> transmission studies of the human T cell leukemia virus, and 5) studies on oncogenes of human choriocarcinoma, and other tumors.</p>		

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Project Descriptions:I. Molecular cloning and characterization of viral DNA derived from amphotropic virus strains isolated in this laboratory.

Two strains of amphotropic virus, YACNX and R5NX, which were isolated from inbred mouse tumors originally induced by Moloney-MuLV and Rauscher-MuLV, were successfully cloned in pBR322 and propagated in E.coli. A prototype strain of amphotropic virus, 4070A, isolated from California wild mouse was also cloned in the same way. Such cloning of viral DNAs greatly facilitated (and economized) the subsequent characterization of these strains in terms of restriction endonuclease map analysis.

Previous studies by competitive radioimmunoassays using specific antisera against p12 and gp70 of these viruses, and also tryptic digest maps of these antigens have indicated that the YACNX strain is related (but not identical) to the prototype virus, 4070A, while the R5NX strain is a recombinant of AKR-MuLV and amphotropic virus.

By elaborate molecular hybridization we established restriction endonuclease maps of these viruses. The results added another set of supporting evidence to the above assumptions. For example, Hinc II cut the DNA of 4070A and YACNX at 3.3, 4.1, and 7.6 kilobase (kb) sites, whereas the same enzyme cut the DNA of R5NX and AKR-MuLV at 2.0, 3.0, 3.3, 4.2, 5.9, and 6.3 kb sites. Pvu II cut 4070A DNA at 0.1, 0.2, 1.9, 4.2, 5.1, 8.2, and 8.4 kb sites, YACNX DNA at 0.1, 1.9, 4.2, 7.5, 8.6, and 8.7 kb sites, R5NX DNA at 4.2, 5.9, and 7.5 kb sites, and AKR-MuLV at 5.9 kb site only. Other enzymes such as Xba I, Sst I, Hpa I, Bcl I, etc. gave patterns of fragments in conformity with the above hypothesis.

The viral genome is considered to be divided as follows, going from left to right: 0 to 0.6 kb, long terminal repeat (LTR); 0.5 to 1.0 kb, leader; 1.0 to 2.8 kb, gag; 2.9 to 5.8 kb, pol; 6.0 to 8.2 kb, env; 8.2 to 8.8 kb, LTR. The data of restriction endonuclease map described above would suggest that the gag and pol genes of R5NX may have been derived from AKR-MuLV by recombination of amphotropic virus with AKR-MuLV. Further work on the nucleotide sequence may define the recombinant origins and cross-over sites more clearly.

II. Characterization and nucleotide sequencing of polyoma virus mutants that can replicate in embryonal carcinoma (EC) and trophoblast (TR) cells.

The mouse trophoblast (TR) cells used in the present studies had multiple characteristics of trophoblast, including a lack of SSEA-1, presence of cytoskeletal proteins Endo A and Endo B, and low expression of H-2 and β -microglobulin. The EC cell line exhibits properties similar to cells of the

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II. Characterization and nucleotide sequencing of polyoma virus mutants that can replicate in embryonal carcinoma (EC) and trophoblast (TR) cells.

The mouse trophoblast (TR) cells used in the present studies had multiple characteristics of trophectoderm, including a lack of SSEA-1, presence of cytoskeletal proteins Endo A and Endo B, and low expression of H-2 and β 2-microglobulin. The EC cell line exhibits properties similar to cells of the

inner cell mass. Thus, these cell lines provide a tool for studying the two types of cell present in blastocyst: trophoctoderm, which emerges from the first differentiation event in murine development, and multipotential inner cell mass. TR cells, like EC cells, were found to be resistant to wild-type polyoma virus growth. Mutant viruses (PyTr mutants) which could overcome this restriction were isolated and found to have genetic changes within a regulatory region of polyoma virus genome, similar to PyEC mutants previously described. The growth properties of six PyTr mutants and four newly isolated PyECF9 mutants were compared using two lines of EC cells, TR cells, and a representative of differentiated fibroblast. It appeared that the mutants from less differentiated (such as EC) cells could grow in more differentiated (such as TR and 3T3) cells but not vice versa.

The nucleotide sequence analysis of the PyTr mutant genomes revealed that both PyTr-91 and -92 mutants examined have an identical 26 base-pair (bp) long deletion from nucleotide (nt) 5131 to nt 5166. This deleted region of the two mutants is replaced by 4 or 54 bp long DNA segments consisting of neighboring viral sequences. This exact 26 bp long DNA segment from nt 5131 to nt 5156, however, is not necessarily deleted in all the PyTr mutants. None of the PyECF9 mutants reported so far contains this type of deletion and duplication at around nt 5131 to nt 5156. Katinka and others (1981) have reported that two PyECF9 mutants which they have examined have insertions of two base pairs in such a way to make the stem of a hypothetical hairpin structure at nt 5170 to nt 5187 bp longer than that proposed for the wild-type sequence. The two PyTr mutants tested here have sequences identical to our wild-type (A3 strain) within this proposed hairpin structure. Therefore, this proposed hairpin structure does not determine the phenotype of the mutants. Fujimura and others (1981) reported that the only mutation they found in three PyECF9 mutants that is common to all is an A to G change at nt 5230. This A to G change was observed in one of the two PyECF9 mutants examined by Katinka and others (1981) and both PyECF9 mutants examined by Sekikawa and Levine (1981). None of the two PyTr mutants we present have this A to G change.

It is not clear what nucleotide changes are characteristic to PyECF9 or PyTr mutations. The sequences of additional mutants will have to be examined to identify general sequence changes unique to each type of mutation.

Our results indicate that viral mutants derived from the passage of virus in EC and TR cells are different in both their phenotype and their genome from wild-type virus. Studies of the mechanism regulating differential growth of these mutants may help elucidate differences in genetic control mechanisms operating in EC and trophoctoderm cells and contribute to an understanding of the processes by which the first morphologically detectable differentiation occurs in early murine development.

III. Role of methylation affecting the expression of the type C retroviruses in embryonal carcinoma (EC) and trophoblast (TR) cells.

Accumulating evidence suggests an inverse correlation between DNA methylation and gene expression. However, the details of this transcriptional control and its physiologic role in differentiation and development are not clear.

We have investigated the basis of nonpermissiveness of these cells to type C retroviruses as a model for studying the regulation of gene expression in murine EC and TR cells. Established lines of the latter were derived from midterm placenta, and their properties have been described.

Infection of EC and TR cells with retroviruses resulted in integration of viral DNA in the cellular DNA as detected by hybridization with p³²-labeled probes. However, no infectious virus was produced from these cells or from permissive cells transfected with DNA from the infected EC and TR cells. The sites of methylation in the proviral DNA were determined by using isoschizomers Hpa II and Msp I. Unlike Hpa II, Msp I can cut 5'-CCGG-3' even if the internal cytosine residue is methylated. The methylation sites were also determined by analyzing the Ava I, Hha I and Hpa II cleavage patterns of proviral DNA before and after treatment of cells with 5'-azacytidine (AzaC), and using 5'- and 3'-viral probes for hybridization. It was found that demethylation occurred more readily in gag genes than in LTR regions, and that poly(A)⁺-containing virus-specific RNA of 4S size which reacted with viral probe was detected in the EC and TR cells treated with AzaC. Methylation of integrated viral DNA was observed earlier (3 days postinfection) in TR cells than in EC cells (9d). Since no infectious virus was produced by these cells before methylation of proviral DNA occurred, and since treatment with AzaC did not always result in infectious virus production, it is concluded that some unknown mechanisms in addition to de novo methylation of viral DNA in the cell are contributing to the block of viral replication in EC and TR cells.

Further work is in progress to isolate mutant type C retroviruses that can replicate in EC and/or TR cells. In addition, Harvey murine sarcoma and other sarcoma viruses are being used to analyze the regulation mechanism for viral oncogen expression after integration into EC and TR cells. Human trophoblast cells are also being examined for the type C virus gene expression and regulation.

IV. In Vitro transmission studies of the human T-cell leukemia virus (HTLV).

Studies have been initiated on the infectivity of HTLV on various human and animal cell lines. The reverse transcriptase activity and viral antigen expression of the infected cells are being used as markers for viral replication. Furthermore, the ability of the HTLV to act as a helper virus for MSV is being investigated. Furthermore, we have cloned HTLV cDNA in pBR322 plasmid, and work is in progress to characterize this clone for its future use as a probe to detect this DNA in various patient tissues.

V. Studies on Oncogenes of Human Choriocarcinoma, and other tumors.

Studies have been initiated to extract DNA from various human and murine tumor, including human choriocarcinoma, hepatoma, and pancreatic tumor, and murine reticulum cell neoplasms from SJL/J mice for transfection experiments in order to isolate and characterize the oncogenes derived from these tumors. The choriocarcinoma and reticulum cell neoplasms are of particular interest because these have relationship with our past studies on trophoblast gene expressions and SJL/J type C retroviruses. These studies will yield some illuminating facts on the oncogene status of these tumors.

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I. Tumor Antigens

1. Studies of Purification and Biological Effects of TSTA.

A. Attempts to purify to homogeneity the tumor rejection antigen(s) of sarcomas, Meth A, CI-4, CII-7 and a control SV-40 induced sarcomas. These are collaborative studies (DuBois, Appella and Law, LCB) and DeLeo (Sloan-Kettering). These studies are now made somewhat easier by our recent finding that the majority of TSTA is found in the cytosol fraction of tumor cells and one need not resort to solubilization of the cell membranes with detergents. The results of our recent studies with the cytosol-derived antigens are as follows: 1) the majority of the TSTA is in the cytosol (but is found also on the membrane); 2) the biochemical properties, that is, behavior in gel chromatography, lectin affinity chromatography responses to proteases, etc. and the immunologic properties such as dose-response to antigen, specificity, lack of any evidence of immune deviations in the host are similar in both the membrane derived and the cytosol-derived TSTA in those studied, Meth A, CI-4 and CII-7; 3) the active fraction following S-200, lectin and Aca54 chromatography is in the 75Kd range; 4) this most enriched cytosol fraction is found also to absorb inhibition of the Meth A specific antiserum that detects TSSA on Meth A cells. Thus TSTA and TSSA appear to be related antigens and these both appear not to be integral membrane proteins but to be peripherally bound to the membrane, and 5) two-dimensional gel electrophoresis of the most active fraction from the final step of chromatography shows that a major component of 75,000 M_r may represent the TA (TSTA and TSSA).

This component has been isolated from the cytosol. The antigen was purified either by preparative electrophoresis in the presence of sodium dodecyl sulfate or immunoaffinity chromatography following hexylamine agarose, gel filtration, and hydroxylapatite chromatography. The 75Kd protein, prepared by either of these methods and presenting as a single band on SDS-PAGE, effectively primed BALB/c mice to reject the Meth A tumor; such priming provided no protection against challenge by other independently derived sarcomas of BALB/c origin. A second protein of molecular weight 75Kd was isolated from the cytosol of the recently derived methylcolanthrene-induced sarcoma, CI-4, by essentially the same chromatographic scheme. This protein was similarly shown to be immunogenic in the tumor rejection assay and, furthermore, provided protection only against CI-4 challenge. The antigens purified from the Meth A and CI-4 sarcomas appear to be closely related proteins. Both of them can be purified from the cytosol fraction and can be recognized by a rabbit antiserum prepared against the Meth A 75Kd protein. The two proteins have approximately the same molecular weight but differ in their chromatographic behavior on hexylamine agarose and hydroxylapatite. These results indicate that the individually specific transplantation antigens found in chemically induced sarcomas may be the products of a single multigene family or somatic derivatives of a single gene.

B. Most recently, two additional 75Kd proteins have been isolated and purified using immunoaffinity chromatography following the procedure used for Meth A and CI-4. These 2 neoplasms include CII-7, having its own unique TSTA, and an unrelated SV-40-induced sarcoma, mKSA. Preliminary assays show the CII-7, 75Kd antigen to be highly immunogenic and still maintain its unique specificity. mKSA assays are now in progress.

C. The anti-75Kd rabbit antiserum prepared from the purified Meth A 75Kd protein has been used in precipitation and binding assays. Extensive binding assays have identified 75Kd in all neoplasms assayed, including mouse, hamster, rat and man but not in normal fibroblasts (man and mouse), nor in non-stimulated mouse thymocytes and spleen cells.

2. Phenotypic Studies of BALB/3T3 Cells Transfected with DNA from MC-induced Neoplasms.

The high frequency of co-transfer of the 2 phenotypes TSTA and transformation (malignancy) reported previously using high MW DNA from Meth A cells was not observed when using DNA cleaved by the Bgl II restriction enzyme. Although transformation was achieved with the 15 and 20 kb fragments, the specific Meth A TSTA activity was observed only in two (2) of the 25 transformants in contrast to the 50-60% frequency of co-transfer observed in assaying the primary and secondary transfectants of high MW DNA.

Hopkins (MIT) has subsequently cloned a partial polyoma virus determinant from one of the cleaved-DNA transfectants. It was then found that this polyoma sequence was incorporated into the genome of the Meth A variant used for preparation of the DNA used in transfection. Interestingly, this polyoma virus sequence was not found in the original Meth A sarcoma, thus the transfecting determinant had no relationship to the induction of the original Meth A neoplasm.

It has been determined that the expression of TSTA of the 4 transfectants studied was Meth A specific and not related to the transferred polyoma virus determinant:

- a) Meth A original (not bearing polyoma) immunized against challenges of all the transfectants,
- b) each transfectant immunized against Meth A (original) challenge, and,
- c) polyoma virus-induced neoplasms containing polyoma specific TSTA did not immunize against any of the transfectants.

We are now in the process of assaying a group of transfectants obtained in BALB/3T3 recipient cells using high MW DNA from the Meth A (original) sarcoma (collaborative work with A. B. DeLeo, Sloan-Kettering).

3. Assignment of the Gene Coding for Meth A TSTA to Chromosome 12.

Through analysis of somatic cell hybrids of Meth A micro cells and the Chinese hamster cell line E-36, we have been able to assign the gene for Meth A TSTA to the distal region of chromosome 12. This is the region of chromosome 12 coding for immunoglobulin (Ig) heavy chains.

Only those microcell hybrids containing X¹² (chromosome 12 translocated to X) were able to specifically immunize against Meth A cell challenge; 4 other hybrid lines were not immunogenic. These specific somatic cell hybrids had previously been shown to completely absorb anti-Meth A cytotoxic activity from the syngeneic serum of DeLeo.

Interestingly, no evidence for linkage of the Meth A coding gene and the H-2 complex was found. None of the hybrids that expressed Meth A antigen had a recognizable Chromosome 17 (which carries H-2) nor did they express glyoxalase 1, assigned to Chromosome 17. This result further strengthens previous work from this laboratory showing that Meth A TSTA is distinct from H-2 products (collaborative investigations with Drs. DeLeo [Sloan-Kettering] and Pravtcheva [Yale University]).

4. Tumor Antigens of the RBL-5 Leukemia.

Collaborative work continues with Rogers (LG) concerning the biochemical and immunologic characterization of leukemia TSTAs, specifically of the strong tumor rejection antigen of the T cell leukemia RBL-5. Unlike the unique TSTA of chemically-induced neoplasms, the RBL-5 TSTA is group specific -- cross reacting with all FMR-induced lymphomas -- but not with other leukemias. The TSTA of RBL-5 has been purified to apparent homogeneity. It has the same MW as Meth A, CI-4, CII-7 sarcoma TSTA, 75Kd, but is a glycoprotein. As little as 50 ng of this protein provides complete protection against TLD₁₀₀ challenges of RBL-5 leukemia cells.

A rabbit antiserum was prepared using the 75Kd protein; this precipitated a single protein of 175Kd MW from metabolically-labeled RBL-5 cells. Through the use of this antiserum as an immunoabsorbent, it was possible to purify a mixture of 175Kd and 75Kd proteins. Upon separation, the 175Kd was found to express RBL-5 TSTA. It was shown that 75Kd was a breakdown product of 175Kd and was indeed a stable breakdown product. Similar to findings with the Meth A 75Kd antiserum, the 175Kd protein was found to be expressed on all neoplasms and on diverse normal cells. As with Meth A, CI-4 and CII-7, this protein of RBL-5 is being sequenced with the hope of preparing synthetic DNA probes that will allow isolation of the determinant encoding this protein.

Significance for Cancer Research

Characterization of tumor antigens of the rejection type is a necessary prerequisite for understanding the mechanisms of immune surveillance, tumor inhibition and facilitation. In addition, the role of these cytosol and membrane components in the mechanisms of initiation and maintenance of malignancy will be studied.

As a basis for any study of membrane bound antigens, it is necessary to study the nature of histocompatibility antigens and their relationship to tumor antigens. Thus our emphasis is on parallel studies of H-2 antigens in order to provide a basis for understanding tumor antigens.

Objective 3; Approaches 4, 5.

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Project Description:

Objectives: 1) Mechanisms for tumor cells induced suppression; 2) Development of specific tumor immunity in tumor bearing hosts; 3) Prostaglandin regulation of immune response; 4) Immunoregulation of T cell-mediated immunity.

Methods Employed: Tumor cell transformed by oncogenic viruses, chemicals or unknown agents are kept in tissue cultures as transplanted tumors in mice. The ^{125}I UdR release assay was used to measure the cell-mediated cytotoxic response in vitro, and the adoptive transfer experiment was used to measure the cell-mediated immunity in vivo. The lg sedimentation technique was used to separate various populations of lymphocytes and tumor cells. The syngeneic mixed lymphocyte tumor cell cultures were used to generate cytotoxic T cells against tumor associated antigens. The mixed lymphocyte cultures were used to generate alloreactive cytotoxic T cells. Interleukin 2 (IL2) was prepared from rat spleen cells and was partially purified by ammonium sulfate precipitation and Sephadex GT 150 column.

Major Findings:

1) Mechanism for tumor cell-induced suppression: We have demonstrated, in the in vitro system with syngeneic mixed lymphocyte tumor cell cultures, that tumor cells triggered a suppressor mechanism through macrophages. This was further mediated by prostaglandins to induce the generation of suppressor T cells. When these experiments were performed in the in vivo tumor neutralization test, it was found that adherent macrophages isolated from a progressively growing tumor (FBL-3 ascites) suppressed the in vivo protective effect of immune T cells. The suppression was acted at the effector phase of the immune response. These findings confirmed our in vitro observation and indicate that tumor cells may also trigger an in vivo suppressor mechanism which mediated through macrophages.

2) Development of specific tumor immunity in tumor bearing hosts: Specific tumor immunity was found to be generally suppressed in tumor bearing hosts. However we have found that highly reactive specific antitumor cytotoxic T cells could be demonstrated at tumor site (FBL-3 ascites tumor). When these T cells were tested in vivo, they failed to give long lasting protection against tumor challenge. This was later found to be caused by the presence of suppressor macrophages as discussed above. In order to solve this problem, we have cultured these lymphocytes in medium supplemented with IL2. It has been shown that only T cells were selectively growing in IL2. After culturing for 2 to 3 weeks, the relatively purified T cell preparation isolated from tumor sites were found to give long lasting protection in the in vivo tumor transplantation experiment. These findings indicate that the in vivo activity of antitumor T cells could be augmented by in vitro culturing in IL2. This method may offer a new approach to the immunotherapy of cancer in autochthonous host.

3) Long term culturing of NK (natural killer) cells in culture: After multiple attempts, we have succeeded in establishing a long term NK cell line which could be maintained in cultured medium supplemented with IL2. Single cell clones were obtained from this cell line by limiting dilution technique, and they were found to be derived from heterogenous populations of NK cells. To maintain NK

cells in long term culture should help the detailed study of the nature and biological activities of NK cells. In the past, these studies were restricted by the limited amounts of NK cells available.

4) Prostaglandin regulation of immune response: In the study of tumor cell triggered macrophage-mediated immunosuppression, we have found that the suppression was further mediated through the production of prostaglandins. Studies were performed to examine the regulation by prostaglandins of the immune response in the absence of tumor cells. We have found that polyclonal activation of cytotoxic lymphocytes was induced by culturing normal spleen cells with exogenous, syngeneic peritoneal macrophages and indomethacin. There was no requirement of antigenic or mitogenic stimulation. The effectors were characterized to be cytotoxic T cells (CTL) whereas the responders were found to be derived from NK cells. It was later found that the polyclonal activation of CTL was mediated through the production of a lymphokine which was distinct from IL1 or IL2. This lymphokine was essential for providing a differentiation signal to induce NK-derived precursors to be differentiated into CTL. These experiments will provide a new approach to examine the "resting" CTL precursors and should help us to understand the mechanisms for the activation and differentiation of CTL.

5) Immunoregulation of T cell-mediated immunity: T cells and NK cells were presumed to play the central role in the immunosurveillance of tumor growth. The mechanisms for the regulation of T cell-mediated immunity is composed of complicated immunoregulatory networks and circuits. They involve the participation of various populations of lymphocytes, macrophages and lymphokines. To examine the origin of the CTL precursors and the mechanisms for their activation is the most fundamental approach to these questions. Efforts are made to isolate and purify the putative lymphokine to regulate the activation and differentiation of CTL-p into CTL.

Significance of biomedical research and the program of the National Cancer Institute.

We have successfully modified and augmented the in vitro and in vivo anti-tumor activity of immune T cells obtained from tumor bearing hosts. This is achieved by culturing the cells in median supplemented with IL2. This method offers a new approach to perform immunotherapy in autochthonous hosts. We have also examined the mechanisms for the activation and differentiation of cytotoxic T lymphocytes. Macrophages and prostaglandins were found to play the central role in the regulation of CTL precursor activation. This was further mediated through the production of a lymphokine which was distinct from IL1 or IL2. These studies should help to understand the fundamental mechanisms of the immune surveillance networks and circuits which regulate the development of specific anti-tumor immunity.

Proposed Course:

- 1) Further studies on the mechanisms for tumor cell-induced immunosuppression.
- 2) Macrophage and prostaglandin regulation of T cell-mediated immunity.
- 3) Lymphokine regulation of CTL activation.

Publications

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OTHER INVESTIGATORS

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1. Effects of interferon on methylcholanthrene-induced sarcomas of BALB/c mice. The rationale for pursuing these studies was predicted on a number of distinct but related factors. First, the clinical literature regarding the interferon treatment of various human sarcomas is less than definitive. Thus one could expect to derive a more interpretable set of data regarding properties of interferon in a controlled animal model system maintained in syngeneic mice. The methylcholanthrene-induced sarcoma, Meth A, meets aforementioned requirements; additionally it can be passed both in vivo and in vitro. There are no known murine retroviruses or other viral agents associated with this tumor and thus one could easily dissect the anticellular effect of interferon from the antiviral ones. Further, by comparing the response in cell culture with that in the intact mouse it is possible to analyze the direct action of interferon on Meth A cells separately from the host mediated effects. It is of importance that there are stable membrane markers such as the tumor specific transplantation antigen (TSTA) and the H-2 antigens which are potentially susceptible to the action of interferon.

Our first series of experiments established that in vitro treatment with a mixture of α and β interferon was effective in preventing the development of tumors in the intact mouse as long as the challenge dose was 10^5 cells or less. This effect could be abrogated by using a challenge dose of 10^6 cells. Cell counts, trypan blue dye exclusion, and ^3H -thymidine uptake were done on cells in culture and indicated that interferon had a cytostatic effect, but not a cytotoxic one. Appropriate studies confirmed that the continuous presence of interferon is necessary for maintaining any cell inhibitory effects. Since these data suggest that the major effects of interferon on Meth A cells do not appear to be mediated through anticellular activity, a number of experiments have been done to define the role of the immune response in this system.

Challenge of nude mice with virus-infected tumor cells serves as a stimulus for natural killer (NK) cell activity and leads to tumor rejection. We elected to compare the tumorigenicity of interferon treated and untreated Meth A cells in nude mice with that seen in syngeneic BALB/c mice to determine whether interferon treatment of Meth A cells, in absence of virus, could serve as a stimulus for NK cell activity. After an inoculating dose of 10^5 cells, both control and experimental groups had a tumor incidence of 100%. With an inoculating dose of 10^4 cells, 100% of the controls and 85% of the mice receiving interferon-treated cells developed tumors. As opposed to BALB/c mice, interferon offers no protective effect in nude mice, indicating that the rejection of interferon treated Meth A cells is thymus dependent, and not mediated by the NK system.

Meth A cells showed an increased expression of H-2K and H-2D antigens following treatment and this was comparable to that previously reported for both human and mouse cells. Since others have shown that H-2 antigens play a role in host recognition of the TSTA of methylcholanthrene-induced sarcomas, it has to be considered that an increased expression of H-2K and H-2D could facilitate the recognition of Meth A cells by precursor T cells of the syngeneic host.

After completing the initial series of experiments, we obtained a preparation of cloned γ interferon which was produced by recombinant DNA technology. Since little is known about the antitumor effects of this class of interferon it seemed appropriate to test it in the Meth A model system. Again, as was the case with the α - β mixture, the results of in vitro treatment with the γ interferon produced a cytostatic effect but not a cytotoxic one. However, the effect on growth of Meth A cells in mice was more striking. Whereas the γ - β mixture had no apparent effect when a challenge dose of 10^6 cells was used, there was both a retardation in appearance and a significant reduction in size of tumors in mice receiving Meth A cells treated with γ interferon.

In order to determine if the primary effect of interferon on Meth A cells is mediated through the immune system BALB/c mice were treated with Cytosan at a dose level of 300mgm./kg. This dose is just below the lethal level but sufficient to produce long-term suppression affecting all classes of antibody-producing cells. Under these conditions there was no difference between control mice and those receiving interferon-treated cells (100% of the animals receiving 1×10^5 or 1×10^6 cells developed tumors). When this result is considered in tandem with the nude mouse experiments, it is apparent that functional T cells are necessary for the rejection of interferon-treated Meth A cells. Using an in vitro cytotoxicity assay, attempts were made to identify a class of T cells in the spleen which would lyse tumor cells. Despite a variety of approaches, including pretreatment of Meth A cells with concavalin A, no lysis could be demonstrated. Preliminary experiments using peritoneal exudate cells instead of spleen cells suggest that there may be sensitized immune cells capable of attacking this particular sarcoma.

A consideration of these results has some noteworthy implications with regard to the clinical use of interferon in the treatment of various human neoplasms. Notwithstanding the limitations inherent in any single animal model system, it has to be considered that the administration of interferon to patients with significant deficits in immune system function, may not be successful. Thus in those clinical situations in which the tumor is widely metastatic and in which there has been prolonged chemotherapy or radiotherapy, one might question the potential efficacy of interferon. A more definitive test of the effectiveness of interferon may require the use of this compound as primary mode of treatment.

2. Effect of interferon on murine retroviruses. Most of the studies in this system are being done with Dr. Paula Pitha and reflect a longstanding interest in the mechanisms which account for the effect of interferon on retroviruses. The effects of interferon on retroviruses occur after the synthesis of viral RNA and structural proteins. Our work and that of other laboratories has demonstrated a mode of action involving the late stages of

virus assembly and maturation. Yet, the precise type of change observed seems to vary among the different classes of retroviruses. With ecotropic viruses such as Gross and Rauscher murine leukemia virus, there is maturational arrest at either the budding or particle release stage depending on the type of host cell used in propagation. Even in the systems where there is a release of virions from interferon-treated cells, the released particles have reduced infectivity. These effects are dependent on the continuous presence of interferon in the in vitro system; following withdrawal of interferon there is an almost complete recovery of virus titer with 18-24 hours.

Experiments have been done with a newer class of murine retrovirus, MCF, an env gene recombinant of ecotropic and xenotropic viruses endogenous to the AKR mouse which seems to accelerate the development of leukemia. Results derived from studying SC-1 cells chronically infected with MCF virus reveal changes which are analogous to that seen for other classes of retroviruses, but which reflect some individual differences. Interferon treatment for 24-48 hours reduced the titer of infectious virus 100-fold but reduced particle production by only 10-fold. There was a 2.5-fold increase in the number of cell surface-associated virions in the presence of interferon. Kinetic studies indicated that there were no demonstrable changes in the intracellular processing of viral polyproteins to mature structural proteins, but there was an accumulation of viral p30 and p12, consistent with the observed increase in cell-associated virions. Virus particles produced from interferon treated cells contained an 85K dalton glycoprotein of nonviral origin, they were nearly devoid of gp70 and contained measurably lower quantities of p30. The paucity of gp70 in these virions is consistent with their lack of infectivity.

Until now the studies of murine retroviruses have been done using a mixture of α and β interferons. Recently we obtained cloned γ interferon and have repeated many of the experiments reported previously. Our results indicate that γ interferon has most of the same antiviral properties of the $\alpha\beta$ mixture.

3. Role of retroviruses in the host rejection of methylnanthrene-induced sarcomas. In an initial experiment mouse Meth A sarcoma cells were deliberately infected with Moloney murine leukemia virus (MuLV) prior to interferon treatment to determine if virus infection would enhance the antiproliferative effects of interferon. The results from one of the control experiments indicated 100% rejection of the virus-infected tumor cells in the absence of interferon. Our working hypothesis is that Moloney MuLV is sufficiently antigenic to induce antibody formation, with this viral immune response acting to enhance the rejection of the infected neoplasm. It is known that several of the laboratory-derived strains of MuLV are more antigenic than the parental virus, Gross MuLV, from which they are believed to be derived. To test our hypothesis, we infected Meth A cells with a B-tropic variant of Gross virus, 1802-B. When these cells were used to challenge BALB/c mice there was no difference between the control and experimental groups. Currently a number of experiments are underway, using a variety of B-tropic viruses to infect Meth A cells to determine if the host response to Moloney MuLV is unique or shared in common with other retroviruses.

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OTHER INVESTIGATORS

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Major Findings:

1. Genomic Maps of RHHV and WR-RHHV

It has been proposed that murine type-C leukemia virus (MuLV) generally of low pathogenicity, when recombined with a subset of host cellular nucleotide sequences (c-src), give rise to competent oncogenic viruses. The laboratory isolates of rat RNA tumor viruses with transforming activity such as Harvey sarcoma virus (HaMuSV) and Kirsten sarcoma virus (K-MuSV) have been considered such recombinants based on homologous oligonucleotide distribution patterns of rat cellular 30S RNA and MuLV genomic RNA. Recently three strictly rat-tropic RNA tumor viruses have been isolated, namely rat hepatoma leukemia-helper virus (RHHV) isolated in our laboratory, wild rat leukemia helper virus (WR-RaLV), and Sprague-Dawley rat leukemia virus (SD-RaLV). These were considered as endogenous rat type-C viruses since they were released from chemically induced tumors. Upon recombination with the c-src sequences some of these have demonstrated cell transformation capacity. The analyses of the genomic complexity of these endogenous rat leukemia viruses would no doubt provide further insights on the "helper" sequence and its function, especially when recombined with c-src sequences in the genome of a transforming rat virus. Recently we have identified the DNA sequences specific for both the endogenous RHHV and WR-RaLV as 8.0-8.8 kilobases in length. These viral DNAs were successfully cloned via the pBR322 vector and cultured in E. coli RRI (EK-1) host.

A genomic map of RHHV proviral DNA has been constructed from the various restriction enzyme cleavages. Approximately 22 restriction endonucleases were initially tested for the construction of RALV genomic map. Among the restriction endonucleases that demonstrated specificities on the RHHV DNA, Alu I, DPN I, MSP I, Bgl II, Nci I, Hae III and Hind III generated numerous small limit DNA fragments, for which the assignments of specific sites on the genomic DNA proved difficult. The presence of at least 13 methylated cytosines and guansines was nevertheless established at the various Msp I and Dpn I sites. The orientation of RHHV 8.8 Kb proviral DNA with respect to the 3' terminus was determined by the greatest degree of homology of the various restricted DNA fragments to poly-(A⁺) mRNA specific for RHHV.

By labeling the 5' terminus of a purified, concentrated and recycled 8.8 kb DNA in a polynucleotide kinase reaction, the positions of the various restriction enzyme cleavage sites with respect to the 5' terminus were ascertained. The following restriction endonucleases including Eco RI, Bam HI, Pst I, Kpn I, Taq I, Pvu II, and Sma I yielded consistent cleavage patterns, and proved to be in-

strumental in deducing the restriction map of RHHV DNA. A striking feature associated with the RHHV restriction map is the repeated pattern of Taq I, Pvu II, and Pst I specificities at both 3' and 5' termini of the 8.8 kb DNA. The polynucleotide sequence flanked by these restriction endonuclease recognition sites measured 600-800 nucleotides in length, and may be considered the most plausible candidate for LTR.

A genomic map of WR-RaLV was deduced from various restriction enzyme cleavages described below. Approximately 25 restriction endonucleases were first tested for the construction of WR-RaLV genomic map. Among the restriction endonucleases that demonstrated specificities on the WR-RaLV DNA, Alu I, Hpa I, Msp I, Bgl II, Nci I, Hae III, Kpn I, Sma I and Dpn I, generated numerous small limit DNA fragments which were not amenable for the construction of WR-RaLV restriction map. The presence of at least 10 methylated cytosines was nevertheless established at the various Msp I sites. Kinetic studies provided some assurance in the assignments of certain Bgl II and Nci I sites. The following restriction endonucleases namely Eco RI, Bam HI, Ava I, Pst I, Kpn I, Hind III, Taq I, Pvu II, Sal I and Eco RV, provided information instrumental in the mapping of WR-RaLV genomic DNA.

2. Heteroduplex analysis of conservative and divergent DNA sequences among rat tumor viruses

Laboratory variants of highly tumorigenic retroviruses are known to have evolved by recombination between a cellular DNA sequence, known as c-src, and the genomic DNA of low pathogenic leukemia helper virus. It thus seems pertinent to study the genomic complexity of the wild rat endogenous virus with respect to the common and/or divergent DNA sequences among the various feral and laboratory variants of rat leukemia helper and sarcoma viruses. These included cloned DNA sequences in total or subgenomic sizes, derived from rat hepatoma helper virus (RHHV), Kirsten murine sarcoma virus (KiSV), Harvey murine sarcoma virus (HaSV), and the rat endogenous 30S RNA. Results obtained from reciprocal Southern blot-hybridization analysis suggested that WR-RaLV genomic DNA shared extensive homology with the various total or subgenomic DNAs derived from these leukemia helper and sarcoma viruses. A more refined approach by heteroduplex analysis was then chosen to examine the divergent sequences within the genomic DNA which might have been so limited that escaped detection by Southern blot-hybridization analysis. Preliminary studies showed that WR-RaLV DNA and the RHHV DNA formed heteroduplex that suggested homology at both the 5' and 3' termini. Limited homology was also observed within the two molecules revealing two particular areas of divergency; one at approximately 2.8 to 4.0 kb, which corresponded in part with the KiSV P21 coding sequence (V-RAS or K-src) and other sequences proximal to the 5' terminus, and the other one at approximately 5.5 to 7.0 kb, which corresponded in part with the env gene of the leukemia helper virus genome. WR-RaLV formed heteroduplex with the subgenomic 1.0 kb DNA of the KiSV P21 coding sequence. In contrast such was not observed between RHHV DNA and the 1.0 kb subgenomic DNA of the KiSV P21 coding sequence. Heteroduplex analysis also suggested at least two areas of divergent sequences between WR-RaLV DNA and the rat endogenous 30S RNA. In general, results of our preliminary heteroduplex analysis suggested that sequences immediately proximal to the 5' terminus of the rat leukemia helper viral DNA were the conservative elements among the various rat C-type tumor viral DNAs investigated. Divergency appeared

to pertain to DNA sequences within the C-type tumor viral genome indicative of recombination with cellular DNA sequences such as the c-src and/or the env coding sequences.

3. Microinjection of RaLV total and subgenomic DNA sequences for DNA transduction analysis

The biological activity of the cloned RHHV DNA en toto or in restricted subgenomic fragments was assessed by intranuclear microinjection into normal rat kidney cells (NRK¹⁵³). Release of rat C-type leukemia helper viruses by the microinjected cells was examined by superinfection on Kirsten transformed non-producer cells (KNRK). Immediate release of helper leukemia viruses at a very low level was observed only in the NRK^{m3.5/cir} cells microinjected with the supercoiled form of RHHV DNA en toto, suggesting that the circular form of the viral DNA might have expedited the replication and expression of viral particles. Genome rescue experiments were also performed by co-cultivating the microinjected NRK^{m153} cells carrying various linear RHHV DNAs, en toto or of subgenomic sizes, with K-NRK cells. Results indicated that both the total and the 5.8-6.2 kb DNA fragment proximal to the 5' terminus of the cloned RHHV 8.8 kb DNA were able to successfully rescue a transforming replication-competent pseudotype virus. Subgenomic DNA fragments derived from the center or the 3' end of the RHHV DNA were ineffective in the genome rescue experiments.

4. Nucleotide sequence of the genomic DNA of RHHV and WR-RHHV.

On the basis of the restriction maps we have begun nucleotide sequencing of both the RHHV and WR-RHHV genomic DNA. This yielded information on the conservative versus the highly mutagenic and/or recombining sequences within the RaLV genome when compared to those of the mouse leukemia helper viruses. Suitable restriction sites such as Bam HI, Eco RI, Kpn I, Pst I, Taq I and Pvu II that offered strong labeling by Klinow reaction were used in preparing radioactive RaLV DNA. Applying computer analysis on our results we further discovered suitable restriction sites such as Xor II, Sst I, and Hpa II further down stream of the nucleotide sequence that permitted sequencing into the genomic DNA. This also provides a confirmative restriction analysis on the nucleotide sequence obtained from our experiment.

5. Isolation and purification of interleukin 2 and a lymphokine and their possible roles in cellular defense mechanism

Interleukin (IL2), a biological modifier produced by rat activated spleen (T-lymphocyte) cells in the presence of Concanavalin A, was purified 50-100 fold by ammonium sulfate fractionation and sephadex G100 column molecular filtration technique. IL2 was found to possess short-term growth-promoting effect on preformed cytotoxic T-lymphocytes for 3-5 weeks. After culturing for 3 to 5 weeks, the relatively purified T cell preparation isolated from tumor sites were found to give long lasting protection in the in vivo tumor transplantation experiment. These findings indicate that the in vivo activity of antitumor T cells could be augmented by in vitro culturing in IL2. This method may offer a new approach to the immunotherapy of cancer (autochthonous)

host. Another fruitful aspect of IL2 research was the establishment of an IL2 dependent natural killer (NK) cell line, designated as IL2-CE1, in long term culture. NK cells were known to be short-term cell culture and were difficult to maintain in culture. After multiple attempts, we have succeeded in establishing a long term NK cell line which could be maintained in culture medium supplemented with IL2. Single cell clones were obtained from this cell line by limiting dilution technique, and they were found to be derived from heterogenous populations of NK cells. Establishment of these NK cell clones in long-term culture should no doubt allow detailed characterization, which may shed information on the mechanism of cellular defense mechanism. Furthermore, this will permit precise evaluation of their significance in the immune surveillance against neoplasia.

As a continuing collaborative project with Dr. C.C. Ting immunoregulation of T cell-mediated immunity, efforts are concentrated on the isolation and purification of putative lymphokine, critical in regulating the activation and differentiation of cytotoxic T-lymphocyte precursors into cytotoxic T-lymphocyte. T cells and NK cells were presumed to play the central role in the immunosurveillance of tumor growth. The mechanisms for the regulation of T cell-mediated immunity is composed of complicated immunoregulatory networks and circuits. They involve the participation of various populations of lymphocytes, macrophages, and lymphokines. To examine the origin of the cytotoxic T-lymphocyte precursors and the mechanisms for their activation is the most fundamental approach to these questions.

Significance to the biomedical research and the program of the National Cancer Institute.

By resolving the genomic maps and complexity of two rat leukemia helper viruses isolated from chemically induced tumors, we successfully identify the helper DNA sequences essential for recombination with c-src in the course of oncogene expression.

We have also successfully isolated and purified IL2 for establishing relatively stable culture of T-lymphocytes resulting in modified and augmented in vitro and in vivo anti-tumor activity of the immune T lymphocytes prepared from tumor bearing hosts. Our finding offers a new approach to perform immunotherapy in autochthonous hosts. We have also examined the mechanisms for the activation and differentiation of cytotoxic T lymphocytes.

Relationship of Research to National Cancer Plan

Project Area - 10; Approach Elements - 2; Approach - 4

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SUMMARY REPORT

MACROMOLECULAR BIOLOGY SECTION, IMMUNOLOGY INTRAMURAL RESEARCH PROGRAM

DCBD, NCI

October 1, 1982 - September 30, 1983

There were changes in the Macromolecular Biology Section last year: Sid Shifrin who collaborates with Dr. L. D. Kohn in the LCP at NIADDK on thyrotropin receptor now reports directly to the Director, DCBD. Samuel Luborsky is spending the year on work/study assignment at the Laboratory of Molecular Biology at Cambridge, England, with Ed Lennox, developing an assay for lactoferin receptor on the cell surface membrane.

Concisely stated, the goal of research of the MBS is to reduce cell surface changes in mammalian cells relevant to cell growth control to molecules which are expressed on the cell surface and which also directly interact with cellular DNA. There are only two known macromolecules in mammalian systems: the SV40 T antigen and the so-called p53 a newly recognized cellular protein, which appear at this time to fulfill both of these requirements.

We have found in the past that the SV40 early gene coded T antigen is also expressed on the surface of SV40 transformed mouse cells and can cause immune recognition and rejection in the mouse. This is the first case, when (in the model mouse system) the tumor associated transplantation antigen is fully defined molecularly. We found in certain transplantation experiments that the immunocompetent syngeneic mouse rejected all SV40 T antigen and the full early SV40 DNA possessing clonal cells. We also found that the tumor cells (clones) which grow arose by a rare recombination event, and lost a substantial portion of the early half of the SV40 DNA necessary for T antigen and tumor transplantation antigen. The necessary pieces of the early SV40 DNA for these two antigens are being defined by the Southern blot technique, using appropriate DNA probes.

The p53 is a phosphoprotein of approximately 53,000 MW which has been known to be in complex in most SV40 transformed cells with the T antigen. We now isolated it from numerous cells not transformed by SV40. These cells include normal murine cells in early stages of embryogenesis, placenta cells including term human placenta cells, a mouse ectoderm derived neuroblastoma cell line (neuro 2A), endoderm derived parietal yolk sac cell line (PYS-2) and mesoderm derived L-cell clones. The methionine labelled tryptic peptides in 2D maps indicated that the SV40 p53, and the various embryogenesis related stage dependent p53 molecules we have isolated is the same protein within the same mammalian species. We have found that 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 and in certain other cells relates to differences in phosphorylation of the p53. We have also shown that the various p53 molecules have (associated) phosphokinase activity. Using cloned DNA probes studies began on the modulation of p53 mRNA biosynthesis in cell division cycle, in the differentiation of the embryonal carcinoma cells, and also of the cells in the developing embryo.

Our current objective is to fully characterize p53 (i.e. determine the complete amino acid sequence of p53) compare it with the structure expected from the existing DNA clones, and then define how changes in p53 (i.e. phosphorylation, mRNA modulation) may relate to the role of this molecule in embryogenesis and in cell division in both normal and malignant cells.

Other Professional Personnel

Krishna Baksi, Guest Worker, Macromolecular Biology Section, IRP, NCI

Project Description

Objectives: To elucidate certain biochemical and biological changes both in the cell surface and in the control mechanisms involved in cell division, which occur during transformation to malignancy in mouse cells.

Methods Employed: Labelling of glycoproteins, proteins and nucleic acids with radioactive precursors during cell growth in culture. Fractionation techniques and isolation of (labelled) subcellular components, including cell surface membranes and macromolecules associated with surface membranes. Isolation and characterization procedures for macromolecules include thin-layer and column chromatography, gel electrophoresis, electrofocussing and autoradiography of gel slabs. Analytical and preparative ultracentrifuge studies. Enzymological techniques pertinent to intermediary metabolism. Cell growth dynamics in culture, and also tumorigenicity in vivo in syngeneic and in nude mice.

Major Findings: I. Changes in Cell Surface Glycosaminoglycans. Our role in this project is only to supply Drs. Winterbourne (London) and Kent (Oxford) with existing selected clonal derivative mouse cells from "families" of clonal cells developed for comprehensive biochemical and biological studies, to provide the biologic data, and to assist in evaluating the results of such comparative studies.

A specific lesion has been found in the past by Dr. Winterbourne in the metabolism of heparan sulfate i.e. a reduction in 6-O-sulfate glucosamine residues which occurs in regions of the carbohydrate chain of heparan sulfate containing relatively few sulfate groups. This lesion was found to be related to the ability of the SV40 transformed cells to grow to high cell densities. He now found the same lesion in numerous spontaneously transformed mutant tumor cell clones, as compared to the parent non-tumorigenic clone. It is known that after SV40 induced transformation there is an increase in the synthesis of cellular DNA, accompanied by increased activities of the appropriate enzymes. It is commonly thought that many tumorigenic transformation events are associated with loss of control of DNA synthesis. The essence of Dr. Winterbourne's finding is that the changes in heparan sulfate metabolism relate to this, and the changes somehow over-ride the normal control of metabolism in both of the two different forms of transformations studied by us; the SV40 induced and the spontaneous tumorigenic transformations.

Dr. Kent uses our cells to study changes during cell transformation in cell surface receptors where glycoproteins appear to play a role, such as in receptors to epidermal growth factor, in uptake of carbohydrates, and also in changes in glucosamine metabolism which is a major pathway of carbohydrate metabolism for glycoprotein biosynthesis.

II. "Spontaneous" Transformation of Cells. In the past we obtained families of genetically well defined and closely related mouse fibroblast clonal cells with very different cellular tumorigenicity. For example from a parent clone (210C), which is essentially non-tumorigenic ($TD_{50} = 10^{6.4}$) we have isolated 10 independent tumor lines by selection through in vivo passage through the syngeneic AL/N mice (work with L. Waters). Each tumor line had $TD_{50} \sim 10^2$, both in the syngeneic and in nude mice, and this phenotypic property was heritable and stable. By recloning (with V. McFarland) three clones obtained from three independent tumor lines, we have obtained more than 3 dozen subclones. All of the subclones expressed the phenotypic property ($TD_{50} \sim 10^2$). Thus the tumorigenicity, when once appeared, did not revert, and did not appear to "segregate". These families of cells now at hand represent an unique collection of clones to begin a study (at a statistically significant level) of the biochemical correlates on various molecular levels (DNA, RNA, proteins etc.) of spontaneous ("chance") transformation.

A screening begun by Dr. F. Mushinski for the expression of the proto-oncogenes or c-oncogenes, the cellular DNA sequences homologous to the transforming genes (v -oncogenes) of acute transforming viruses. The parent non-tumorigenic 210C clone and one of the highly tumorigenic derivative tumor lines was screened for expression of mRNA. Using v -mos, v -myc and v -fes DNA probes on Northern blots both the 210C clone and the derivative tumor line were found negative, but using v -abl both were found positive. The latter was also found by others recently on other established "normal" fibroblast mouse cell lines (D. Baltimore, personal communication). Screenings will continue by Dr. Mushinski using other probes.

Dr. D. Winterbourne found that 11 out of 11 of the first generation tumor lines tested had the specific change in cell surface heparan sulfate biosynthesis (see above) as compared to the parent normal clone. From two other normal clones all the two derivative tumor lines tested also had the specific change. No other detectable difference in carbohydrate metabolism related to cell surface glycosaminoglycan biosynthesis was found in any of the cells tested. Dr. Winterbourne is continuing this collaborative work and is attempting to characterize the enzyme (and DNA) changes (mutations) which appears to occur in all spontaneous cellular transformation to tumorigenicity in the above system.

III. SV40 T Antigen and "TSTA". A major achievement of this laboratory was the establishment that the SV40 T antigen contains the amino acid sequences necessary and sufficient for this virally defined cell surface antigen (the T antigen is encoded in the early half of the SV40 genome) for immunologic recognition and rejection of SV40 transformed cells in the mouse.

We found in the past that when spontaneously transformed highly tumorigenic mouse cells (clones) are further transformed by SV40, the resulting T antigen positive cells (clones) have reduced tumorigenicity because of the "TSTA" effect of the T antigen. When the tumors were re-established in tissue culture, the resulting tumor lines (and clones) did not express T antigen activity, and recovered the original high tumorigenicity of the spontaneously transformed clones. We recognized that rare T antigen negative revertant tumor cells may have lost the early half of the inserted SV40 DNA by some reorganization of the cellular DNA, apparently through random excision and recombination events.

These cells may be then selected out as the growing tumor cells under propitious immunologic conditions.

To prove this we detected the inserted and residual SV40 DNA in the various cells (with Dr. Baksi and C. Parrott). We generated by restrictions and then isolated the appropriate labelled DNA probes possessing various parts of the SV40 genome including those corresponding to the N terminal and the C terminal of the T antigen (original DNA clones were from the laboratories of M. Singer and C. Weissman). The results fully confirmed our predictions: the revertant tumor lines and clones have lost the insertion of the whole early SV40 DNA. We also found retention of another SV40 DNA insert representing DNA coding for the extreme C terminal end of the T antigen, the Bam I - Bcl I fragment, which is thus insufficient for the TSTA activity in mice.

Work on the biochemical detection of the T antigen on the surface of the SV40 transformed mouse cells was completed and published by us earlier. Our findings now have been confirmed by several independent groups (cf. Dr. Butel at the University of Texas; Dr. Durfler at the University of Ulm, W. Germany; Dr. Gurney at the University of Utah). No further work is planned in our laboratory. Collaborative studies on cell surface detection of the SV40 T antigen is now restricted to that of giving out cell lines, reagents and information. Other investigators collaborating in this fashion include: Dr. T. Rose, Cancer Research Center, Seattle; Drs. Pierre and Evelyn May and Dr. K. Chandrasekaran, Villejuif, France; Dr. A. DiMarco, Milan, Italy; Dr. Chungming Chang, Taiwan; Dr. Javier Coll, Madrid, Spain.

Significance to Biomedical Research and the Program of the Institute: Studies of biochemical changes in cells, particularly in the cell surface, which relate to acquisition of tumorigenic potential caused by some rare event, such as somatic mutation, and the studies on immunologic recognition and rejection in vivo are of interest, especially when they may lead to understanding of the changes in molecular mechanism which appears to be reducible to heritable changes of control processes on the DNA level.

Proposed Course of the Project: Attempts will be continued to further clarify the relevance of the various changes in cell membrane biochemistry, to spontaneous and to viral induced transformation of cells. We also plan to further relate these changes to phenotypic changes in cell immunogenicity and in tumorigenicity in various mouse systems. Studies on the molecular level will include the delineation of the structural and regulatory parts of the SV40 DNA which is minimal but sufficient for the TSTA activity in mice. In collaborative work we will also attempt to identify and if possible to isolate (clone) the transforming DNA pieces (alleles) in spontaneous transformations. We hope that the information and methodology developed in the mouse system will be of use in studies on human tumor cells.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01CB05546-03 IRP
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.) A Common Protein in Embryonic Differentiation and in Cellular Transformation		
PRINCIPAL INVESTIGATOR (List other professional personnel on subsequent pages.) (Name, title, laboratory, and institute affiliation) Peter T. Mora Chief, Macromolecular Biology Section, IRP, DCBD, NCI		
COOPERATING UNITS (if any) Daniel Simmons, University of Delaware; E. G. Gurney, University of Utah; Javier Coll, Instituto Nacional de Prevision, Madrid, Spain; J. Chou, NICHD		
LAB/BRANCH Immunology Research Program		
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CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The purpose of this project is to characterize an SV40 induced 53,000 MW (p53) cellular protein in embryonal carcinoma cells (EC), embryonic cells and in various transformed cells. Undifferentiated EC cells and primary cells prepared from midgestation mouse, rat and hamster embryos all expressed the protein p53 without SV40 infection. The 2D tryptic peptide maps obtained from EC and embryo cells showed that this protein is similar, if not identical to SV40 induced p53 and is conserved evolutionarily. The p53 was also isolated and partially characterized from various tumorigenic mouse cells such as L cells, neuroblastoma cells, 3T12 cells. The p53 was also present in human placental cells. The turnover of this protein was found to be rapid in tumorigenic and in non-tumorigenic cells whereas in most SV40 transformed cells the protein was found to be stable due to its interaction and complex formation with the large T antigen of SV40. However, it was found that certain SV40 transformed mouse cells possess stable p53 not in complex with the T antigen. Also in SV40 transformed term and first trimester human placental cells the p53 is not in complex with the T antigen.</p>		

Other Professional Personnel:

T. S. Usha, Visiting Fellow, Macromolecular Biology Section, IRP, NCI

Project Description

Objectives: To isolate and characterize a specific cellular phosphoprotein of γ 53,000 MW (p53) which is present up to midgestation stage in embryo primary cells and also in many cells transformed by various agents, and to determine the function of the p53.

Methods Employed: The biochemical and tissue methods are outlined in Project Report Z01CB05526-15 IRP. In addition, complex immunochemical techniques, such as detection of cell (surface) antigens both in cell culture and in situ in embryos with pertinent specific and monoclonal antibodies, dissection of mouse embryos of various ages for organ precursors.

Major Findings: 1) The embryo protein is a phosphoprotein. From 14 day old rat embryo primary cultures, labelled with inorganic ^{32}P , p53 was specifically precipitated with a monoclonal antibody prepared against p53 from SV40 transformed mouse fibroblast (monoclonal Ab from E. Tucker Gurney, University of Utah). A related 53K phosphoprotein from SV40 transformed mouse cells was found to possess phosphoserine residues and small amount of phosphothreonine residues, but no detectable amounts of phosphotyrosine residues (with C. Parrott and T. Martensen, NHLBI). We (C. Parrott) also showed that an cAMP independent $\gamma^{32}\text{P}$ ATP phosphokinase activity is associated with p53. We are now involved in determining what type of phosphorylation and phosphokinase activity is present in early embryo cells from various murine species, and also in special mouse cells such as Neuro 2-A cell, and in Balb/c 3T12 cell and in clonal derivatives of this cell with or without SV40 transformation. With D. Simmons, we are comparing the phosphorylation (2D tryptic peptide fingerprints) of the p53 molecules which are not in complex with a stabilizing protein, with that of the complexed and the SV40 T antigen stabilized p53.

2) Cell division rate and p53. Careful cell growth rate measurements and quantitations of the ^{35}S -Met labelled p53 are in progress in numerous established normal and transformed cell lines and clones, and also on embryonal carcinoma cells in which cell growth rate (and also state of differentiation) can be controlled to certain extent (cAMP, retinoic acid, etc.). Up to this time no general correlation has been observed with cell growth rate. It is important to emphasize that our experiments on embryo primaries (as in any other cells) are all in rapidly dividing cells, at their optimal (exponential) growth. Under such conditions the effects of other parameters (such as the age of the embryo, difference between primary and secondary cultures, the presence of SV40 T antigen, transformation (and selection) of certain cells are all much greater (50-100 x higher), than the small effects (<10%) of cell division rate on the amount of the 53K protein in the cells. Our published results (with Dr. Chandrasekaran and Dr. W. Anderson) on the absence of significant correlation in embryonal carcinoma cells with cell growth rate, but strong correlation with the state of differentiation was confirmed by others (A. Levin, N.Y.U., and M. Oren, Weizman Institute, Israel).

3) The detection of p53 in human tumors. As many (but not all) established human tumor lines were shown by others to contain the p53 in well detectable amounts, we embarked on a collaborative screening project on the detectability and quantitation of this protein in freshly established (primary) cells from various human tumor biopsies at a large research hospital in Madrid (Dr. J. Coll, Head of the Cancer Cell Membrane and Immunology Research Section, Instituto Nacional de Prevision). We are supplying Dr. Coll with the specific antibodies necessary for the detection of p53 and with information on optimizing quantitation.

Dr. Chandrasekaran, previously of this laboratory, now in Villejuif, Paris, is screening sera of human cancer patients for antibody against human p53 by a method developed in our laboratory. In agreement with others (L. Crawford, ICRF, London) about 10% of patients with breast cancer have antibody.

4) The p53 in mouse tumor lines, mouse embryonal carcinoma cells, and in mouse placenta cells. Diverse mouse tumor lines, such as the neuroblastoma (Neuro 2A) line, the L-cell (clone 929), and also of mouse embryonal carcinoma (EC) cells, such as the undifferentiated EC F9, the differentiated EC PYS2 were used to isolate and characterize the p53. All these p53 molecules are phosphoproteins and have similar methionine labelled tryptic peptides when resolved by 2D electrophoresis-chromatography to that of the p53 in SV40 transformed mouse cells in complex with the T antigen (C. Parrott and D. Simmons). All these p53 molecules (from tumor and the EC cells) have a rapid turnover (half life 30'-3 hrs.) as compared to the T antigen complexed p53 (>24 hrs.).

Quantitation of the p53 in the above cells (clones) showed that established cell lines (clones) derived from all three major germ layers of the differentiating mouse embryo contain substantial amount of p53: These include the ectoderm derived neuroblastoma cell line (Neuro 2A), the endoderm derived parietal yolk sac cell line (PYS-2) and the mesoderm derived L cells. There is also substantial amounts of p53 in extraembryonic cells: In throphoblasts derived from the mid-gestation mouse embryonal placenta. The amounts of p53 detected by a sensitive immunoprecipitation technique decreased in the following order: p53 in Neuro 2A > F9 > placenta > PYS2 > L cells >> established normal mouse embryo fibroblasts. The amount of p53 detected appears to be a function of the stability of the p53 in the various cells in tissue culture. In Neuro 2A cells the induced "differentiation" in culture by 8-bromocyclic AMP decreased both the amount of p53 and also the growth rate of the cells.

5) There is no general correlation with cellular tumorigenicity and p53 levels. Careful analysis was carried out in our laboratory using newly established clonal mouse cell lines with known pedigree and close familiar relationships. In mouse fibroblasts from three "families" of cells no correlation was detectable with the TD_{50} values in syngeneic (AL/N or Balb/c) or in nude mice. Several highly tumorigenic clones ($TD_{50} \leq 10^2$) form rapidly (≤ 8 weeks) lethal fibrosarcomas in contrast to the normal parent clone ($TD_{50} > 10^6$). All these cells possessed very little p53 (1/50th to 1/100th) when compared to an SV40 transformed clonal derivative cell.

6) Certain SV40 transformed mouse cells have free p53 not in complex with the T antigen. Quantitative amounts of the cellular p53 and of simian virus 40 (SV40) coded large T antigen and the degree of complex formation between these proteins were studied in a group of related SV40 transformed and non-transformed mouse embryo fibroblast cells from AL/N or Balb/c strain mice. The cells were labelled metabolically with ³⁵S-methionine or ³²P. The amount of p53 and large T antigen was estimated by comparing the incorporated radioactivity recovered by immunoprecipitation with PAb122 hybridoma cell supernatant and SV40 induced tumor bearing hamster serum respectively, separating the p53 and the T antigen, and expressing the radioactivities as the percentage of the total trichloroacetic acid precipitable radioactivity. The degree of complex formation was estimated by immunoprecipitating with antibody specific for one of the proteins and determining the amount of other protein co-precipitating. The amount of p53 and of the T antigen varied greatly in these cells. In the SV40 transformed cells derived from Balb 3T12 cells only ~20% of the p53 was found to be in complex with T antigen. In contrast in cells derived from AL/N strain embryos substantially all of the p53 was in complex with the T antigen, in agreement with past observations. Comparison of the ³⁵S tryptic peptide fingerprints revealed no differences between the uncomplexed p53 from an SV40 transformed Balb 3T12 derived clone and the complexed p53 from an SV40 transformed AL/N derived clone, nor between the uncomplexed T antigens of these clones (with D. Simmons). The results demonstrate that complete p53 complex formation does not exist for all SV40 transformed mouse cells, and indicate that p53-large T antigen complex formation may not be necessary for SV40 transformation of mouse cells. The reason for reduced complex formation in the SV40 transformed 3T12 derived cells is unknown. The phosphorylation of the p53 appeared to be lower in cells with reduced degree of complex formation.

7) Human placenta cells, with or without transformation by SV40, possess free p53 (with J. Chou, NICHD). Established human placenta cell lines from term placenta synthesize p53. Term or first trimester placenta cell line when transformed by a temperature sensitive SV40 mutant synthesize both p53 and also SV40 T antigen at about equal amounts at both permissive and non-permissive temperatures. The p53 at both temperature in both cell lines is substantially (>90%) is not in complex with the T antigen.

Significance to Biomedical Research and the Program of the Institute:

Re-expression of certain embryonic antigens in some cancers have been used by others in biochemical and immunological studies concerning development of certain tumors. Analysis and identification of the embryo protein(s) we have discovered could provide more insight into the mechanism of transformation; also the phase specific expression of this protein could be used at the molecular level to study embryonic differentiation.

Proposed Course of the Project: A DNA clone of coding for part of the p53 (from M. Oren, Weizman Institute) has been obtained. With this probe it is hoped to characterize the cellular gene which is constitutively express in normal embryogenesis, and which also is modulated in many (but not all) cellular transformations. Various potential molecular mechanisms for the modulation of the p53 gene expression will be explored in embryogenesis and in embryonal carcinomas. The nature of the phosphokinase activity in embryo cells and in embryonal carcinomas will be further studied.

Publications:

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