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NATIONAL CANCER INSTITUTE

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October 1, 1981 through September 30, 1982

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LABORATORY OF PATHOLOGY
DIVISION OF CANCER BIOLOGY AND DIAGNOSIS
NATIONAL CANCER INSTITUTE
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The Laboratory of Pathology is responsible for the diagnostic services in Pathological Anatomy for the Clinical Center of the NIH and has research programs in various areas of experimental pathology. The Laboratory is divided into 9 sections:

A. Surgical Pathology and Postmortem Section
B. Cytopathology Section
C. Ultrastructural Pathology Section
D. Biochemical Pathology Section
E. Comparative Oncology Section
F. Viral Oncology and Molecular Pathology Section
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A. Surgical Pathology and Postmortem Section

Dr. Jose C. Costa together with Drs. Reichert and Barsky, the residents, staff pathologists from the Laboratory of Pathology plus Dr. A.W. Cheever (NIAID), Dr. W.C. Roberts (NHLBI), Dr. K.J. Stromberg (NCI), Dr. L.A. Liotta (NCI), and Dr. P.E. McKeever (NINCDS) were responsible for the surgical pathology and autopsy diagnostic services. 5,666 surgical specimens or biopsies were accessioned in the past year. 141 autopsies were accessioned. These specimens involved the preparation of 53,408 slides, including 36,023 H&E stained sections, 6,238 special stains and 605 frozen sections. Clinicopathological studies in pulmonary vasculitis, breast cancer, pancreatic carcinoma, esophageal carcinoma, recurrent hyperparathyroidism and soft tissue sarcoma, are in progress.

Many specimens of fresh human tissues, including the eyes which are regularly removed during a complete autopsy, are furnished to NIH scientists in various laboratories. A tissue procurement nurse works closely with the staff and helps in the distribution of specimens to scientists.

Conferences with individual clinicians and surgeons from the various Institutes are an important part of the care and study of patients in the Clinical Center. Regular gross specimen reviews of organs removed at autopsy are conducted by the staff and most surgical specimens are discussed at the weekly Surgical Pathology Conference conducted by Dr. Costa, residents and staff. In addition, the staff participates in other regular conferences including Pediatric Oncology Tumor Conference (Dr. Triche), Hematopathology and Lymphoma Staging Conferences (Dr. Jaffe and staff), a monthly Neuropathology Conference (Dr. Valsamis) and conferences on Sarcoma Staging, Surgical Morbidity and Mortality, Medicine-Radiation Therapy (Dr. Costa and staff). The staff and residents present selected autopsies at monthly clinicopathologic conferences (e.g. NCI - Dr. Costa, NHLBI - Dr. Roberts). Weekly

slide presentations by the residents using current surgical and autopsy cases are held as a part of their training program.

B. Cytopathology Section

This section provides diagnostic services in cytology (both exfoliative and fine needle aspiration) and medical genetics. The chromosomal analysis includes conventional and special banding techniques (C-, G-, and R-bands) for the examination of individual chromosome pairs. During the year, approximately 3800 cytology specimens were accessioned. These represent over 7550 smears examined and reported. A little less than 1/4 were cervical and vaginal specimens; 3/4 were other types of specimens (including ascitic, pleural, gastric, spinal fluids, bronchial specimens (both brushing and washing), sputum and fine needle aspirations.

In addition to the diagnostic reports, the staff of this section collaborates closely with the clinical and surgical staff in various research projects. This section also provides diagnostic training for residents, visiting fellows and guest workers. Dr. H.-Z. Zhang, a Visiting Fellow from the People's Republic of China, has been learning diagnostic cytology and studying the feasibility of applying immunoperoxidase techniques to cytological materials.

Dr. Chu also serves on the staff of Tutorial on Clinical Cytology, sponsored by IAC, ASC, and AARM. She gives lectures and workshops on Fine Needle Aspiration Cytology.

C. Ultrastructural Pathology Section

This section provides diagnostic electron microscopy services for a diverse group of Clinical Center physicians, including NCI, NIAMDD, NHLBI, NIAID, and NINCDS, as well as submitted cases from outside physicians. This past year 326 cases were accessioned; over 300 were processed and diagnosed. The service is housed in a separate module and is essentially self-contained, which provides a convenient reference point for all diagnostic EM cases, including facilities to examine the cases as well as all records, tissues, etc. This facility also provides diagnostic training and clinical research opportunities for residents and fellows. Dr. William Green, a Visiting Fellow from the University of North Carolina, has been learning diagnostic EM while investigating the production of matrix proteins and tissue-specific proteins such as Factor VIII by Ewing's sarcoma and other childhood tumors. Dr. Andrea Modesti, a Fogarty Fellow from the University of Rome, Italy, has been employing investigative ultrastructure, including EM immunocytochemistry, on several projects, including a study of the basic organization of basement membranes, using the amniotic membrane model employed by Dr. Lance Liotta in conjunction with monospecific antisera for various basement membrane components. Using the protein A-gold technique, he has localized laminin, type IV collagen, and type V collagen to the proximal lamina densa, distal lamina densa, and proximal collagen fibre matrix, respectively;

these findings are somewhat at variance with previous studies but are of greater validity due to the specific reagents and techniques employed.

Dr. Triche has continued to collaborate with Drs. Henry Metzger and Chaviva Isersky-Carter of NIAMDD. An extensive study of the disposition of the IgE Fc receptor in the basophil membrane, employing monomeric ferritin-labelled IgE and basophil membrane ghosts, has been completed. In conjunction with biochemical assays, no evidence for a cytoplasmic protrusion of the Fc receptor was found in reversed membrane vesicles, suggesting that an intramembranous process must be responsible for the transfer of responses associated with binding and crosslinking of IgE, as in the allergic response. There seems to be no direct link between the receptor and a cytoplasmic mediator of degranulation. This mechanism is currently being investigated. Related collaborative work with Dr. Isersky-Carter on the biological fate of cross-linked IgE (as in allergen binding) is complete and in rough draft form. These studies have demonstrated that only cross-linked IgE with its associated receptors is internalized; this is followed by rapid lysosomal degradation and exocytosis. Both IgE and its receptor are degraded; no surface re-expression of either was found, unlike most other systems documented in the literature. The biological fate of both IgE and its receptor thus appears unique.

Drs. Modesti and Triche have completed a collaborative study on cystinosis with Dr. Joe Schulman and associates of NICHHD. Correlative morphological and biochemical studies on homo- and heterozygous cystinotics and normal controls have demonstrated that heterozygotes, though clinically normal, have a diminished capacity to lysosomally process cystine; such impaired lysosomes are detectable in peripheral blood neutrophils and the defect is exacerbated by pretreatment with cystine (dimethyl cystine). Normals show no abnormality; homozygotes display an even more conspicuous defect. This may allow detection of suspected or unknown carriers by examination of peripheral blood neutrophils.

Studies of matrix synthesis by Ewing's tumor have been completed by Dr. Dickman. These studies have clearly established that this tumor is a sarcoma, since it synthesizes types I and III (stromal) collagens, but it is also very primitive, since it also synthesizes type IV collagen, on "epithelial" collagen. These results suggest that this tumor is undifferentiated, since the only known precedent for such dichotomous lines of synthesis is in embryonic tissues such as placenta and embryonal carcinoma (ie, teratoma). Arguments relating Ewing's tumor to a specific normal tissue thus seem superfluous.

A similar approach has been extended to other so-called "round-cell" tumors of childhood by Dr. Triche, with the assistance of Ms. Sharon Mims (research biologist) and Ms. Barbara Silsby (graduate student and biologist). These tumors (neuroblastoma, primitive soft tissue sarcoma, and lymphoma) each have unique profiles of matrix protein synthesis and can be distinguished thereby from one another. Since the usual diagnostic criteria are incapable of reliably doing so, a derivative approach to the detection of matrix protein synthesis by these tumors in vivo may be diagnostically useful. In addition, preliminary results suggest these tumors may be useful models for the study of the synthesis and function of specific matrix proteins such as laminin,

entactin, fibronectin, type IV collagen, basement membrane proteoglycan, and stromal collagens. The in vitro assembly of an identifiable basal lamina may be possible; the contribution of each component to this structure can then be studied.

Dr. Triche's chapter on Pediatric Pathology in "Cancer in the Young" and review on Round-cell Tumors of Childhood in "Perspectives in Pediatric Pathology" were both published in May, 1982. A review on round-cell tumors by Drs. Triche and Askin has been solicited for a special symposium on pediatric pathology in Human Pathology, to be published in spring, 1983.

Dr. Triche and Dr. Green continue to provide all diagnostic electron microscopy for Clinical Center patients outside the NHLBI. Both also participate fully in the routine service work (Dr. Triche - surgical pathology; Dr. Green - autopsy pathology). Both also interface directly with the Pediatric Oncology Service, including the weekly tumor board. Dr. Triche also offers a graduate level medical education course (Medi 407 - Ultrastructural Pathology) for the FAES and a short course (Selected Neoplastic Diseases of Childhood) with Dr. Bruce Webber (St. Jude Research Hospital) for the IAP; he is also a member of the NIH faculty for the new FAES course on Surgical Pathology, "New Approaches to Old Problems in Surgical Pathology".

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. Methods for performing sugar and methylation analysis on whole glycoproteins in amounts less than 1 mg starting material have been developed and are being applied to studies of the structures of human polypeptide hormones.

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. The antibodies obtained bind a purified glycolipid that contains the same carbohydrate structure. This glycolipid, 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. Studies on the origin of $(G)_4$ indicate that the oligosaccharide arises by the combined actions of plasma α amylases and glucosidase on glycogen. The normal urinary excretion rate of $(G)_4$ results from degradation of about 0.01% of total body glycogen by this pathway.

E. Comparative Oncology Section

The investigative work of this section is conducted by a member of the Senior Pathology Staff, Dr. Dawe; a Biologist GS-12, Mr. Morgan; a Biologist GS-11, Mrs. Williams; a Bio. Lab. Technologist GS-9, Mr. Summerour; and a Bio. Lab. Technologist GS-7, Mrs. Brown. Mr. Frank Massie is responsible for care of animals on experiment and Mr. Nathaniel Nelson of the NCI Central Animal Facility, is responsible for care of mice in the breeding colonies.

During the past year, research has been centered mainly upon collaborative projects with Dr. Mark Israel of DCT and NIAID, and Dr. Tom Benjamin, Department of Pathology, Harvard Medical School. These and other projects are as follows:

I. Genetic and Epigenetic Factors in the Mouse Polyoma Virus Tumor System.

Studies of epigenetic factors were concluded with completion of Dr. Suzanne Fisher's study of isozyme profiles of the various polyoma tumors, as reported last year. One of 2 papers in preparation is nearing final draft.

Dr. Israel has done molecular genetic analyses on 5 cloned lines of polyoma virus, while Dr. Dawe has done pathological studies to correlate variations in viral genome with variations in the biological effects of infection in the mouse. After a preliminary survey of the differing responses to the 5 viral clones, 2 were selected for full genetic analysis. One, Py (LID), causes a high mortality (over 90%) in the 30-day period after birth, and high tumor incidence in survivors. The other, Py (A_3), causes only a low mortality in the perinatal period and very few tumors in survivors followed 9 months. Death from Py (LID) infection is caused by a combination of severe renal damage and intracerebral hemorrhages. Blood urea nitrogen levels become markedly elevated, and closely correlate with extent of anatomic renal damage.

Dr. Israel, by restriction enzyme mapping and base pair sequence analysis, has found that a single difference between Py (LID) and Py (A3) exists in a short non-coding segment of the Py genome where Py (LID) contains a tandem repeat not present in Py (A3). He has excised this segment from Py (LID) and inserted it into Py (A3), and Dr. Dawe is currently testing the recombinant virus to determine whether it has biologic properties characteristic of Py (LID). The mortality curve should be available within 60 days, and tumor data within the next year.

Dr. Dawe's collaboration with Dr. Benjamin is similar to that with Dr. Israel, except that 2 additional virus clones are under study. One, Py (59RA), produces low mortality and low tumor incidence, while the other, Py (PTA), produces low mortality and high tumor incidence in mice. Dr. Benjamin has identified a genomic difference between the two clones and is preparing appropriate recombinant viruses to test for biologic correlation. Mouse bioassay of the recombinant lines has not been started as yet.

A by-product of the collaboration with Dr. Israel is a pathological study of the sequential effects of 3 cloned Py virus lines in $C_3H/BiDa$ mice, starting from the day of infection at birth and extending through the time of appearance of Py-induced tumors. The study by Dr. Dawe and Dr. Howley of the VOMP section, makes use of the immunoperoxidase technique for identification of Py antigens in lytically infected cells. This sensitive method has made it possible to recognize sites of virus infection not previously known, and to see changes transitional from lytic foci to tumor foci. The study will result in a more complete chronologic picture of the events in vivo following Py infection, and will examine the variability of this picture in relation to genomic variants of the virus.

II. The Identity and Biological Significance of "X Cells" in Lesions Simulating Neoplasms in Fishes.

In previous work, Dr. Dawe has obtained evidence that certain lesions simulating neoplasms in marine fishes are in fact composed largely of cells of protistan parasitic nature ("X cells"). Such lesions include the cutaneous "papillomas" in pleuronectids and percids, and the pseudobranch and gill-chamber "tumors" in gadids. It is of interest to cancer researchers that the X cells, tentatively classified as a hartmannellid amoeba, have the ability to stimulate reversible proliferation in cutaneous epithelium and pseudobranch (gill) epithelium.

In a survey of Atlantic cod conducted aboard the F.S.S. Anton Dohrn at the invitation of biologists at the University of Hamburg (West Germany), Dr. Dawe found examples of the pseudobranch lesions in 1% of cod from the German Bight area of the North Sea. X cells in the lesions were identical to those in similar lesions in Pacific cod. A significant finding was that involvement of the thymus by X cells was found in a quarter of the fish with pseudobranch lesions. Thus X cells are now seen to have a tropism for skin, gill, and thymus. Developmentally, these sites are of ectodermal and pharyngeal endodermal origin, as are the organs of mice in which epithelial tumors are induced by polyoma virus. Dr. Dawe finds it curious that a protistan parasite and a small DNA virus each possess a mechanism to stimulate mitogenically a group of cell types that have a common embryologic and phyletic derivation. He suggests it may be of value to see if mitogens with features in common are present in these two etiologically different cell proliferation systems.

III. Myoepithelial Contractions in Mammary Glands in Organ Culture.

Mr. Morgan has continued his time-lapse cinematographic study of contractions effected by myoepithelium in mouse mammary gland. He has nearly com-

pleted characterization of these contractions with respect to contraction and relaxation times, synchrony of contractions within and among lobules, and endocrine influences. Contractions are vividly demonstrated in his films.

IV. Time-lapse Cinematographic Study of Lysis of Unsensitized Chicken Red Cells by Human Leukemia-60 (HL-60) in the Presence of Tetradecanoyl Phorbol Acetate (TPA).

Dr. Michael Blaese and associates have found that HL-60 myeloid leukemia cells destroy unsensitized chicken Rbc in culture if TPA is added to the medium. Mr. Morgan has collaborated with Dr. Blaese by performing time-lapse motion picture studies of the system. Films show that TPA first causes clustering of the HL-60 cells in the cultures, a step apparently essential for expression of the killing action. Subsequently, Rbc in the vicinity of HL-60 clusters are observed to undergo collapse and conversion to "ghosts". Mr. Morgan is now tracing the paths and contacts of Rbc with HL-60 to determine whether cell-cell contact is required for Rbc lysis in this system.

F. Viral Oncology and Molecular Pathology Section

The investigative studies ongoing in the section are carried out under the direction of three principal investigators: Drs. Howley, Costa, and Rabson. These studies 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. In addition, Dr. Costa has continued to establish and characterize human xenografts carried in nude mice. He has studied the induction of endogenous murine viruses by the human tumor and the transformation of murine cells in vivo (recruitment ?) by the human xenografts.

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. These transformants have altered growth properties in that they are able to grow in low serum with no added supplements besides hydrocortisone, whereas the non-transformed human epidermal counterparts required 10% fetal calf serum plus a number of additives. These altered growth properties of the transformants were due to a functional A gene product in that

they reverted to a normal phenotype at the non-permissive temperature. With regard to the properties of epidermal cell differentiation, the SV40 transformants quantitatively made less keratins than their non-transformed counterparts, and a lower percentage of the cells could be induced to form cross-linked envelopes by an ionophore. It is the expression of the A gene product which interferes with the expression of these differentiated properties in epidermal cells in that at the restrictive temperature, a normal pattern of differentiation returns in the transformants.

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. The bodies of these messages are complementary to sequences in the non-transforming 31% of the BPV-1 genome. 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. The transformants were analyzed and found to contain the hybrid DNA. Each independent clone was found to be synthesizing rat preproinsulin messenger RNA and making rat proinsulin which was secreted from the cells. More recently, Dr. Law, in collaboration with Dr. Bruce Howard (Laboratory of Biochemistry, NCI), has been examining the expression of selective markers cloned into a variety of transcriptional units in bovine papillomavirus. Specifically, they have inserted the pSV2-gpt segment containing the E. coli XGPRT gene cloned into a modified SV40 early region transcriptional unit into the transforming region of the bovine papillomavirus and have shown that the two selective markers (malignant transformation and ability to grow under selective media conditions) are closely linked. Analysis of the DNA shows that the hybrid DNA is present extrachromosomally but that a high degree of rearrangements have occurred. More recently, Dr. Law has shown that this selective marker, when cloned into a BPV-pML2 hybrid, is able to transform cells at high efficiency and that the input DNA is stable. Dr. Sarver has demonstrated that the complete bovine papillomavirus genome, when cloned into a deletion derivative of pBR322 called pML2, is highly efficient at transforming mouse cells and can be used as a shuttle vector for transporting DNAs between animal cells and prokaryotic bacteria. Dr. Rosenbaum has inserted the human beta interferon gene

into a bovine papillomavirus vector and has shown that mouse cells selected for their malignant phenotype synthesize a low amount of human beta interferon constitutively. Of interest is that there is approximately a 5-10 fold induction of interferon production utilizing either poly IC or inactivated NDV induction. In collaboration with Dr. Michel Revel of the Weizmann Institute in Israel, it was shown that the messenger RNA in the induced cells is coming from the authentic interferon promoter. While the complete BPV genome cloned into pML2 is able to transform mouse cells at high efficiency, the pML2 sequences are inhibitory to the transformation of mouse cells by the 69% fragment when they are covalently linked. Interestingly, a number of eukaryotic segments have been identified which provide a facilitation function when covalently linked to a BPV_{69T}-pML2 hybrid DNA. Drs. Rosenbaum and Sarver have identified three of these in the laboratory and they include the rat preproinsulin gene, the human growth hormone gene, and a rat intergenic segment.

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. Interestingly, after 10 passages in the presence of interferon, approximately 1-10% of the cells carried in the presence of interferon, have a flat morphology. These cells are no longer anchorage-independent and are susceptible to retransformation by the bovine papillomavirus. Analysis of the DNA of these flat revertants indicates that they no longer contain any BPV DNA sequences at the level of .2 copies per cell.

G. Hematopathology Section

Drs. Jaffe, Cossman, Crabtree, Kant, Neckers, Fornace, Braziel 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 Braziel 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. The above staff participate in two weekly conferences for the clinical staff of the Medicine Branch and Radiation Oncology Branch, DCT, NCI. These include a weekly Clinicopathologic Conference on current cases as well as a more formal weekly Staging Conference for malignant lymphomas. In addition, Drs. Jaffe and Cossman participate in the diagnostic services of the Laboratory of Pathology, taking responsibility for both Surgical Pathology and Postmortem Pathology, and as such, supervise residents involved in these services. 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.

Laboratory of Pathology, DCBD, NCI

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. We 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 inconsistly 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.

In collaboration with Dr. Robert C. Gallo, Division of Cancer Treatment, we are 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. Unusual epidemiologic aspects of these cases are being investigated in collaboration with Dr. William Blattner, DCCP. There appear to be certain areas of the world in which HTLV is endemic and HTLV-associated malignancies are seen with increased frequency. These areas include Southern Japan, perhaps Shangai, China, Peru, the Caribbean, and perhaps other areas with similar environmental and climatic conditions.

We have previously 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 Braziel in the Hematopathology Section. Functional studies are being done in collaboration with Drs. Geraldine Schecter and John Horton. Hypercalcemia appears to be a particularly prevalent complication in patients with HTLV-associated T-cell malignancies and the production of OAF is being investigated in HTLV-positive lymphoid lines in collaboration with Dr. Gallo.

Laboratory of Pathology, DCBD, NCI

We have 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. 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.

Other studies partially completed and in progress have investigated immunoglobulin gene rearrangement in malignant lymphomas and other lymphoid proliferations. These studies have been done in collaboration with Drs. Stanley Korsmeyer, Thomas Waldmann, and Philip Leder. Hairy-cell leukemia or leukemic reticuloendotheliosis, a disease in which immunoglobulin synthesis is only inconsistently demonstrated, has been consistently shown to be of B-cell derivation based on immunoglobulin gene rearrangement.

Studies done in collaboration with Drs. Zeller and Tsichlis, Laboratory of Tumor Virus Genetics, DCBD, have investigated expression of the MYC gene in malignant lymphomas. Using a MYC gene probe consisting of the MC29 viral transforming gene and a very small piece of the viral envelope, the messenger RNA from a variety of well-characterized human lymphomas and normal lymphoid tissues was screened for MYC expression. There was a statistically significant 3- to 5-fold increase in the level of MYC expression in nodular lymphomas as compared to normal tissues including normal tonsil, liver, thymus, and spleen.

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.

It has been shown that B-cell lymphomas can be subclassified based on expression of different antigens. Well-differentiated lymphocytic lymphomas and chronic lymphocytic leukemia express LEU1 but rarely express the common ALL antigen (J5). Malignant lymphomas of intermediate lymphocytic differentiation have a unique phenotype and express both J5 and LEU1. Nodular or follicular lymphomas express J5 but fail to express LEU1. The above observations confirm that malignant lymphomas of intermediate lymphocytic differentiation are a distinct entity which can be recognized both morphologically and immunologically.

We have also shown that the above studies can be extremely useful in the staging of patients with non-Hodgkin's lymphomas. Analysis of small numbers of cells obtained via lymph node needle aspiration can be examined and identified as to their involvement by the malignant process. These techniques are also applicable to fluids such as pleural effusions and ascites. Additional studies have investigated the role of the transferrin receptor and growth of normal and neoplastic cells. Studies have shown that expression of OKT9 correlates with growth. Furthermore, 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 determing 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.

Secondly, they have obtained the first sequence of fibrinogen signal polypeptide and have found that the sequence has several interesting features which suggest a mechanism for coordinating the processing and secretion of fibrinogen molecules from the hepatocyte.

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 Bldg. 37. The section also has a working laboratory located at 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

Approximately 80% of our effort was expended on the support of substantive biologic work employing our image processing and other advanced computer techniques. The majority of this work was collaborative in nature, ranging from the

characterization of proteins which are associated with various directions of stem cell differentiation to methods of characterizing the secondary structure of nucleic acid molecules of known base sequence.

All of these activities place immense demands on our computer facility, and the major fraction of our remaining effort has been devoted to solutions to these problems which can be reached by modest or even relatively small hardware and software expenditures and by avoiding the trap of expanding mainframe capacity to meet computational and storage demands.

Computer Aided 2 Dimensional Electrophoretic Gel Analysis (GELLAB)

Much biomedical research involving 2D gels produces multiple (3 or 4 as many 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 both 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.

1. One of several collaborative biomedical projects involving the use of GELLAB to follow cell differentiation, is one with Drs. McGuire & Thorgeirsson (C-LCHPH). The HL60 cell line is a promyelocytic cell line which can be induced to differentiate along granulocytic or macrophagic pathways by a variety of chemical agents. In this system GELLAB is applied to analyze changes in the levels of messenger RNAs associated with differentiation along the separate pathways. Briefly, mRNA is extracted from cells which are untreated, dimethylformamide-treated (granulocyte differentiation), or phorbol ester-treated (macrophage differentiation). The mRNA is translated in vitro and the resultant proteins are separated by 2D gel electrophoresis. GELLAB used in this way gives direct information on the relative amounts of RNA present in the translation mix. Our work has shown many changes in relative amounts of particular mRNAs and particularly exciting is the presence of an mRNA protein product represented by a robust spot found by GELLAB, present only after dimethylformamide treatment and thus, presumably, an expression of granulocytic differentiation.

2. An analysis of differences in malarial proteins synthesized by Plasmodium knowlesi parasites of different variant antigen phenotype is a collaborative effort with Drs. R.J. Howard and S.D. Aley of NIAID-LPD using GELLAB to characterize the proteins associated with or even perhaps definitive of the phenotypic variants. Success in this direction would represent a major step along the road to further understanding some of the complex immune responses in malarial infection. A large number of 2D gels derived from cloned P. knowlesi parasites of different variant antigen phenotypes have been analyzed. These gels each show of the order of 700 proteins synthesized by each clone and as

would be expected, most of these are identical. However, several proteins specific to each variant phenotype has been identified by GELLAB. These are currently being submitted to immunoprecipitation analysis using variant specific antisera and concurrently, studies of their specific subcellular locations are underway.

3. Another directable cell differentiation system, the murine erythroleukemia cell, infected by Friend virus is the subject of an intensive collaborative effort, this time with Drs. P. Wirth and S. Thorgeirsson (C-LCHPH). Erythroid differentiation is inducible by a variety of simple compounds such as DMSO and HMBA. The differentiation is marked by hemoglobin production and characteristic morphologic changes. The system of course must also involve the suppression of alternate genic effects related, for example, to the continued replication of the proerythroblastic element. A large number of gels representing various treatments and controls have been analyzed and not so surprinsingly, no new spots were found. Numerous spots showed quantitative alterations in protein after either DMSO or HMBA treatment, reflecting the complexity of the intracellular events triggered by the simple chemical stimuli. The value of GELLAB here lies in its particularly strong set theoretic and statistical internal routines allowing the user to assure himself of the consistency of an observed change.

4. Our most extensive and long-lasting collaboration is with Dr. E. Lester at the University of Chicago. The University is one of the sites to which we have successfully exported GELLAB. The extent of the biological data bases involved in our collaboration with Lester is so great that continued use of our System 20 as well as the one at Chicago is necessary. We have published several papers on the effects of phytohemagglutinin on lymphocyte proteins. Such stimuli are also part of the major project to "fingerprint" several of the morphologically seemingly identical leukemias. The collaboration with Lester also involves the HL60 cell line, except that here we are examining whole cell extracts. This will certainly supplement the results we are obtaining with McGuire.

5. Some of our earliest experiments using what later became GELLAB, involved the P388D1 cytotoxicity observed with asbestos and asbestiform fibers. Using some suggestions by Dr. Barry Margolin of NIEHS, we concentrated on gels prepared from cells exposed for 72 hours rather than shorter intervals. Our preliminary results indicate that different quantities of protein are produced by cytoxic fibers, while others in varying amounts as a result of simple non-cytotoxic phagocytosis. These gels were prepared in collaboration with Drs. Merril and Goldman of NIMH.

In addition to the foregoing well-established substantive collaborative projects, a series of short feasibility studies were performed with many workers including Dr. Darrel Davidson (NCI), Dr. Richard Henneberry (NINDS), etc. These produced results which enable both the investigator and ourselves to evaluate the applicability of GELLAB to their problem, and for us to form some idea of the significance of the problem. As a result of many of these, there is little prospect for much available GELLAB time on our present processor for the forseeable future.

Analysis and Synthesis of Nucleic Acid Secondary Structure

Realizing with our early work on the analysis of electron micrographs of nucleic acid molecules, that additional non-pictorial information was necessary for a complete analysis, we turned to the problem of generation of secondary structure given the sequence of bases that make up the molecule. It was hoped that the two complementary problems could be, in fact, brought together and in the process, both simplified.

The combinatoric complexity of computing secondary structure from sequence data, employing thermodynamic data available on various base pairs and sequences, verges on the practically intractable. However, we have made significant inroads on selected aspects of this problem in collaboration with Drs. J. Maizel (Child Health) and Ravi Dhar (Lab. of DNA Transforming Viruses). The initial programs required run times in proportion to some exponential power of the number of bases, so that even small fragments of a few hundred bases would require months of CPU time. Bruce Shapiro has adapted a program so that only cubed time is required. Addressing characteristics of DECsystems have limited the number of bases in a molecule to a few hundred. Our improvements to the core program now allows molecules of 420 bases to be run. We have further developed the algorithm so that much larger molecules can be run using a disk paging technique. Molecules of 1500 or more bases now are possible. However, the resources consumed by such programs are almost prohibitive.

An additional molecular drawing program was developed which has some interesting advantages over the drawing program developed previously. This new program eliminates the problem of overlap of complex structures which were topologically inherent in the older algorithm. Now the output of the secondary structure predicting programs can be viewed without the intervening interactive untangling process. Both programs have been used quite successfully by Dr. Maizel.

The drawings that are produced from the new program also contain the inherent facility for secondary structure comparisons amongst different sequences. This makes it possible to do higher order similarity determinations beyond the level of simple sequence data. A circle graph algorithm was also developed that permits similarity comparison but from a different viewpoint.

Collaboration with Dr. Ravi Dhar on the examination of the secondary structure of a Harvey sarcoma virus sequence showed that the start codon of 3 mRNAs were in position of high destabilization thus, perhaps, making them accessible for expression.

Our ability to handle larger molecules is the basis for an extension of our collaboration with Dr. Maizel. A 14000 base fragment of adenovirus mRNA is being examined for secondary structure in an overlapping piecewise manner. Comparisons are being made between the predicted structures with EM studies of the same portion of the molecule. The results of this experiment will be significant in regard to the eventual merger of the analysis and synthesis techniques of the secondary structure problem.

Laboratory of Pathology, DCBD, NCI

We have continued to process selected fiber samples through our cytotoxicity system, including some coated crysotile fibres provided by Dr. Ann Wylie of the University of Maryland. Thus far in exotic material, such as this and in routine material such as that sent us from the French Academy, we have found no disagreements between the Stanton Hypothesis and P388D1 cytotoxicity on the other.

Our image processing work on living cell images has been deferred due to the overwhelming demands on our resources for 2D gel analysis and the secondary structure work. One algorithm for allowing multiple sequences of digital time lapse image acquisition, using multiple fields at computer-determined intervals and acquisition rates, was developed. Work on the critical algorithm for automatic cell boundary generation has been suspended for lack of resources.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00853-29 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Surgical Pathology and Postmortem Section

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.C. Costa	Chief, Surgical Pathology & Postmortem Section	LP NCI
OTHERS:	S.H. Barsky	Expert	LP NCI
	C.M. Reichert	Expert	LP NCI

(See next page for additional staff)

COOPERATING UNITS (if any)

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SECTION
Surgical Pathology and Postmortem Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
20	20	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

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.

PHS-6040

Project Description

Staff:

+Dr. Gail J. Bryant
+Dr. Ilona Linnoila
+Dr. Charles R. Simrell
+Dr. Carl W. Rettenmier
+Dr. Dr. Dana M. Fowlkes
+Dr. Maria Tsokos
Dr. Sue Ellen Martin
Dr. Timothy J. O'Leary
Dr. Lubomir P. Turek
Dr. Elaine S. Jaffe, Chief, Hematopathology Section
Dr. Clyde J. Dawe, Chief, Comparative Oncology 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 Pathophysiology, 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 pathologic anatomy; and (4) to collaborate with investigators in research involving the use and study of human material.

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

(a) 141 autopsy examinations. The residents perform nearly all of the postmortem dissections under supervision of the staff. A gross review is held weekly by the staff and residents. The residents review the microscopic slides of each autopsy 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) 5,666 specimens were accessioned in the Surgical Pathology and Postmortem Section. These specimens are initially examined and described by a resident and their reports are checked by the staff. Associate pathologists from other laboratories or sections are frequently consulted about problems in diagnosis in surgical pathology and/or assume responsibility for handling certain tissue removed for specific research projects. 605 pre-operatory consults were rendered.

(c) Histological preparation and special procedures: The functions are carried out under the direction of Mrs. Ruby Howard. 53,408 slides were prepared. Of these, 36,023 were H&E stained and 6,238 were special stains.

(d) Photographic services of the department: A photographic record is made of the large amount of the 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. A total of 329 black and white negatives and 644 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 Isenburg, who provides professional assistance and facilities for the entire staff of the Laboratory of Pathology.

2. Independent research. The staff is engaged in the following projects:

(a) Evaluation of the role of pathological classification and histological grading in the therapy of soft tissue sarcomas (Costa).

(b) Characterization of angiocentric lymphoproliferative disease (Costa, Jaffe).

(c) Characterization of lymphadenopathy in immunodeficient homosexual patients (Costa, Falk, Spier, CDC).

(d) Characterization of the pathological lesions seen in the toxic oil syndrome in epidemic in Spain (Shearer, Costa).

(e) Derivation of monoclonal antibodies to identify and characterize antigens present in Schwann cell tumors and other sarcomas of the soft somatic tissues (Reichert, Costa).

(f) Study of pancreatic cell hyperplasia (O'Leary, Costa).

(g) Transplantation in nude mice of human tumors (Costa).

(h) Study of invasion and metastases (Barsky, Liotta).

3. Collaborative research

(a) Pathological characterization of excisional biopsies in breast carcinoma subsequent mastectomies (Reichert, DeMoss).

(b) Characterization of pachydermotactyly (Reichert).

(c) Study of thyroid nodules (Barsky/Robbins).

(d) Pathological features of testicular and extratesticular germ-cell tumors (Barsky, Ozols, Javadpour).

(e) Morphology of myositis ossificans (Zasloff, Barsky).

(f) Study of cis platinum neuropathy (Reichert).

(g) Study of vasculitis (Fauci, Costa).

Mrs. Margaret Flanagan and Mrs. T. d'Angelo, Tissue Procurement Nurses, have provided human specimens to different laboratories at NIH.

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

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.

5. 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

1. Reichert, C.M., Rosenberg, S.A., Webber, B.L., and Costa, J.C.: Malignant melanoma: A search for occult lymph node metastases. Hum. Pathol. 12: 449-451, 1981.

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13. Costa, J.C.: Nasopharyngeal cancer update - XII International Symposium of Dusseldorf. Hum. Pathol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00852-29 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Exfoliative Cytology Applied to Human Diagnostic Problems and Research Problems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.W. Chu	Chief, Cytopathology Section Medical Officer (Path.)	LP	NCI
OTHERS:	S.E. Martin	Jr. Staff Pathologist	LP	NCI
	H.-Z. Zhang	Visiting Fellow	LP	NCI
	T.A. Wood	Res. Biologist (Cytol)	LP	NCI
	L. Galito	Res. Biologist (Cytol)	LP	NCI
	A.M. Honig	Res. Biologist (Cytol)	LP	NCI
	K. Nakahara	Res. Biologist (Genetics)	LP	NCI
	L. Stroud	Biologist (Cytol)	LP	NCI

COOPERATING UNITS (if any)

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Cytopathology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9	4	5

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Provides complete diagnostic service in exfoliative cytology, medical cytogenetics, and fine needle aspiration cytology. In addition, collaborates in various clinical research projects utilizing special techniques including special staining and tissue culture techniques. Investigates and applies new cytochemistry techniques to improve and enhance cytological diagnostic efficacy.

Project Description

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).

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. Setting up immunoperoxidase staining techniques to be used on cytological smears and/or research materials.
6. To use Pap stain on oral smears from patients who are fitted with the intra-oral fluoride device.
7. Maturation index in precocious babies.

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.

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-O, mucicarmine 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.

Biopsy materials of skin, gonads, tumors are cultivated by tissue culture technique and cells harvested for chromosomal analysis.

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. Using chromosomal hybridization in situ technique, certain genes associated with malignant B lymphocytes may be mapped to their chromosomal bands with precision.
6. Providing cytologic methods to research projects specifically, fine needle aspiration in thyroid nodules.

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.

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. Intensive pursuit of cytochemical techniques to improve cytological diagnostic efficacy.

Activities: 1. Under the mandate of U.S.-China agreement, visited China as participant in the Exchange Program, October 1981.

2. While in China, a series of lectures were given on the subjects of cytology.

3. Lectures and workshop on fine needle aspiration of thyroid lesions, Chicago, Ill., May 9-16, 1981.

4. Lecture on Urinary Cytology, Cairo, Egypt, June 1981.

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2. El-Bolkainy, M.N., Chu, E.W., and Ibrahim, A.S.: Organization of a Screening Project for the Detection of Bladder Cancer. In El-Bolkainy, M.N., and Chu, E.W. (Eds.): Detection of Bladder Cancer Associated with Schistosomiasis. Cairo, Egypt, Al-Ahram Press, 1981, pp. 19-28.

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

7. Mazumder, A., Grimm, E.A., Zhang, H.-Z., and Rosenberg, S.A.: Lysis of fresh human solid tumors by autologous lymphocytes activated in vitro with lectins. Cancer Res. 42: 913-918, 1982.

8. Martin, S.E., Dwyer, A., Kissane, J.M., and Costa, J.C.: Small-cell osteosarcoma. Cancer, in press.

9. 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.

10. 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., in press.

11. Pretorius, H.T., Kinsella, T.J., Barsky, S.H., Brennan, M.F., Chu, E.W., and Robbins, J.: Thyroid nodules following high-dose radiotherapy. JAMA, in press.

12. Chu, E.W., and Martin, S.E.: Fine Needle Aspiration Cytology. In Liotta, L., and Hart, I. (Eds.): Tumor Invasion and Metastases. Martinez-Ninghof Pub., 1982, in press.

13. Grimm, E.A., Mazumder, A., Zhang, H.-Z., and Rosenberg, S.A.: The lymphokine activated killer cell phenomenon: Lysis of NK resistant fresh solid tumor cells by IL-2 activated autologous human peripheral blood lymphocytes. J. Exp. Med., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00518-04 LP

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Fate of IgE Bound to Mast Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. Isersky	Senior Investigator	A&R	NIAMDD
OTHERS:	T.J. Triche	Chief, Ultrastructural Pathology Section (Surgeon)	LP	NCI
	S.J. Mims	Biologist	LP	NCI
	J. Rivera	Biologist	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:	PROFESSIONAL:	OTHER:
12	6	6

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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

Objectives: To study the effect of IgE cross linking on its fate after cell surface binding.

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

Major Findings: 1. Oligomerization of IgE with a chemical cross linker (dimethyl suberimidate) and subsequent binding of defined oligomers ($n \geq 2,3$, 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 ($n \geq 3$). 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 $Fc\varepsilon$ 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

1. Isersky, 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., in press.

SMITHSONIAN SCIENCE INFO ATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00520-04 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Surface Disposition of Cell Bound IgE with Time

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section (Surgeon)	LP	NCI
OTHERS:	C. Isersky	Senior Investigator	A&R	NIAMDD
	S.J. Mims	Biologist	LP	NCI
	J. Rivera	Biologist	A&R	NIAMDD

COOPERATING UNITS (if any)

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Laboratory of Pathology

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Ultrastructural Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	2	2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 (Isersky, 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.

Project Description

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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00530-03 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Analysis of Bile from Cases of Gallbladder Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D.E. Henson Medical Director LP NCI

COOPERATING UNITS (if any)

Dr. Roger Soloway, University of Pennsylvania

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Laboratory of Pathology

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NCI, NIH, Bethesda, Maryland 20205

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2	2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Determine the composition of bile and gallstones from patients with carcinoma of the gallbladder. The study includes racial and ethnic groups, e.g. American Indians, who are known to have a high incidence of gallbladder cancer.

Results indicate that Indians with gallbladder cancer have more glycolithicholic acid in bile than controls without cancer and that these patients do not completely sulfate the cholic acids. Reasons for incomplete sulfation are not presently known, but will be investigated. Non-sulfated cholic acids are toxic.

PHS-6040

Project Description

Objectives: Determine if there are differences in the composition of bile or stones from patients with cancer of the gallbladder compared to patients without cancer and determine reasons for the incomplete sulfation.

Methods Employed: Analytical chemistry methods, such as gas chromatography, thin layer chromatography, column chromatography and immunocytochemistry.

Significance to Biomedical Research and the Program of the Institute: Cancer of the gallbladder is common in some ethnic groups such as American Indians. Investigation of this disease in a defined population group could tell us about effects of diet and other factors on bile composition which ultimately would have a bearing on preventive measures. If the initial findings that patients with cancer do not sulfate completely prove to result from a defect in liver enzyme activity, then this will be the first demonstration of a metabolic defect in humans associated with cancer.

Proposed Course: Continue to analyze bile from patients concentrating on types of bile acids, mechanisms of sulfation, and mechanisms of malignant transformation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00545-04 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Extracellular Matrix Synthesis by Human Tumors in Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section (Surgeon)	LP	NCI
OTHERS:	W.R. Green	Medical Staff Fellow	LP	NCI
	A. Modesti	Visiting Fellow	LP	NCI
	B.M. Silsby	Biologist	LP	NCI

COOPERATING UNITS (if any)

National Institute of Dental Research

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Ultrastructural Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	6	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The type and amount of matrix proteins synthesized by human tumor cells in vitro appears to parallel that of cultured normal cell counterparts to some extent. We wish to broaden these observations to a variety of human tumors, and determine whether differentiation in vitro induced by differentiating agents will cause a reversion to more normal patterns of collagen synthesis, and whether these patterns might allow more precise categorization of the tumor's origins. In addition, the analysis will include a search for any new or atypical matrix proteins unique to these tumors. Such proteins may be useful in studying extracellular matrix assembly and function.

Project Description

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. Four 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.

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

1. Dickman, P.S., Liotta, L.A., and Triche, T.J.: Ewing's sarcoma: Characterization in established cultures and evidence of its histogenesis. Lab. Invest., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00594-06 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Pathology of Family Cancer Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.E. Henson	Medical Director	LP	NCI
OTHERS:	J.J. Mulvihill	Head, Clinical Genetics Section	CEB	NCI
	J.F. Fraumeni, Jr.	Chief, Environmental Epidemiology Branch	EEB	NCI
	W.A. Blattner	Clinical Investigator	EEB	NCI
	M.H. Greene	Clinical Investigator	EEB	NCI

COOPERATING UNITS (if any)

Epidemiology Branch, NCI

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Ultrastructural Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	5	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Review histopathology material from families who have a high incidence of cancer. These families are being followed by the Epidemiology Branch. Material is reviewed to confirm diagnosis and determine unusual aspects of the histology of the cancer in view of the family history and genetic background.

PHS-6040

Project Description

Objectives: Review and confirm the histological diagnoses on families that have a high incidence of cancer and which are being followed by the Epidemiology Branch.

Methods Employed: Light microscopy, special histochemistry stains.

Significance to Biomedical Research and the Program of the Institute: Confirm diagnoses of cancer and apply uniform terminology and classification to the lesions so that patterns may be found.

Proposed Course: Continue to review surgical pathology or autopsy material submitted from referring hospitals and report results to the Epidemiology Branch.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00884-01 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Ultrastructural Organization of Basal Lamina

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.J. Triche	Chief, Ultrastructural Pathology Section (Surgeon)	LP	NCI
OTHERS:	L.A. Liotta	Senior Investigator	LPP	NCI
	A. Modesti	Visiting Fellow	LP	NCI

COOPERATING UNITS (if any)

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Laboratory of Pathology

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Ultrastructural Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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

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

Methods Employed: 1. 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; ± .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 to some extent 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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00508-05 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immune Response of CBA/N Mice to Oligosaccharides Coupled to Protein Carriers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Surgeon	LP	NCI
OTHERS:	K. Stein	Senior Staff Fellow	DBP	BOB, FDA
	W.E. Paul	Chief	LI	NIAID

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:	PROFESSIONAL:	OTHER:
0.1	0.1	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

Project Description

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 isoelectric 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 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: This project has been completed. A second manuscript describing results is in preparation.

Publications

1. Stein, K.E., Zopf, D.A., Johnson, B.M., Miller, C.B., and Paul, W.E.: The immune response to an isomaltohexosyl-protein conjugate, a thymus-dependent analogue of $\alpha(1\rightarrow 6)$ dextran. J. Immunol. 128: 1350-1354, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00510-04 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Glucose-containing Tetrasaccharide in Human Urine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Surgeon	LP NCI
OTHERS:	M. Ugorski	Visiting Fellow	LP NCI
	P.A. Pizzo	Surgeon	PO NCI

COOPERATING UNITS (if any)

Department of Clinical Chemistry, University of Lund, Lund, Sweden
(Dr. Arne Lundblad)

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Laboratory of Pathology

SECTION
Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies raised against a glucose-containing tetrasaccharide-Glcα1-6Glcα1-4Glcα1-4Glc-coupled to KLH have been used in a radioimmunoassay to measure urinary excretion of the oligosaccharide in urine of patients with glycogenoses, pregnant women, and pediatric patients with soft tissue sarcomas. Preliminary data suggest that the rate of urinary excretion of this tetrasaccharide may be a useful indicator of the tumor mass present in certain patients. The hypothesis that the oligosaccharide may originate as a product of digestion of glycogen by amylase is under investigation.

Project Description

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 radioimmunoassay method was devised using rabbit antiserum prepared by immunization with a synthetic glycoconjugate containing $(G)_4$ coupled to KLH. Specificity of the assay was established by comparing relative activities of $(G)_4$ and several other glucose-containing oligosaccharides with related structures as inhibitors in the assay. The assay has greatest sensitivity and specificity for reduced $(G)_4$.

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 dextrins that include $(G)_4$ (0.7 mg). Treatment of the higher limit dextrins (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 $(G)_4$ 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: Measurements of $(G)_4$ excretion in patients with neuromuscular, metabolic, and neoplastic diseases will continue to further explore the utility of this assay and correlation with clinical status. Studies on the biochemical origin of $(G)_4$ will be carried out to determine the plasma enzyme responsible for production of $(G)_4$ from α-limit dextrins.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00511-04 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Carbohydrate Heterogeneity in Alpha Subunits of Human Polypeptide Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Nilsson	Visiting Scientist	LP	NCI
OTHERS:	D.A. Zopf	Surgeon	LP	NCI
	S.W. Rosen	Senior Investigator	CE	NIAMDD
	B. Weintraub	Senior Investigator	CE	NIAMDD

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:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

Project Description

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 - trifluoroacetolysis - 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: Analytical methods for GC/MS of oligosaccharides on a microscale are under development and will be applied to obtain further detailed information about the structures of the oligosaccharide chains of the peptide hormones.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00523-03 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Complex Carbohydrate Released from Mammalian Cells by Trifluoroacetolysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Surgeon	LP	NCI
OTHERS:	B. Nilsson	Visiting Scientist	LP	NCI

COOPERATING UNITS (if any)

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Laboratory of Pathology

SECTION
Biochemical Pathology Section

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NCI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

PHS-6040

Project Description

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 trifluoroacetolysis 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: Oligosaccharides are quantitatively released intact from glycosphingolipids whose ceramide moieties contain 4-sphingenine. The mixture of oligosaccharides released from a total neutral glycosphingolipid extract of a human colorectal carcinoma can be separated into more than twenty components. Major constituents include the following:

Oligosaccharide	Structure
Lactose	Galβ1-4Glc
Lacto-N-triose II	GlcNAcβ1-3Galβ1-4Glc
Lacto-N-neotetraose	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Globoside	GalNAcβ1-3Galβ1-4Galβ1-4Glc
Lacto-N-fucopentaose III	Galβ1-4GlcNAcβ1-3Galβ1-4Glc 3 Fucα1

Several minor constituents also give distinct peaks and mass spectra. Some of these are novel oligosaccharide structures, not previously recognized by standard methods. The presence of large amounts of lacto-N-fucopentaose III (LNF III) is interesting since this is a very minor component of normal colonic mucosa. This determinant has been detected recently as the tissue antigen for hybridoma antibodies that bind human tumors.

Significance to Biomedical Research and the Program of the Institute: There is abundant evidence that cell surface carbohydrates can mediate specific binding of hormones, toxins, immunoglobulins, lectins, and other macromolecules. This project is designed to survey the similarities and differences in oligosaccharide patterns produced by cells at various stages of cellular differentiation in order to develop chemical and immunologic markers for cellular differentiation. In cases where major differences exist between normal and tumor tissues, oligosaccharides may be useful as tumor-specific markers.

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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00525-03 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Analysis of Oligosaccharides by Combined Gas Chromatography-Mass Spectrometry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.A. Zopf	Surgeon	LP NCI
OTHERS:	B. Nilsson	Visiting Fellow	LP NCI
	J. Cashel	Med. Technician	LP NCI

COOPERATING UNITS (if any)

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Laboratory of Pathology

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Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 retention time and decreases efficiency of 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 useful separation of oligosaccharides containing up to six monosaccharde 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.

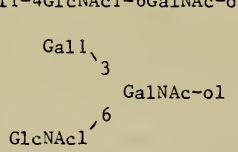
Project Description

Objectives: To develop methods for separation and analysis of oligosaccharides by gas chromatography and mass spectrometry.

Methods Employed: Following trifluoroacetolysis, oligosaccharides are treated with sodium borohydride and methanolic ammonia and finally are permethylated. N-trifluoroacetylated and permethylated oligosaccharide alditols are separated by gas chromatography using a fused silica capillary column 50 meters long coated with methyl silicon. The column effluent is passed without separation into a mass spectrometer.

Major Findings: Analysis of oligosaccharides as permethylated, N-trifluoroacetylated alditols can be accomplished by combined gas chromatography-mass spectrometry for molecules containing up to seven monosaccharide units including two hexosamines. Standards prepared by trifluoroacetolysis of purified glycolipids and glycoproteins with known carbohydrate structures enable identification of compounds according to retention time on GC under standard conditions and mass spectra. Oligosaccharides from the core regions of blood group substances, proteoglycans, and glycoproteins have been analyzed. For example, 1) The following oligosaccharides were separated and identified from 100 micrograms of a mixture that could not be fractionated by HPLC or paper chromatography: GlcNAc1-3Gal1-3GalNAc-ol

Gal1-4GlcNAc1-6GalNAc-ol



2) The biantennary and triantennary core regions of asparagine-linked carbohydrate chains of glycoproteins have been analyzed as intact permethylated and N-trifluoroacetylated pentasaccharide or hexasaccharide alditols. They can be identified by retention times and mass spectra. 3) The core region of the keratan sulfate chain from proteoglycans of rat chondrosarcoma have been analyzed and shown by GC/MS of the derivatized core oligosaccharide to consist of the following structure:

Galβ1-4GlcNAcβ1-6GalNAc
3
Galβ1

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

1. Nilsson, B., and Zopf, D.A.: Gas Chromatography and Mass Spectrometry of Hexosamine-containing Oligosaccharide Alditols as their Permethylated, N-trifluoroacetyl Derivatives. In Ginsburg, V. (Ed.): Methods in Enzymology. New York, Academic Press, 1982, vol. 83, pp. 46-58.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00529-03 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Structure of the Carbohydrate Portion of Human Haptoglobin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Nilsson	Visiting Scientist	LP	NCI
OTHERS:	D.A. Zopf	Surgeon	LP	NCI
	G.C. Ashwell	Chief	LBM	NIAMDD
	M.E. Lowe	Staff Fellow	LBM	NIAMDD

COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Pathology

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Biochemical Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

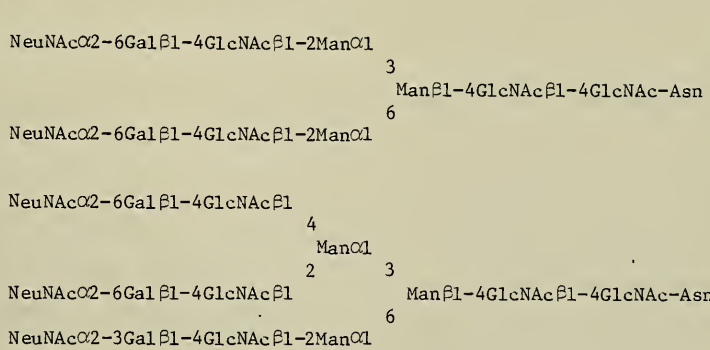
Haptoglobin is a plasma glycoprotein whose half time in the circulation of the rat is 19 days. When haptoglobin forms a complex with hemoglobin the complex is removed from circulation by the liver with a half time of only 4 minutes. Uptake of the complex by hepatocyte membranes is specifically blocked by glycopeptides derived from exhaustive pronase digests of haptoglobin and is inhibited by prior treatment of the complex with periodate or glycosidases. The haptoglobin complex does not inhibit uptake of asialo-orosomucoid by hepatocyte membranes, nor vice versa. Since the above evidence suggests that uptake of the haptoglobin-hemoglobin complex by hepatocyte membranes is mediated by a carbohydrate specific membrane receptor different from that responsible for uptake asialo-orosomucoid, we have undertaken structural analysis of the carbohydrate portion of haptoglobin.

Project Description

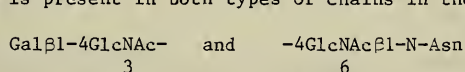
Objectives: To determine the chemical structure of the carbohydrate portion of haptoglobin.

Methods Employed: Following sugar analysis of whole purified haptoglobin, the carbohydrate chains are released by trifluoroacetolysis. Further specific chemical degradative steps including Smith degradation, nitrous acid deamination, chromium trioxide oxidation, and selective acid hydrolysis produce products which are analyzed by sugar analysis, methylation analysis, and permethylation of oligosaccharides. Analyses at all stages are carried out by combined gas chromatography-mass spectrometry.

Major Findings: Each beta subunit of haptoglobin 1-1 contains 4 asparagine-linked carbohydrate chains, two biantennary, and two triantennary, which in aggregate comprise 22.6% of the haptoglobin molecule by weight. The structures of these chains are as follows:



2) Fucose is present in both types of chains in the sequences



3) The distribution of fucose between triantennary and biantennary chains is about 14:1.

Significance to Biomedical Research and the Program of the Institute: Determination of the carbohydrate structure of haptoglobin may define the chemical structure responsible for specific uptake of the hemoglobin-haptoglobin complex by hepatocytes. As this uptake system appears to be functionally distinct from other glycoprotein uptake systems, it provides an opportunity for the study of independent function and regulation of two membrane-bound receptors on the same cell.

Proposed Course: The project is completed. A preliminary report was presented at the Sixth International Symposium of Glycoconjugates, Tokyo, Japan, September, 1981. A manuscript for publication is in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00549-02 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Hybridoma Antibodies to Oligosaccharide Haptens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.R. Schroer	Surgeon	LP	NCI
OTHERS:	D.A. Zopf	Surgeon	LP	NCI
	R.E. Levinson	Biologist	LP	NCI

COOPERATING UNITS (if any)

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NCI, NIH, Bethesda, Maryland 20205

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2.0	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hybridoma proteins (HP) with binding characteristics of high specificity and affinity for oligosaccharides would be invaluable reagents in the study of oligosaccharide localization, synthesis and excretion. Additionally, such antibodies might be used to purify or assess homogeneity of oligosaccharides from biological samples (of urine, plasma, tumors, etc.). High affinity characteristics of HP might be advantageously utilized for quantitative radioimmunoassays via isotope dilution techniques. Glycoconjugates of a urinary glucose-containing tetrasaccharide-Glcα1-6Glcα1-4Glcα1-4 Glc and, also, a milk oligosaccharide lacto-N-fucopentaose III (LNFIII) linked to keyhole-limpet hemocyanin (KLH) or edestin have been used to examine the feasibility of this hybridoma approach to carbohydrate-structural analysis.

Project Description

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, $(Glc)_4$, has been purified, phenethylamine derivitized and conjugated to keyhole-limpet hemocyanin for use as an immunogen in mice. 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: A. Anti $(Glc)_4$ Antibodies

1. The antibody response of mice to $(Glc)_4$ is quite diverse, but of very small magnitude, 1-10 μg/ml. The hybridomas reflect this diversity but routinely generate 1-10 mg/ml of antibody (a 100-1000 fold increase). More than 20 such hybrids have been established.

2. The HP of these hybridomas are variously IgM, IgG1, IgG2b, IgG3 and IgA; show spectrotypic diversity by isoelectric focusing; additionally considerable variation in affinity (amongst the hybridomas) for the free oligosaccharide in a radioimmunoassay (RIA) is demonstrable.

3. One hybridoma, anti-(Glc_4), shows a remarkable increase in affinity for the free hapten carbohydrate upon cooling to 4°C. This increase in binding energy is a feature of the Fab region since papain digestion does not ablate the effect. Chromatographic preparations of oligosaccharide are possible with sepharose derivitized antibody and thermal elution to obtain heretofore difficult purifications in a batchwise simple procedure.

4. Further RIA comparisons are in progress to map the binding characteristics of each protein. Such an analytical approach is essential to confidently use these HP in chromatographic preparations of crude oligosaccharide mixtures.

B. Anti-(LNFIII) Antibodies

Antibodies against LNFIII, a carbohydrate moiety expressed abundantly on glycolipids of carcinoma cells, have been prepared by immunizing with LNFIII edestin. Two such antibodies, one IgM and one IgG2b, will be compared to the existing hybridoma anti-LNFIII antibodies elicited by immunizing with intact tumor cells.

Significance of 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 $(Glc)_4$ will be sought to replace currently-used rabbit serum antibodies for RIA analyses of urinary excretion of this oligosaccharide. Anti-LNFIII antibodies prepared via a synthetic vaccine may offer a means of easy preparation of anti-carbohydrate hybridomas for oligosaccharides of tumor glycolipid origin.

Proposed Course: The utility of this model system to enable one to assay for specific oligosaccharides will be further evaluated as a prelude to analysis of more complicated oligosaccharide structures.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00521-12 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Epithelial-Mesenchymal Interactions in Neoplastic Development

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.J. Dawe	Chief, Comparative Oncology Section - Med. Officer (Path.)	LP	NCI
OTHERS:	S.E. Fisher	Post-doctoral Fellow	LP	NCI
	W.D. Morgan	Biologist	LP	NCI
	J.E. Williams	Biologist	LP	NCI
	M.A. Israel	Senior Investigator	I	LBV
	M.L. Meltzer	Microbiologist	I	IRP
	S.K. Arya	IPA (Roswell Park Mem. Inst.)	LP	NCI
	P.M. Howley	Chief, Viral Oncology and Molecular Pathology Section	LP	NCI

COOPERATING UNITS (if any)

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Laboratory of Pathology

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project seeks answers to the questions: 1) How can the tumor-inducing selectivity of polyoma virus (Py) for a specific constellation of organs in the mouse be explained? 2) How do epithelial-mesenchymal interactions (EMI) influence the effect of Py on target epithelium? 3) How do genetic variations in Py alter tumor-site selectivity and biologic response of potential target cells? Experiments in which EMI are perturbed show that such perturbations alter the tumor phenotypes resulting from Py transformation of salivary epithelium. E.g., isozyme profiles of salivary tumors induced in vivo (unperturbed EMI) are highly constant, whereas isozyme profiles of tumors induced in salivary epithelium isolated from salivary mesenchyme are variable and differ from those of tumors induced in intact glands. Evolutionary, embryological, and genetic evidence indicates an evolutionary and developmental kinship among the epithelial organs responsive to Py. Py genetic variants have sharply differing target cell specificity. Attempts are underway to identify Py DNA segments relating to organ tropism. It is postulated that activity of a specific regulatory gene family is common to Py tumor organs and is "used" by Py to induce proliferation.

Project Description

Anticipating retirement on June 26, 1982, the PI initiated no new projects strictly fitting the Project Title. One project, concerned with the cell and tissue response to polyoma virus infection in mice, was initiated primarily by the PI but represents a "target of opportunity" that presented itself during collaborative works with Drs. Israel, Benjamin, and Howley. Projects in which the PI participated are described individually below.

I. Genetic modifications of polyoma virus and their influence on the biological effects of the virus in mice.

(a) Collaborative project with Dr. Mark Israel.

In this project, 3 virus strains are involved. Two were provided by Dawe (Strains 1 and 2) and one by Dr. Israel (Strain 3). In preliminary studies of the mouse response to each of these strains, no two of the response patterns were found to be the same within a homogeneous host response system, the $C_3H/BiDa$ mouse. The basic assumption, therefore, on which the experiment was designed was that differences in response were related to genetic differences in the 3 virus strains. The objectives of the experiment were, in step-wise order: 1) To obtain genetically homogeneous populations of the 3 virus strains. This was to be accomplished by plaque-cloning each strain on mouse kidney cells and preparing homogeneous virus stocks from the cloned viruses, on P388D1 mouse cells. Genetic homogeneity of virus preparations was to be tested by endonuclease restriction enzyme digestion followed by analysis of electropherograms of the viral DNA fragments. 2) To carry out bioassays of each plaque-cloned virus strain after inoculation into newborn $C_3H/BiDa$ mice. Parameters were to be: a) Mortality during the first 30 days of life; b) Blood urea nitrogen curves on each set of test animals and a control set during the first 18 days of life; c) Tumor incidence rates for the 9-month period following inoculation; d) Rank-order of tumors in the various organs during the same 9-month period; e) Histopathological differences in the host mice as determined by complete necropsy studies of mice in each group at days 2, 4, 8, 10, 12, 14 and 18 after inoculation. 3) After the above two objectives were achieved, Dr. Israel would determine by molecular biological methods just where genetic differences exist among the 3 cloned virus strains; 4) Using methods for excising, cloning, and reinserting specific map segments of viral DNA, Dr. Israel would recreate viral genomes with or without those DNA sequences considered essential for one or more of the biological effects listed above; 5) the recombinant viruses would then be plaque-cloned, grown into stocks, the stocks would be tested for homogeneity, and finally the biological activity of these stocks would be bioassayed in parameters (a), (c), and (d) under (2) above. If biological responses were as predicted, the responsible genomic segments could be considered identified, and this information could then be further studied to determine how the critical segments bring about their respective effects; e.g., do they code for a previously unidentified protein, and if so, how does that protein affect host cell viability or proliferative activity?

Major Findings: For 2 of the 3 selected virus strains, all steps of the objectives outlined above have been accomplished excepting the final biological characterization of the 2 cloned recombinant viruses. Molecular analysis by

Dr. Israel shows that we have a uniform population of one viral strain from which a presumably critical DNA segment was deleted. The reciprocal viral strain, into which the presumably critical DNA segment was inserted, does not appear to be absolutely homogeneous, but both members of the genetically altered pair of viruses have been inoculated into sufficient numbers of newborn mice to provide the needed mortality data within 1 month of this writing. Data on tumor incidence and tumor organ rank order will not be complete for another 8 months.

Data demonstrating differences among the 3 original plaque-cloned virus strains are shown in Table I with respect to: 1) Mortality effects; 2) Tumor-inducing activity; and 3) Rank order of organs in which tumors were induced. Virus #3 in the Israel experiment was initially found to be non-homogeneous after plaque-cloning, and was therefore omitted from the genetic redesign phase of the experiment. However, it is remarkable that in all 3 parameters tabulated, its biologic effects were nearly identical to those of clone of virus cloned by Dr. Tom Benjamin from the same original wild (mixed population) virus strain. A closely similar collaborative project with Dr. Benjamin is outlined below.

Taking the available data from both collaborative projects, they show:

1) That clones of polyoma virus with widely different biological effects can be selected and characterized. The genome can remain stable through many replication cycles.

2) That the characters determining degree of lethality and degree of tumor-inducing activity in mice are apparently dissociable. The four-fold table below shows that we have seen at least one cloned virus line with fit for 3 of the 4 possible combinations that would indicate independent assortment. Evidence that high lethality can exist in absence of high tumorigenicity is not seen for any of the 5 viral lines so far bioassayed, while 2 lines have shown the combination of low lethality and low tumorigenicity, and 2 the combination of low lethality with high tumorigenicity.

Tumorigenicity \ Lethality	High	Low
Low	? None yet found	+ (Is #2) (Be #1)
High	+ (Is #1)	+ (Is #3) (Be #2)

3) That the ranking order of organs in which tumors are induced can differ for 2 viral clones, one of which has high lethality and high tumorigenicity, the other low lethality and high tumorigenicity.

The findings suggest not only that lethality and tumorigenicity are determined by different regions of the viral genome, but also that there can be variations within the genome that account for different ranking order of organs developing tumors. From the data available, it seems more likely that tumor rank order is determined by variations within the region determining degree of tumorigenicity, rather than in the region determining lethality. However, since only 1 cloned line with high mortality has been bioassayed, the second possibility is not ruled out. It is even possible that a third region exists that is solely responsible for determining tumor rank order (organ tropism).

The histopathological studies and blood-urea nitrogen studies have allowed us to assign the cause of death in mice infected with the high lethality clone to 2 factors working either individually or in combination. One is lytic damage to the renal cortex, correlating closely to greatly elevated BUN levels. The other is hemorrhage within the brain. Usually both of these anatomic lesions were present in individual mice of the group with high mortality in the period of 8-14 days post-inoculation.

(B) Collaborative project with Dr. Tom Benjamin, Harvard Medical School.

This project is nearly identical in its methods and objectives to Dr. Israel's. The main differences are that Dr. Benjamin is working with two clones of polyoma virus differing in history from the 2 clones Dr. Israel has focused upon, and that neither of the Benjamin clones has high lethality in $C_3H/BiDa$ mice. Dr. Benjamin's clone #2 in Table I was selected from our high tumor strain $2PTA_4$, as was Dr. Israel's clone #3 in Table I. There is therefore some overlap, but Dr. Israel has not used his clone #3 for genetic modification tests, as stocks from this clone did not appear to be sufficiently homogeneous.

The 2 clones in Dr. Benjamin's study have been characterized with respect to lethality, tumorigenicity, and rank order of organs developing tumors (Table I). He is currently developing recombinant cloned lines of virus to be bioassayed for effects of specific insertions and deletions.

Significance to Biomedical Research and the Program of the Institute: Although a great deal has been learned in the past 2 decades about the mechanism of action of polyoma virus, the terminal steps in viral activation of cell proliferation have not yet been identified and characterized in detail. These experiments take advantage of the availability of low and high tumor-inducing variants of the virus. By identifying the genetic differences relating to the corresponding biological differences, it should become possible to determine how the critical segment(s) of viral code, or their products, interact with cellular regulatory mechanisms to yield neoplasms. It is generally claimed that the T antigens, particularly middle T, coded for by PV are essential for tumor induction, but events downstream from T antigen appearance have not been clarified. I have postulated that for the epithelial PV tumors in mice there is a family of regulatory genes that are phylogenetically and developmentally closely interrelated and that are capable of activation by PV, either directly or via one of its protein products. The phylogenetic, developmental, and presumably molecular interrelatedness of the family of genes in point (ectodermal-endodermal

pharyngeal derivative genes) would explain why PV or its products interact with the specific group of organs (PV organ constellations) to yield epithelial tumors. This hypothesis is one of the products of many years of investigation under this project title. The experiments described above offer an approach to testing the hypothesis. If the critical viral component of the reaction system (viral genes or gene products + cellular genes or gene products $\longrightarrow$ neoplasms) can be identified, then the cellular components can be identified more easily. One of the long-standing objectives of the NCI Program has been to trace the molecular events in such a system until all the critical steps are known. Methods of intervention can then be developed and applied toward prevention and/or control of tumors in this experimental system.

II. Tracing the events of polyoma virus infection in mice at cytologic and histologic levels.

Studies in the past on the pathogenesis of polyoma tumors in mice have been published by the PI and others. These have provided essential information on the morphology and cell of origin of the various tumors, but are deficient with respect to providing a complete picture of the step by step events following laboratory infection of the newborn mouse. The previous works were limited by the then available technology and a lack of knowledge concerning variability among different virus preparations, dose-dependent differences in response, and host animal variations.

This project is a study at the light microscope level of the sequential changes that can be seen at 48 hour intervals starting at the second day after infection of newborn $C_3H/BiDa$ mice, and extending to the 18th day, a time at which lytic viral effects have begun to subside and development of some early tumors has begun. A small number of additional animals at ages 20 to 50 days will also be studied, as well as a group of animals bearing large tumors at all the organ sites from which polyoma tumors are known to arise. These mouse tissues are available as a by-product of Project I above, and will include sets of mice that received equal doses of 3 respective cloned virus lines, and uninfected controls. In each case adjacent tissue sections will be stained with H&E and with the immunoperoxidase technique, using rabbit antibody against the polyoma group antigen VP1 from disrupted SV40 virus. In preliminary work we have found this antibody preparation is very effective in localizing cells undergoing lytic effects of PV. Lytic cells are accurately identified by the PAP method as judged by correlation with virus titers obtained by the hemagglutination technique on live kidney tissue taken at comparable intervals after infection with the respective virus clones. Some 60 complete necropsies will be examined, requiring approximately 3000 histologic sections.

Observations already made indicate this study can achieve the following objectives:

1. Confirm the PV lytic causation of lesions previously only assumed to be caused by PV on the basis of changes observed in standard H&E-stained material. Examples where this has proved to be possible are lesions in renal cortex and medulla, salivary glands, pancreas, thyroid gland, thymus, tooth organ, hair follicles, testis, adrenal medulla, cardiac muscle, smooth muscle of arteries, and bone. Tissues where confirmation of PV lysis is still needed are

testis (Sertoli cells?), endometrium and myometrium, mesothelium of pleura, pericardium, and peritoneum, capillary endothelium in brain, and others.

2. Detect, because of the greater sensitivity of the PAP method, lytic lesions and/or the presence of PV antigens in cells where they were not previously suspected to be. New cell types already found to be infected or to contain viral antigen are: glial cells in brain, reticular cells in lymph nodes, Kupffer cells in liver, hepatic parenchymal cells, cartilage and precartilage, and megakaryocytes in spleen.

3. Identify those fully developed tumors in which PV continues to exist in infectious form or at least produces viral antigen.

4. Determine the changes that occur with respect to viral antigen production in lesions that appear to be part of a series of events transitional between lytic infection and neoplastic development.

5. Through a synthesis of the information in the above categories, to assemble a more accurate concept of the sequential events starting with spread of the initial infection and ending either in healing, persistent virus infection, or tumor development. For example, we have noted that during the first two weeks of infection with the highly lethal clone of virus, the kidneys are the site of very extensive lytic lesions and virus production. For this virus, kidney, thyroid, testis, and thymus may represent "booster sites", where virus production is amplified and virus is disseminated to other organs (via bloodstream and lymphatics). Kupffer cells in liver and macrophages in lymph nodes, spleen, and other tissues may be agents of virus removal, as they contain viral antigen in their cytoplasm. Lytic lesions in bone and cartilage appear to be sufficient to explain the "runting phenomenon", which has heretofore been cloaked in unnecessarily complicated theories. At the completion of the work, a correlation of lytic response with tumor development in virtually every specialized cell type in the mouse will be attempted. Information gathered so far indicates 1) that tumors do not develop from cell types that give no evidence of lytic infection; 2) many cell types that do show abundant lytic infection do not develop a neoplastic response (e.g., renal tubular epithelium, testicular Sertoli cells, pancreatic exocrine cells). Our working postulate is that in the mouse (though not in the hamster) lytic infection is essential, but not sufficient of itself, for neoplasia to follow. Our currently preferred concept is that only those cells in particular states of differentiation (phenotypic expression) are capable of emerging from the lytic condition and transforming to neoplastic condition. This capability relates, we further postulate, to a family of proliferation-regulating genes specifically in an activated state.

Significance to Biomedical Research and the Program of the Institute: The above concept under examination runs contrary to current dogma, which says that lytic infection and transforming infection are antithetical. The dogma appears to be correct for PV infection in hamsters and rats, which are not natural hosts of PV, and in which lytic infection rarely occurs. However, in the mouse, the natural host of PV, lytic infection is abundant and in some but not all lytic sites neoplasms can be seen emerging. If it can be established that lytic infection can, under special conditions as in the newborn or immune-suppressed

mouse, lead to neoplasia, it would seem that common lytic viral diseases in man should be investigated more thoroughly to see if they may not, under exceptional circumstances, lead to neoplasia. This already appears to be true for infection with the BK and JC human polyoma viruses, which have been associated with neoplasia in persons manifesting immune-deficiency states.

III. Characterization of polyoma tumors in mice by isozyme analysis.

This project in which Dr. Suzanne Fisher was the principal investigator was completed in October, 1981, and the results were reported last year. Two papers are in preparation. One is in 3rd draft and will be submitted to a journal very shortly. The other requires further rewriting.

Proposed Course: Although retiring on June 26, 1982, I intend to complete those parts of the above projects for which I am responsible. Some of the pathological work (necropsies) and materials (histological slides) will be completed by staff of NCI during the coming year, and I will complete interpretations at home (either in Bethesda or in Woods Hole, MA, where I plan to move).

Table I

Israel

Israel ($2PTA_5$)

Virus	$PLID_3F$		PA_3F		$PPTA_5F$	
	#1	%	#2	%	#3	%
Lethality in NB period (18d.)	High 39/51	76	Low 5/54	9	Low 6/59	10
Tumor-inducing activity	High 24/26 ($3x10^2$ dil.)	92	Low 2/48	4	High 47/53	89
Main tumor types	Tooth-19/26	73			Hair fol. 39/53	74
	Hair f. 16/26	62	Sal. g.-1	50	Thymus 37/53	70
	Renal s. 15/26	58	Mam.-1	50	Sal. 16/53	30
	Sal. 13/26	50			Mam. 6/53	11
	Mam. 3/26	12			Skin sarc. 6/53	11
	Thymus 2/26	8			Renal sarc. 4/53	8
	Adrenal 1/26	4			Bone 3/53	6
	Bone 1/26	4			Tooth 2/53	4
					Adrenal 2/53	4

Benjamin

Virus	P59RAF		$PPTA_4F$	
	#1	%	#2	%
Lethality in NB period (18d.)	Low 3/50 (3 non-viral)	0	Low 15/51 (6 non-viral)	18
Tumor-inducing activity	None 0/45	0	High 32/32	100
Main tumor types			Hair f. 32/32	100
			Thymus 26/32	81
			Sal. 23/32	72
			Mam. 20/32	62
			Renal s. 8/32	25
			Bone 5/32	16
			Tooth 3/32	9

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00538-17 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Identity of X Cells in "Tumors" of Fishes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.J. Dawe	Chief, Comparative Oncology Section - Med. Officer (Path.)	LP	NCI
OTHERS:	C.M. Poore	Biologist	LP	NCI
	A.J. Garvin	Surgeon	LP	NCI
	S.E. Fisher	Guest Worker	LP	NCI
	M.C. Habbersett	Biologist (Cytol.)	LP	NCI

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2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

X cell lesions (papillomas and pseudobranch tumors) have been reported in the literature to involve fishes of 3 Orders (Pleuronectiformes, Gadiformes, and Perciformes) covering about 40 species. For some 70 years these lesions have been considered neoplastic or pre-neoplastic. In imprint preparations in this study, X cells stained by Wright's, Feulgen, and a DNA-binding fluorescent dye (bisbenzimidazole), the features of the mitotic cycle of X cells were visualized in detail and found to be closely similar to those of hartmannellid amoebae. Mitoses of X cells differed markedly from mitoses of host fish cells. In multinucleated plasmodia-like forms, mitoses of the several nuclei in a given cell were synchronized. Isozyme studies revealed starch gel electrophoresis bands extractable from X cells but not from host fish cells, active in at least 4 to 6 enzyme systems. Flow fluorometric quantitation of DNA showed the X cell population to be sharply distinct from that of host fish cells, with X cell/fish cell DNA ratio of about 1:3. It was concluded that X cells are parasitic rather than neoplastic in nature.

Project Description

Objectives: The objective of the study of X cell lesions in fishes is to determine whether X cells are neoplastically or otherwise transformed cells of host origin, or are xenogenous cells of parasitic nature.

The methods used were described in last year's report and consist of 1) standard histological examination of lesions; 2) electron microscopy; 3) cytochemical procedures performed on sections and imprint preparations; 4) flow microflourometry for determination of relative quantities of DNA per cell; 5) isozyme analysis using 26 enzyme systems; 6) immunoperoxidase techniques.

Major Findings: Histological and ultrastructural characteristics of the pseudobranch "tumors" in Pacific cod were published in an earlier report (J. Natl. Cancer Inst. 59: 377-398, 1977), and the more recent observations are included in a report in press listed below. In summary, in imprint preparations of X cells stained by Wright's, Feulgen, and a DNA-binding fluorescent dye (bisbenzimidazole), the features of the mitotic cycle of X cells were visualized in detail and found to be closely similar to those of amoebae in the family Hartmannellidae. Mitoses of X cells differed markedly from mitoses of any known vertebrate. In multinucleated plasmodia-like forms, mitoses of the several nuclei in a given cell were synchronized, but did not take the form of multipolar figures, as in neoplasms.

Isozyme studies by Dr. Fisher revealed starch gel electrophoresis bands extractable from X cells but not from host fish cells, active in at least 4 to 6 enzyme systems. Flow fluorometric quantitation of DNA per cell showed the X cell population to be sharply distinct from that of host fish cells, with X cell/fish cell DNA ratio of about 1:3.

On October 12-23 the PI participated in a survey of diseases of fish in the North Sea. The survey was conducted aboard the F.S.S. Anton Dohrn, under the auspices of the West German National Fisheries Institute. Fish were taken by trawl net from 54 mapped sampling stations. X cell lesions were found in 12 Atlantic cod of approximately 1200 examined. Findings of interest were:

1. Cytologically the X cells in lesions in the pseudobranch region were identical to those previously studied in Pacific cod.

2. In 3 of the 12 fish with X cell lesions in the pseudobranch region, the thymus was also involved. Involvement of thymus had not previously been recognized in either Pacific or Atlantic cod.

3. In 1 cod with X cell lesions in pseudobranch and thymus, envelope cells were found to have Type A intranuclear inclusion bodies suggestive of virus infection. Electron microscopic examination (by H. Michelitch and Dr. Triche) failed to show a virus in X cells. It is believed that the presumptive viral infection of envelope cells represents coexistent but causally unrelated coinfection of this cod both with a virus and with X cells.

4. In several of the X cell-infected cod the lesions were in a state of regression associated with granulomatous reaction by the host. This parallels our previous observation of regression and granulomatous reaction to X cell lesions in Pacific cod.

5. All but 1 of the cod with X cell lesions came from a limited area within the German Bight. All were in the size group of 20-45 cm, the size at which cod approach or reach sexual maturity. These findings further support the impression that the disease is epizootic in nature and is related to habitat.

Significance to Biomedical Research and the Program of the Institute: X cell lesions have been reported in the literature to involve fishes of 3 Orders (Perciformes, Gadiformes, and Pleuronectiformes), covering approximately 40 species. For some 70 years these lesions have been considered to be neoplasms and much effort has been invested by cancer researchers. Although the life cycle and exact taxonomic position of X cells remain to be worked out, our findings leave no doubt that they are xenogenous organisms. They may remain of interest to cancer researchers to a limited degree, since they do appear to elicit a mild proliferative response (non-neoplastic) in the epithelia in which they reside.

This project will be concluded after completion of a final paper covering previously unreported findings described above.

Publications

1. Dawe, C.J.: Polyoma Tumors in Mice and X Cell Tumors in Fish, Viewed through Telescope and Microscope. In Dawe, C.J., Harshbarger, J.C., Kondo, S., Sugimura, T., and Takayama, S. (Eds.): Phyletic Approaches to Cancer. Proceedings of the 11th International Symposium of the Princess Takamatsu Cancer Research Fund, Tokyo, 1980. Tokyo, Japan, Japan Scientific Societies Press, 1981, pp. 19-49.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00533-09 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Study of Virus Cell Interaction and Biological Characterization of Human Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.C. Costa	Chief, Surgical Pathology & Postmortem Section	LP NCI
OTHERS:	A.S. Rabson	Acting Chief, Laboratory of Pathology	LP NCI
	C.L. Yee	Biologist	LP NCI
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COOPERATING UNITS (if any)

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4 1/2	3	1 1/2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Viruses belonging to the human herpesvirus family induce a receptor for the Fc portion of IgG during their lytic cycle. We are studying the receptors induced by herpes simplex virus in mammalian cells. We are also studying the induction of Fc receptors in lymphoblastoid cell lines by Epstein-Barr virus. Definition and characterization of the receptor will allow us to gain insights on the possible biological role of the receptor during lytic infection.

We are also studying biological properties of human tumors carried in nude mice and of tissue culture cell lines derived thereof. The synthesis of specific tumor products that serve as markers and their regulation is being studied.

Project Description

Virus-cell Interaction

Studies on the induction of Fc receptors by herpes simplex virus show that IgG protein A complexes can specifically interact with a glycoprotein present in viral infected cells and absent in control uninfected cells. This glycoprotein interacts with mouse and rabbit IgG independently of the specificity of the antigen combining site. This glycoprotein can be partially purified by immunoprecipitation and affinity chromatography. Analyzed on SDS polyacrylamide gel electrophoresis, the protein has an apparent molecular weight of 120,000 daltons.

Protein A IgG complexes appear to bind to herpes infected cells with increased avidity when compared to IgG in its monomeric state.

Biology of Human Tumors

Studies of human xenografts in nu/nu mice have shown three types of interaction of murine viruses or viral-like agents with the xenografts. 1) These types are induction of endogenous murine virus in the tumor stromal cells. 2) Replication of C-type virus, presumably murine, in human tumor cells. 3) Replication of intercisternal A particles (IAP) in human cells. Studies of one isolate of a C-type virus replicating in a transplantable human chondrosarcoma indicate that the virus is an endogenous NIH duotropic C-type particle. There is no indication that human DNA has been incorporated in the genome of the replicating C particle.

Publications

See Report Z01 CB 00853-29 LP for publications.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00542-05 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on Oncogenic Primate DNA Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Howley	Senior Surgeon	LP	NCI
OTHERS:	M.-F. Law	Expert	LP	NCI
	S.P. Schlegel	Expert	LEP	NCI

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study involves the characterization of the primate polyomaviruses BK, JC, SV40, STMV, and SA12. In addition, zur Hausen has recently identified a lymphotrophic polyomavirus in African Green monkeys and serologic evidence suggests that a human lymphotrophic 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 human 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.

Project Description

Objectives: Our recent efforts have focused in two directions. The first has been concerning the JC virus which is difficult to propagate in tissue culture. We have molecularly cloned the genome of the prototype Mad-1 strain of JC virus in E. coli using the plasmid vector pBR322. In addition, we have recently cloned the entire genome of wild-type JC strains directly from PML brains. We have compared these wild-type strains with the laboratory Mad-1 strain and mapped the regions of variance. These studies are designed to understand the genetic organization of the genome and to evaluate the role of genetic variance in the tissue specificity and pathologic properties of this virus. Our second area of interest 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.

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

Major Findings: 1. We have purified JC DNA directly from two PML brains utilizing the differential salt precipitation technique of Hirt. The DNA present in these two brains is quite homogeneous in size. 2. These two new viral DNAs have been cloned into pBR322 and have been mapped utilizing restriction endonucleases and found to be similar in their structure to the wild-type Mad-1 strain. 3. Areas of variance among these genomes have been mapped using multi-cut restriction endonucleases and have been found to map in the non-coding region to the late side of the origin of replication. These findings have been published in the Journal of Virology 4. Human epidermal cells have been transformed by wild-type SV40 DNA and by the DNA of a 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 an 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°C), however, the tsA209 transformed cells revert to a normal phenotype in terms of morphology, growth parameters, and differentiative properties.

Significance to Biomedical Research and the Program of the Institute: JC virus is a known human pathogen and is capable of transforming a variety of rodent cells. A better understanding of the molecular biology of this virus will allow an evaluation of whether this virus has a role in human neoplasia. Most viral transformation systems have been done on fibroblasts and the ability to use SV40 and its conditional mutants to transform human epithelial cells provides a mechanism to study the properties of malignantly-transformed epithelial cells.

Proposed Course: 1. To further study the genetic organization in control regions of the JC viral genome. 2. To use 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

1. Rentier-Delrue, F., Lubiniecki, A., and Howley, P.M.: Analysis of JC virus DNA purified directly from human PML brains. J. Virol. 38: 761-769, 1981.

2. Howley, P.M.: Papovaviruses - Search for Evidence of Possible Association with Human Cancer. In Phillips, L.A. (Ed.): Viruses Associated with Human Cancer. New York, Marcel Dekker, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00543-04 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Characterization of the Papillomaviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	N. Sarver	Staff Fellow	LP	NCI
	Y.C. Yang	Visiting Fellow	LP	NCI
	S.M. Rosenbaum	Visiting Fellow	LP	NCI
	D.R. Lowy	Medical Director	D	NCI
	S.P. Schlegel	Expert	LEP	NCI

COOPERATING UNITS (if any)

Dermatology Branch, NCI; Laboratory of Experimental Pathology, NCI

LAB/BRANCH
Laboratory of Pathology

SECTION
Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

There are currently recognized to be 12 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 fibropapillomas 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.

Project Description

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 complete sequencing of the BPV-1 genome. 6. The analysis of the viral mRNAs expressed in BPV-1 transformed and in productive fibropapillomas excised from cattle. 7. Analysis of the "early" papillomavirus products expressed in BPV-1 transformed cells. 8. An analysis of the viral specific products required for the extrachromosomal replication of papillomavirus genomes in persistently-infected cells. 9. A determination of the cis and transfunctions 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. In collaboration with Elsen Chen, Peter Seeburg, and Art Levinson, all of Genentech Corporation, the complete sequence of the BPV-1 genome has been determined. The genome consists of 7,945 base pairs. All of the open-reading frames are found from one strand. A paper has been submitted to Nature with these results.

2. Dr. Law has established that mouse cells transformed by the bovine papillomavirus type 1, its cloned DNA, or its cloned subgenomic transforming region, all contain extrachromosomal plasmid DNA molecules. He has ruled out integration of a portion of the viral genome by a reconstruction experiment. This data has been published in PNAS.

3. Dr. Heilman has mapped the viral transcripts present in bovine papillomavirus transformed cells. She has localized 5 transcripts whose 3' ends all map at 0.34 map units. A polyadenylation site (AATAAA) is localized at this point in the genome. The 5 transcripts are colinear and the 5' ends of the bodies map at .47, .485, .54, .785, and .85 map units. Within the 69% transforming region, 2 "TATA" boxes are localized at .87 map units and .98 map units. These TATA boxes are diagnostic of the eukaryotic promoter and may serve as the promoter for this early transcriptional unit. Additional or authentic promoter sites localized elsewhere in the genome, however, have not been ruled out. In addition, Dr. Heilman and Ms. Engel have mapped 2 species of RNA which they can purify from productive bovine fibropapillomas. These also have a common 3' end at .98 map units, and they are currently mapping the 5' ends of the bodies of these messages as well as the promoters active in the synthesis of these messenger RNAs. These messenger RNAs have been shown to encode the major capsid protein of the bovine papillomavirus by in vitro translation of the mRNA into the 53 K VP1 protein following selection of the messenger RNA to a fragment of DNA containing the late region of the bovine papillomavirus.

4. Because of the unique plasmid nature of the BPV-1 genome in transformed mouse cells, we examine 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 were done in collaboration with Dr. Douglas Lowy and Dr. Israel Dvoretzky of the Dermatology Branch, as well as with Dr. Robert Friedman of the Department of Pathology at the Uniformed Services University for Health Sciences. It was 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 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, it was found that in the ID14 cell line approximately 10% of the individual colonies now had a flat morphology and in the ID13 cell line approximately 1.2% of the colonies had a flat morphology. Independent colonies were grown up and shown morphologically to be quite similar to the non-transformed C127 cells in appearance. In addition, these cells were no longer anchorage-independent as their transformed counterparts are, and were susceptible to retransformation by the bovine papillomavirus. Analysis of the DNA within these cells show that they had been "cured" of all viral DNA sequences.

Publications

1. Law, M.-F., Lowy, D.R., Dvoretzky, I., and Howley, P.M.: Mouse cells transformed by bovine papillomavirus contain only episomal viral DNA sequences. PNAS 78: 2727-2731, 1981.

2. Heilman, C.A., Engel, L.W., Lowy, D.R., and Howley, P.M.: Virus-specific transcription in bovine papillomavirus-transformed mouse cells. Virology 119: 22-34, 1982.

3. Howley, P.M.: The human papillomaviruses. Arch. Pathol., in press.

4. 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 Merrigan, 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, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00547-02 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Use of Papillomavirus DNAs as Eukaryotic Cloning Vectors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.M. Howley	Senior Surgeon	LP NCI
OTHERS:	N. Sarver	Staff Fellow	LP NCI
	M.-F. Law	Expert	LP NCI
	S.M. Rosenbaum	Visiting Fellow	LP NCI

COOPERATING UNITS (if any)

Laboratory of Molecular Virology, NCI

LAB/BRANCH
Laboratory of Pathology

SECTION
Viral Oncology and Molecular Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 cloned the rat preproinsulin gene together with the transforming region of the bovine papillomavirus and assessed the ability of the papillomaviruses to be used as cloning vectors. Recently, 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.

Project Description

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 selectible 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 selective 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 selective 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, DNA sequencing, and DNA transfection.

Major Findings: 1. We have demonstrated the utility of the bovine papillomavirus transforming segment as a eukaryotic cloning vector. We have hooked up the 69% transforming segment of the BPV-1 to a 1.6 kb segment of the rat preproinsulin gene. We cloned this hybrid DNA into pBR322. After cleaving the DNA away from the prokaryotic sequences, we have taken the hybrid DNA and used it to transfect susceptible mouse cells. The 48 independent clones have been studied and each has been shown to contain the BPV-rat preproinsulin DNA hybrid and that each of these is synthesizing rat preproinsulin messenger RNA and rat proinsulin which is secreted into the media. These data have been published in references 1 and 2 below. These studies were done in collaboration with Drs. Peter Gruss and George Khoury of the Laboratory of Molecular Virology.

2. Dr. Law has cloned the E. coli GPT gene arranged in a modified SV40 early transcriptional unit into a BPV_{69T}-pBR322 hybrid. In collaboration with Bruce Howard of the Laboratory of Molecular Biology, he has used this DNA to transform susceptible mouse cells and select the transformants either for their malignant phenotype or for their ability to survive in selective media containing mycophenolic acid. Dr. Law has shown that the mouse cells selected for their malignant phenotype are capable of growing in selective media. In addition, the mouse cells selected for their ability to grow in the otherwise poison media demonstrate a malignant phenotype. Thus, the two phenotypic markers are closely linked. One problem that became obvious in the analysis of the DNA in these transformants, is that 34 out of 36 of the lines contained hybrid DNA which had undergone some rearrangement and/or acquisition of sequences. Two of the lines appear to contain the input DNA in an unmodified state. A manuscript describing these studies is in press in a book entitled Viral Vectors, to be published by Cold Spring Harbor Laboratory.

3. Dr. Sarver has completed a study in which she has shown that the complete BPV genome cloned into a deletion derivative of pBR322 called pML2. It is capable of transforming mouse cells at high efficiency. She had previously shown that the complete pBR322 sequences are inhibitory to BPV transformation when they are cloned into either the 69% transforming region or into the complete BPV-1 genome. The 69% fragment when cloned into pML2 is not able to transform mouse cells at high efficiency. The sequences which are deleted from pBR322 in the pML2 molecule, are sequences which are inhibitory to the replication of covalently-linked SV40 sequences in monkey cells. Thus, these same sequences are inhibitory to the sis replication of BPV sequences in mouse cells. Dr. Sarver has shown that the BPV-pML2 hybrid DNA is present in an unmodified form in the mouse cells and that this can be rescued out of the eukaryotic cells using the Hirt supernatant when transfected back onto susceptible E. coli. Thus, the BPV-pML2 is a useful vector for shuttling defined sequences between animal cells and prokaryotic cells.

4. While the BPV_{69T}-pML2 DNA does not transform mouse cells at high efficiency, a number of eukaryotic DNA inserts when cloned into this molecule do facilitate the transformation of mouse cells. Drs. Sarver and Rosenbaum in the laboratory have shown that a 5.3 kb genomic segment containing the rat preproinsulin DNA, a 2.6 kb genomic sequence containing the human growth hormone gene, and a 4.5 kb segment of rat genomic DNA between the alpha and gamma rat fibrinogen genes, is capable of providing this facilitory function. This function is similar to the one that has been defined by Drs. Maniatis and DiMaio at Harvard for the human beta hemoglobin gene. Studies are under way to determine what the nature of this biologic facilitation is at a molecular level.

Publications

1. Sarver, N., Gruss, P., Law, M.-F., Khoury, G., and Howley, P.M.: Bovine papillomavirus DNA - a novel eukaryotic cloning vector. Molec. Cell. Biol. 1: 486-496, 1981.

2. Sarver, N., Gruss, P., Law, M.-F., Khoury, G., and Howley, P.M.: Rat Insulin Gene Covalently Linked to Bovine Papillomavirus DNA as Expressed in Transformed Mouse Cells. In Brown, D., and Fox, C.R. (Eds.): ICN-UCLA Symposia on Molecular and Cellular Biology. Developmental Biology Using Purified Genes. New York, Academic Press, 1981, Vol. XXIII, pp. 547-556.

3. 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. Viral Vectors, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., in press.

4. Howley, P.M., Sarver, N., and Law, M.-F.: Eukaryotic cloning vectors derived from bovine papillomavirus DNA. In Wu, R., Grossman, L., and Moldave, E. (Eds.): Methods in Enzymology. New York, Academic Press, Inc., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00546-02 LP

PERIOD COVERED
October 1, 1981 through December 31, 1981

TITLE OF PROJECT (80 characters or less)

Specific Incorporation of a Fluorescent Probe by Transformed Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L.M. Neckers	Expert	LP	NCI
OTHERS:	J. Cossman	Sr. Assistant Surgeon	LP	NCI
	E.S. Jaffe	Chief, Hematopathology Section	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0		

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been discontinued. We have observed that merocyanine 540 is not specifically taken up by leukemic lymphocytes. Its rate of uptake is correlated to metabolic state of the cell. Thus, mitogen stimulated lymphocytes take up the dye as well. The dye appears to concentrate in mitochondria of rapidly growing or metabolizing cells. Since other markers exist for these populations, we have discontinued this project.

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00548-02 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Monoaminergic Receptors on Lymphocytes Visualized by FACS Analysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: L.M. Neckers Expert LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In the last year, we have obtained monoclonal antibodies to the beta-adrenergic receptor and have demonstrated the presence of these receptors on lymphocytes using the fluorescence-activated cell sorter. We have also obtained antisera to opiate receptors and have been unable to show their presence on lymphocytes using similar techniques. We are currently attempting to study cholinergic receptors on lymphocytes and lymphoblastoid cell lines and to make an antibody to the cholinergic receptor.

Project Description

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

Methods Employed: Fluorescent derivitization 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.

SMITHSONIAN SCIENCE INFORMATI EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00550-02 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immunologic Characterization of Malignant Lymphomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.S. Jaffe	Chief, Hematopathology Section	LP	NCI
OTHERS:	J. Cossman	Sr. Assistant Surgeon	LP	NCI
	R.I. Fisher	Senior Investigator	M	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

Project Description

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, acid phosphate, tartrate resistant acid phosphatase, alpha-napthyl butyrate esterase, acid alpha-napthyl acetate esterase, alkaline phosphatase, beta-glucuronidase.

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.

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

1. Jaffe, E.S., Strauchen, J.A., and Berard, C.W.: Predictability of immunologic phenotype by morphologic criteria in diffuse aggressive non-Hodgkin's lymphomas. Am. J. Clin. Pathol. 77: 46-49, 1982.

2. Jaffe, E.S., Smith, S.A., Magrath, I.T., Freeman, C.B., Alabaster, O., and Sussman, E.H.: Induction of complement receptors in human cell lines derived from undifferentiated lymphomas. Lab. Invest. 45: 295-301, 1981.

3. 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, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT C HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00551-02 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Stimulation of Phagocytosis by a Peripheral T-Cell Lymphoma-derived Lymphokine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.S. Jaffe	Chief, Hematopathology Section	LP	NCI
OTHERS:	C.R. Simrell	Resident	LP	NCI
	J. Cossman	Sr. Assistant Surgeon	LP	NCI
	L.M. Neckers	Expert	LP	NCI
	G. Crabtree	IPA	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Certain patients with malignant lymphomas originating from peripheral T cells develop a rapidly fatal syndrome which mimics malignant histiocytosis. It is suspected that the pathogenetic mechanism of this phenomenon may involve a lymphokine produced by the neoplastic T cell which can stimulate the phagocytic cells of the reticuloendothelial system. In order to test this hypothesis, neoplastic cells from fresh biopsies of patients with malignant lymphoma are placed in overnight culture, and supernatants are tested for the presence of soluble factors which are able to affect human phagocytic cells in vitro.

Project Description

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 u 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.

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 reticuloendothelial 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: Continued screening of malignant lymphoma patients to accumulate more cases of factor producing peripheral T-cell lymphoma, attempts to establish a cell line from these cases, preliminary characterization of the factor(s) involved.

Publications

1. 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, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00552-02 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Malignant Lymphomas: Analysis with Monoclonal Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Cossman	Sr. Assistant Surgeon	LP	NCI
OTHERS:	L.M. Neckers	Expert	LP	NCI
	E.S. Jaffe	Chief, Hematopathology Section	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A variety of monoclonal antibodies (hybridomas) have been recently developed that distinguish among classes of normal human lymphocytes and identify discrete stages of differentiation. In addition new monoclonal antibodies against human lymphocytes are being developed in our laboratory. We are using a battery of these antibodies to determine the phenotypes of human malignant lymphomas using a Fluorescence Activated Cell Sorter (FACS-II). 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.

Project Description

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, human neutrophils and a human promyelocytic cell line.

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 antibody. 2. Produce specific anti-human lymphocytic monoclonal antibodies. 3. Produce antibodies specific for primitive differentiation antigens. 4. Determine the inducibility of lymphomas to a more differentiated stage using phorbol esters and other inducing agents. 5. Examine the biosynthesis of CALLA in human B-cell lymphomas and granulocytes.

Publications

1. Cossman, J., and Jaffe, E.S.: Distribution of complement receptor subtypes in non-Hodgkin's lymphomas of B-cell origin. Blood 58: 20-26, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 00553-02 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Control of Fibrinogen Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.R. Crabtree	IPA	LP NCI
OTHERS:	J.A. Kant	Expert	LP NCI
	A.J. Fornace	Expert	LP NCI
	D.M. Fowlkes	Sr. Asst. Surgeon	LP NCI

COOPERATING UNITS (if any)

Laboratory of Biochemistry, NCI

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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. 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 by study of the structure and nucleic acid sequence of the three mRNAs and the genes which give rise to the three fibrinogen chains. We have also begun studying the human afibrinogenemias and dysfibrinogenemias by cloning and examining the structure of the human fibrinogen genes.

Project Description

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.

Major Findings: We have found that mRNA levels for fibrinogen increase 10-38 fold following defibrination. This response may be mediated by a feedback mechanism involving fibrin split products. We have mapped the genes for the α and γ chains of fibrinogen and find they are tightly linked 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: In the future we hope to look for homologous regulatory 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

1. Crabtree, G.R., and Kant, J.A.: Molecular cloning of cDNA for a family of coordinately-regulated genes; the α, β, and γ chains of fibrinogen. J. Biol. Chem. 256: 9718-9723, 1981.

2. Crabtree, G.R., and Kant, J.A.: Coordinate activation of the rat fibrinogen genes by the venom of the Malayan pit viper and glucocorticoids. J. Biol. Chem., in press.

3. Crabtree, G.R., Fowlkes, D.M., Fornace, A.J., Rauch, C.A., and Kant, J.A.: Regulation and structure of the rat fibrinogen gene. Ann. N.Y. Acad. Sci. (invited review to be published in 1982).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00880-01 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Malignant Lymphomas and Hypercalcemia: Analysis with Antibody Against OAF

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.M. Braziel	Medical Staff Fellow	LP	NCI
OTHERS:	E.S. Jaffe	Chief, Hematopathology Section	LP	NCI
	J. Cossman	Sr. Assistant Surgeon	LP	NCI
	S.M. Hsu	Medical Staff Fellow	LP	NCI

COOPERATING UNITS (if any)

Dr. Richard Luben, University of California, Riverside, California

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A lymphokine, called osteoclast activating factor (OAF), has been identified by bioassay, and is postulated to be the cause of hypercalcemia in some hematologic malignancies. A monoclonal antibody to OAF has been produced, and we are using this antibody, in conjunction with immunoenzymatic staining techniques, to determine the role of OAF in the hypercalcemia seen in a significant number of T-cell lymphoma patients. This antibody will also be used to investigate the production of OAF by other normal and neoplastic lymphoid cells.

Project Description

Objectives: To determine the role of OAF in the development of hypercalcemia of T-cell lymphoma patients.

Methods Employed: Immunohistochemical staining; tissue culture; immunologic characterization of lymphoid cells.

Major Findings: We confirm the bioassay findings that OAF is produced by stimulated normal lymphocytes, and some human malignant lymphoid cell lines. Smaller amounts of OAF are present in unstimulated normal lymphocytes.

Significance to Biomedical Research and the Program of the Institute: Increased understanding of the etiology of hypercalcemia in patients with hematologic malignancies would affect treatment of these patients, and possibly patients with other bone-destroying diseases. Hypercalcemia also appear to be a frequent feature of T-cell malignancies associated with HTLV - a unique human retrovirus. The role of HTLV in lymphocyte activation and associated production of OAF and other lymphokines will also be investigated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00881-01 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Cell Growth by Transferrin Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L.M. Neckers	Expert	LP	NCI
OTHERS:	J. Cossman	Sr. Assistant Surgeon	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

All cells studied to date require transferrin for growth. We and others have shown that antibodies to the transferrin receptor block the growth of lymphoblastoid cell lines. In mitogen-stimulated lymphocytes, these antibodies block proliferation. We are studying the processes which regulate the appearance of these receptors in lymphocytes and lymphoblastoid cell lines, and the function of these receptors in cell growth and metabolism.

PHS-6040

Project Description

Objectives: To discern the regulation of transferrin receptor appearance in mitogen-stimulated peripheral blood lymphocytes 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. 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 antireceptor antibody.

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: To continue to study the role of monocytes in transferrin receptor expression in mitogen-stimulated lymphocytes and the reasons why lymphoblastoid cell lines constantly express the receptor with no monocyte requirement.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00882-01 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Use of the Cell Sorter to Clone Hybridoma Cells Secreting Idiotype

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L.M. Neckers	Expert	LP	NCI
OTHERS:	J.B. Trepel	Bio Lab Tech	I	NCI
	D. Sachs	Section Chief, Transplantation Biology	I	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.5	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

Project Description

Objectives: To be able to rapidly identify and clone idiotype-secreting hybridomas from a mixed population of hybridoma cells.

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.

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-idiotype 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-idiotype. Production of relevant antibody would thus be greatly speeded up.

Proposed Course: To try this approach in other systems to see if our technique works for other antigens.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00883-01 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Malignant Lymphomas: Analysis with Monoclonal Antibodies on Tissue Sections

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.-M. Hsu	Medical Staff Fellow	LP	NCI
OTHERS:	E.S. Jaffe	Chief, Hematopathology Section	LP	NCI
	J. Cossman	Sr. Assistant Surgeon	LP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Hematopathology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A variety of monoclonal antibodies (hybridomas) have been recently developed that distinguish among classes of normal human lymphocytes and identify discrete stages of differentiation. In addition, new monoclonal antibodies against human B cells are being developed in our laboratory. 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.

Project Description

Objectives: To identify and characterize human malignant lymphomas.

Methods Employed: Immunohistochemistry; 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.

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 antibody. 2. Produce specific anti-human B-cell monoclonal antibodies. 3. Produce antibodies specific for primitive differentiation antigens.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00517-41 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Report from the Pathological Technology Section

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: B.J. Coolidge Chief, Path. Tech. (Supv. Bio. Lab. Tech. (Micro) LP NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Pathology

SECTION
Pathological Technology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	0	5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

October 1, 1981 - September 30, 1982

Number of investigators	86
Number of pieces of tissue	35,635
Number of bottles of tissue	10,210
Number of blocks cut	20,779
Number of blocks cut serially	672
Number of frozen blocks cut	50
Number of blocks recut	841
Number of slides stained H & E	45,492
Number of slides stained special	2,447
Number of unstained slides	20,267
Number of slides, H & E, special and unstained	68,206

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00885-01 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Automated 2D Gel Analysis Using Computerized Data Base Methods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.F. Lemkin	Computer Scientist, Image Processing Sec.	LP	NCI
OTHERS:	L.E. Lipkin	Chief, Image Processing Sec.	LP	NCI
	M.L. Schultz	Senior Engineer, Image Processing Sec.	LP	NCI
	E.M. Smith	Expert, Image Processing Sec.	LP	NCI
	S.S. Thorgeirsson	Chief, Lab. Carcinogen Metab.	LCM	NCI
	P.J. Wirth	Research Fellow	LCM	NCI
	R. Maguire	Research Fellow	LCM	NCI
	R.P. Howard	Expert, Malaria Sec.	LPI	NIAID
	S. Aley	Research Fellow, Malaria Sec.	LPI	NIAID

COOPERATING UNITS (if any)

NCI, Div. Cancer Treatment, Lab. Carcinogen Metabolism; NIAID, Lab. Parasitic Disease; University of Chicago, Dept. of Med. (E.P. Lester, Asst. Prof. Med.)

LAB/BRANCH
Laboratory of Pathology

SECTION
Image Processing Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.4	2.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 gels prepared by our collaborators. Systems yielding significant differences include the HL60 cell line, Friend virus infected murine erythroleukemia cells (both of which show differences depending on whether an erythroid or non-erythroid direction is taken by chemical induced differentiation) as well as the differences in red cell surface proteins correlating with partial immune response to Plasmodium knowlesi. A series of differences in protein synthetic rates which serve to distinguish T-cell lines from a B-cell line has also been demonstrated.

Additional refinement of the component algorithms is expected to render GELLAB even more effective a tool for cell biological investigation.

Project Description

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 congeneric spots in 2D gels is complicated by local inhomogeneities 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 with establishing some local intergel 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 for congeners. The keystone of this analysis is the R-gel, the representative gel image. This acts as the major framework to link spots across the set of gels. It is the referent 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

1. Lemkin, P.F., and Lipkin, L.E.: GELLAB: A computer system for two-dimensional gel electrophoresis analysis. III. Multiple two-dimensional gel analysis. Comput. Biomed. Res. 14: 407-446, 1981.

2. Lemkin, P.F., and Lipkin, L.E.: GELLAB: Multiple 2D Electrophoretic Gel Analysis. In Allen, R.C., and Arnaud, P. (Eds.): Electrophoresis '81. Berlin, Walter de Gruyter & Co., 1981, pp. 401-411.

3. 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.

4. Lemkin, P.F., and Lipkin, L.E.: Data Base Techniques for 2D Electrophoretic Gel Analysis. In Geisow, M.J., and Barrett, A. (Eds.): Computing in Biological Science. Amsterdam, Elsevier/North Holland, 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00886-01 LP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Analysis and Synthesis of Nucleic Acid Secondary Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.A. Shapiro	Computer Scientist, Image Processing Sec.	LP	NCI
OTHERS:	L.E. Lipkin	Chief, Image Processing Sec.	LP	NCI
	M.L. Schultz	Senior Engineer, Image Processing Sec.	LP	NCI
	E.M. Smith	Expert, Image Processing Sec.	LP	NCI
	J.V. Maizel	Chief, Molec. Structure Sec.	LMG	NICHD
	R. Dhar	Visiting Scientist	LDNATV	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:	PROFESSIONAL:	OTHER:
1.7	1.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The 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 the nucleic acid molecule, 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 structure by removal of overlapping elements, without disturbing base relationships. Exploration of algorithms which will measure non-simple similarity has also begun. 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, and which will eliminate the ambiguities of the electron-micrographic images of the partially denatured nucleic acid molecules.

Project Description

The observation of various features of nucleic acid molecules such as hairpin turns, bulge loops, internal loops and multibranch loops which recur in a relatively constant position is indicative of specific structures of given lengths within the molecules. These structures are believed to have functional significance for the expression of genetic information.

The analysis of nucleic acid structure as shown in EM photographs is quite complex. After image acquisition via our real time image processor (RTPP) considerable preprocessing is necessary, especially to remove shading errors. The latter is largely completely accomplished by means of the DC notch filter technique improved in this laboratory for this purpose. The images may then be segmented, that is the molecules can be separated from background and larger "noise" fragments. Next, the contour of each molecule is extracted following which the extracted contour is smoothed. We have developed algorithms to "describe" such irregular shapes, and there are those which facilitate the finding of the tips of the hair pin features. Thus, a series of features has been derived which can represent the secondary structure visible in the denatured images subject to EM. If secondary structure maps are to be automatically generated, a molecular skeleton must be generated from the contour to provide a base line for the measurement of feature position.

The converse of the foregoing is that, given the sequence data compute the most energetically stable molecular structure. By using the well-known rules for base pairings, it is possible to generate a list of all "potential" regions of a sequence. A region is represented by a 4 element set consisting of the 5' starting base position, the 3' ending base position, the size of the region and the stabilizing energy of the region (derived from published tables. Nucleic acid sequences of about 600 bases may produce 9000 potential regions, so that it is clear that the combinatoric difficulties increase rapidly with molecular size, placing both a computational and storage burden on any machine. Workers such as Salser have published data from which the unpaired bases which may form loops of various kinds may be calculated, so that given sufficient resources and time, an optimum (free energy minimizing) structure may de derived.

The combinatoric complexity of computing secondary structure from sequence data, employing thermodynamic data available on various base pairs and sequences verges on the practically intractable. However, we have made significant inroads on selected aspects of this problem in collaboration with Drs. J. Maizel (Child Health) and R. Dhar (Laboratory of DNA Transforming Viruses). The initial programs required run times in proportion to some exponential power of the number of bases, so that even small fragments of a few hundred bases would require months of CPU time. Bruce Shapiro has adapted a program so that only cubed time is required. Addressing characteristics of DECsystems have limited the number of bases in a molecule to a few hundred. Our improvements to the core program now allows molecules of 420 bases to be run. We have further developed the algorithm so that much larger molecules can be run using a disk paging technique. Molecules of 1500 or more bases now are possible. However, the resources consumed by such programs are almost prohibitive.

An additional molecular drawing program was developed which has some interesting advantages over the drawing program developed previously. This new program eliminates the problem of overlap of complex structures which were topologically inherent in the older algorithm. Now the output of the secondary structure predicting programs can be viewed without the intervening, interactive untangling process. Both programs have been used quite successfully by Dr. Maizel.

The drawings that are produced from the new program also contain the inherent facility for secondary structure comparisons amongst different sequences. This makes it possible to do higher order similarity determinations beyond the level of simple sequence data. A circle graph algorithm was also developed that permits similarity comparison but from a different viewpoint.

Collaboration with Dr. Ravi Dhar on the examination of the secondary structure of a Harvey sarcoma virus sequence showed that the start codon of 3 mRNAs were in position of high destabilization, perhaps making them accessible for expression.

Our ability to handle larger molecules is the basis for an extension of our collaboration with Dr. Maizel. A 14000 base fragment of adenovirus mRNA is being examined for secondary structure in an overlapping piecewise manner. Comparisons are being made between the predicted structures with EM studies of the same portion of the molecule. The results of this experiment will be significant in regard to the eventual merger of the analysis and synthesis techniques of the secondary structure problem.

Publications

1. Shapiro, B.A., Maizel, J., and Lipkin, L.E.: Computer analysis and synthesis of nucleic acid secondary structure. 4th Annual World Conference for Medical Informatics, Strasbourg, France, April 1981, in press.

2. Shapiro, B.A., and Lipkin, L.E.: Nucleic Acid Morphology: Analysis and Synthesis. In Geisow, M.J., and Barrett, A. (Eds.): Computing in Biological Science. Amsterdam, Elsevier/North Holland Biomed. Press, in press.

ANNUAL REPORT OF THE METABOLISM BRANCH

SUMMARY OF SIGNIFICANT ACTIVITIES

NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

The clinical research program of the Metabolism Branch is directed toward two major goals. The first is to define host factors that result in a high incidence of neoplasia. In this area, a broad range of immunological investigations are carried out in patients with malignancy, in patients with immune deficiency diseases and a high incidence of neoplasia as well as in experimental animal systems to determine the nature of immune processes that are of importance in the surveillance against neoplastic cells and to define the nature of defects in the immunological system that result in an increased incidence of neoplasia. Major efforts in this area are also directed toward: 1) Defining events of cellular maturation, cellular interaction and cellular biosynthesis involved in the normal circulating antibody response. Specific emphasis is placed on studies of the immunoglobulin genes and their rearrangements and deletions that control immunoglobulin synthesis and on the action of immunoregulatory cells including helper T cells, suppressor T cells and macrophages that regulate antibody responses and on studies of disorders of these immunoregulatory cell interactions in patients with immune dysfunction. 2) Identification of unique cell surface determinants on subpopulations of lymphoid cells with different functional capabilities using antibodies developed with hybridoma technology. 3) The genetic control of the immune response especially as related to the immune response genes associated with the major histocompatibility complex. 4) Studies of the mechanism of action of various cytotoxic cells and their contribution to host defense. (5) The isolation and characterization of biological modifiers that suppress the human immune response that are produced by T cell lines and T-T cell hybridomas. The second major goal of the Branch is to determine the physiological and biochemical effects that a tumor produces on the metabolism of the host. Both patients with neoplastic disease as well as those with non-neoplastic disorders that facilitate the development of techniques for the study of cell membranes, homeostatic mechanisms and metabolic derrangements of biochemical control mechanisms are being investigated. Within this area a special emphasis is laid on the biochemical events accompanying normal growth and the hormonal control of this growth as it relates to our understanding of malignant growth and on the metabolism of proline and the porphyrins.

Immunoregulatory Cell Interactions in Immune Response

A major effort of the Metabolism Branch over the past few years has been directed toward defining the major events of cellular differentiation, cellular interaction and cellular biosynthesis involved in the specific circulating immune response. These studies have placed special emphasis on defining the defects of B cell maturation and of regulatory T cell and macrophage interaction with B lymphocytes and plasma cells that occur in patients with immunodeficiency diseases associated with a high incidence of malignancy,

in patients with autoimmune disorders as well as in patients with malignancies of the T or B lymphocyte systems. Overall these studies were directed at defining the factors in normal and abnormal states controlling the production of antibodies and the synthesis of the immunoglobulin molecules. Cells that ultimately produce antibodies undergo sequential maturation from stem cells in the marrow to B lymphocytes and then from B lymphocytes into immunoglobulin synthesizing plasma cells.

As a stem cell matures into a B cell there is a well orchestrated rearrangement of the genes coding for immunoglobulin molecules. The human immunoglobulin light chain genes in their embryonic or germ line state are organized in a discontinuous system of multiple germ line variable (V_L) regions, alternative joining (J_L) segments and a single or even multiple constant (C_L) regions. Heavy chains are similarly organized, but have an additional diversity (D_H) segment incorporated between the V_H and J_H regions. During the process of differentiation of a stem cell into a mature B cell, there is a rearrangement of the cellular genome to combine a single V_H with a single D_H and a single J_H to form an active heavy chain gene. Subsequently, a single light chain variable region (V_K or V_L) combines with an appropriate J_K or J_L region to activate a light chain gene. ^{32}P-labelled clones of human constant, joining, diversity and variable region genes were used as probes to study the gene arrangements in T cell, B cell and non-T/non-B forms or human lymphocytic leukemia or cell lines. Genomic DNA was extracted from these cells, digested with the restriction endonucleases known to demonstrate both rearranged and germ line alleles and hybridized with the probes. A single germ line C_{kappa} region gene was found to be present on a 12.0 Kb Bam Hl fragment in all non-B cells. All kappa expressing B leukemic cells and lines demonstrated at least one kappa gene rearrangement when compared to the germ line configuration. The second C_{kappa} allele remained in the embryonic configuration in 5% of the cases, but showed a DNA rearrangement or deletion in the remaining cases. Therefore, three different patterns corresponded to the phenotypic phenomenon of allelic exclusion. The "excluded" allele may be germ line, may be deleted or may be rearranged presumably in an aberant fashion. Eight constant lambda genes and a processed dispersed psuedo lambda gene exist in at least two germ line patterns due to polymorphism. EcoRl digests of all lambda expressing B cells showed a rearranged allele not seen in fibroblasts or T cells from the same individuals. Thirty percent of the individuals displayed a second aberent rearrangement as well.

The arrangement of the genes coding for the opposite isotype were then examined in kappa and lambda expressing B cells. In all kappa expressing B cells, the EcoRl lambda gene containing fragments remained in the germ line configuration. In contrast to the germ line arrangement of lambda genes in kappa expressing B cells, both C_{kappa} alleles were deleted or in rare cases rearranged in lambda expressing human leukemic B cells or lines. The J_{kappa} segment was also deleted in these lambda expressing clonal B cell populations. However, the particular V_{kappa} gene family examined was retained. The lambda genes in normal polyclonal human lambda expressing B cells showed a loss of kappa genes. The fact that lambda genes appear to remain in the germ line configuration in kappa producing cells, whereas kappa genes are rearranged or deleted in lambda producing cells, suggests a hierarchy of events involving light chain gene activation in which kappa gene rearrangements precede those of lambda.

In contrast to the immunoglobulin gene rearrangements in B lymphocytes, each of the T cell leukemias and lines studied displayed germ line patterns of both kappa and lambda light chain genes and in 90% of the cases had germ line heavy chain constant, joining and diversity chain genes as well.

Since all of the immunoglobulin gene reorganization and regulatory events appear to be initiated early in B lymphoid differentiation, we studied the gene rearrangements of the leukemic cells of patients with the most frequent form of childhood acute, the non-T/non-B, lymphocytic leukemia, a leukemia that consists of cells at early stages of differentiation. A remarkable amount of heavy chain gene activity was present with 25 of 26 patients studied showing rearrangements for J_H. In addition a series of categories of immunoglobulin gene rearrangements were present in these cells that had not been seen in mature B cells or T cells. For example, some individuals had rearranged J_H genes but retained both kappa and lambda genes in the germ line configuration. In other individuals there was a rearrangement of mu genes and deletion of kappa genes with lambda genes still in the germ line configuration. In yet another group there were rearrangements or deletions of both mu constant region genes, deletions of both kappa constant region genes and rearrangements of both lambda region genes without the production of immunoglobulin chains. These studies suggest that in the majority of patients the leukemic cells of this form of childhood acute lymphocytic leukemia represent monoclonal expansions of early cells in the B cell/plasma cell series and that the previous requirement for cytoplasmic mu chain synthesis appears to be a far too stringent criteria for placing cells within this precursor series. In addition, certain cells may be "frozen" in their state of maturation as far as immunoglobulin synthesis is concerned since they show deletions and aberrant rearrangements of both heavy and light chain genes without producing an effective immunoglobulin molecule. Finally, the patterns of immunoglobulin gene rearrangements in pre-B cells as well as B cell leukemias and lines suggests an ordered hierarchy of gene rearrangements that occurs as a stem cell matures into a B cell with mu genes preceding light chains and kappa light chain genes generally preceding lambda.

Recently these techniques have been applied to other malignancies of controversial origin such as the hairy cell leukemia. All nine hairy cell leukemias examined showed rearrangements of both the heavy and light chain genes indicating that these cells are in the B cell series.

The maturation of B cells into antibody producing plasma cells is carefully regulated both positively and negatively by distinct subpopulations of lymphoid cells. Specifically many antigens and mitogens require the presence of both helper lymphocytes of thymic origin (helper T cells) and macrophages as well as the B cells to induce a full antibody response. More recently it has been recognized that a separate class of thymic derived cells, suppressor T cells, may act as negative regulators of B cell maturation inhibiting this process. We and others have shown that suppressor T cells emerge from the thymus as inactive prosuppressor cells that require an interaction with another T cell (termed a suppressor inducer or activator) in order to become a final effector of suppression. To study these events we have developed a series of techniques including techniques to study the terminal differentiation of B cells into immunoglobulin synthesizing and secreting cells, techniques to assess helper T cell function, and to detect

both increased and decreased functional activities of suppressor effector T cells and their precursors and activators.

A major accomplishment of the laboratory has been the development of a culture and assay system for the sensitization of human peripheral blood mononuclear cells with a T cell dependent antigen, sheep erythrocytes, in the absence of nonspecific stimulatory agents and with the subsequent generation of macroscopic hemolytic plaques. We have shown that the antibody production by the plaque forming cells generated in this culture system is genetically restricted and is specific for the sensitizing antigen. We have further shown that the antigen specific response measured by this system is dependent on adherent cells and two distinct helper T lymphocyte populations. One helper T cell population is nylon adherent and is sensitive to 2000R irradiation whereas the other is radioresistant and is not adherent to nylon columns. When an antigen is added at high concentrations radioresistant antigen specific suppressor cells are induced. This system is simple, sensitive, and should serve as an effective tool for the analysis of cellular interactions involved in the generation of human antigen specific PFC, the genetic control of the human immune response, and the pathophysiology of altered immunoregulation in disease. This system has been used to show that patients with ataxia-telangiectasia have a helper T cell defect as well as an intrinsic defect of their B cells.

A second approach for specific antibody production by peripheral blood mononuclear cells in vitro involves various strains of influenza virus as the antigen and the cumulative secretion of specific antibody into culture supernatants as measured by an enzyme linked immunosorbent assay (ELISA) as the assay system. Previously Dr. Nelson had demonstrated that this specific antibody response required the cooperative interaction of T-cells, B-cells and monocytes. Studies were undertaken to define the T-cell subset(s) required for specific antibody production in vitro. In these studies Dr. Nelson found that $OKT4^{+}OKT8^{-}$ T-cells but not $OKT4^{-}$ T-cells provided "help" for specific anti-influenza virus antibody production in vitro. These studies taken in conjunction with those discussed below therefore demonstrate two helper T-cell subsets for anti-influenza virus immune reactivity in vitro, an $OKT4^{+}OKT8^{-}$ subset which can provide help for both CTL generation and antiobdy production and an $OKT4^{-}OKT8^{-}$ subset which provides help for CTL generation but not antibody production.

Studies were also initiated using the in vitro specific antibody response to influenza viruses to evaluate immunodeficiency disease patients. Among patients with hypogammalobulinemia, 5 of 11 common variable hypogammaglobulinemia patients produced specific antibody to influenza virus in vitro. This was not true of any of the three patients studied with x-linked hypogammaglobulinemia and growth hormone deficiency in spite of their being able to generate CTL to the same virus. Thus, a subset of common variable hypogammaglobulinemia patients who lack the capacity to produce antibody in vivo can make antibody in vitro. Specific antibody responses in vitro were absent in 6 of 7 patients with A-T. Studies into the mechanism of this non-responsiveness in A-T patients demonstrated that both T-cell and B-cell defects were invloved and among those with T-cell defects, the patient's lacked one subset of T-cells capable of providing help to autologous T-cell while they possessed T-cells capable of providing help to allogenic B-cells. This observation is similar to

that for cord blood T-cells and again demonstrates a lack of maturation of the A-T immune system.

Another area of major activity in the Branch over the past few years has been an attempt to define the nature of the cellular structures involved in self/self and self/non-self recognition and in intercellular communication in immune and non-immune host defense processes. Dr. Blaese and Dr. Muchmore previously identified a system of recognition of foreign target cells by non-immune mononuclear phagocytes based on the interaction of effector cell surface lectin-like receptors with carbohydrate determinants on the target cells. This recognition system appears to be phylogenetically ancient in that it can be found in primitive invertebrates such as the starfish as well as in mammals and man. We have extended these studies to explore the possibility that such lectin carbohydrate interactions might also be involved in immunoregualtory signals between lymphoid cells. T cells of patients with infectious mononucleosis are profoundly inhibitory to the process of B cell activation in vitro by mitogens or by EBV. To test for a possible lectin-sugar interaction in this immunoregulatory process, suppressed lymphocyte cultures containing normal B cells, infectious mononucleosis T cells, and pokeweed mitogen, were supplimented with a variety of oligosaccharides and subsequent immunoglobin production was measured. Over 30 tested sugars had no effect in this system. However, 2 related monosaccharides were found which had a striking effect. D-mannose and methyl D-mannoside almost totally reversed the suppression caused by the infectious mononucleosis T cell permitting immunoglobulin production to occur in these cultures. At a concentration of 25 mM, these sugars reversed the suppression by an average of 85% and substantial activity was detected with as little as 2mM sugar. In preliminary studies, a variety of synthetic mannose analogs have been tested and several compounds show activity at concentrations as low as 50 uM. We have also studied 2 other situations in which similar suppressor phenomena are observed, the human newborn and in some patients with agammaglobulinemia. The activity of suppressor T cells present in human cord blood is also reversed by mannose and methyl mannoside. In suppressed indicator cultures containing cells from agammaglobulinemic patients, additional evidence for sugarlectin mediated immunoregulation was found. Suppressor T cell activity from 2 patients was reversed by mannose or yeast mannan and suppressor monocyte activity in another was reversed by N-acetyl glucosamine. These data appear to support the hypothesis that the sugar-lectin recognition system which evolved in primative invertebrate mononuclear phagocytes as a means of recognizing "self and foreignness" has been preserved and expanded in higher vertebrates to form the basis of certain immunoregulatory cellular interactions as well.

The Epstein Barr Virus (EBV) is the agent responsible for infectious mononucleosis and greater than 90% of normal adults have been infected with this virus. In spite of the ubiquitous nature of this infection, the malignancies associated with EBV, African Burkitt's lymphoma and nasopharyngcal carcinoma, are restricted to very specific ethnic groups or geographic regions. Increasing evidence is appearing however, that this virus may be associated with a common disease found in all ethnic and geographic groups, Rheumatoid Arthritis. Patients with Rheumatoid arthritis have elevated serum antibody titers to certain EBV associated antigens, EBV infected lymphocytes will produce rheumatoid factor in vitro, and EBV

rapidly induces B cell lines to grow from RA patients blood lymphocytes while such lines only grow slowly or not at all from normal subjects. To determine whether these patients might have a defect in immunoregulatory T cells which ordinarily control EBV induced B cell activations, we have studied over 50 patients with this disease. Usual assays for immunoregulatory functions such as helper activity for pokeweed mitogen induced B cell activation and suppressor T cell function induced by exposure to alloantigen was normal in these patients. Strikingly, however, suppressor T cell activity specific for EBV stimulated B cells, which is found in normal EBV immune subjects, was absent in the adult RA patients even though they were immune to EBV as determined by serum antibodies to the virus. This suppressor defect was restricted to reponses induced by EBV and was present in every case studied. Pediatric age patients also develop rheumatoid arthritis and clinically juvenile rheumatoid arthritis (JRA) has been subdivided into a group of patients with single joint involvment and another group with multiple joints involved. Of a group of JRA patients with single joint involvment, about 50% showed no evidence of previous EBV infection (i.e. EBV seronegative) and the remaining 50% were seropositive for EBV and had normal EBV suppressor T cell activity in vitro. By contrast, all the JRA patients we have studied with multiple joint involvment have been EBV seropositive and have demonstrated the defect in EBV specific suppressor T cell activity seen in adult RA patients. These studies support the hypothesis that this ubiquitous viral pathogen may play an important but still poorly understood role in a common form of arthritis in adults and further that a definable subgroup of children with arthritis also share this abnormality.

Dr. Bruce Littman has successfully formed human-human hybridomas from the peripheral B cells of patients with systemic lupus erythematosus (SLE). Not only were findings obtained using a classic HGPRT deficient drug marked human B cell line but more exciting is the successful fusion of SLE B cells with a normal human B cell line using diethyl pyrocarbonate. This is the first example of successful B cell fusion using this very useful and general technique.

Using the in vitro biosynthesis procedures with polyclonal activation we developed we have identified patients with disordered immunoglobulin synthesis due to a variety of mechanisms including disorders of intrinsic B cell activity, disorders of helper T cell function, disorders of the network of interacting T cells involved in immune suppression as well as disorders of monocyte function. Patients with hypogammaglobulinemia with primary B cell defects that we have defined include patients with x-linked agammaglobulinemia, patients with certain types of common variable immunodeficiency, with selective IgA deficiency or with the immunodeficiency characterized by elevated IgM levels and reduced IgG and IgA levels.

The technique for assessing helper T cell activity were applied to homogenous populations of T cells from patients with T cell leukemias. One of 20 acute T cell leukemias and seven of 12 patients with Sezary T cell leukemias retained the capacity to help normal B cells in pokeweed mitogen stimulated cultures. It is of interest that these patients had high IgA and IgE levels in 11 cases. Furthermore the Sezary cell leukemias were associated with a circulating monoclonal immunoglobulin in 11 cases.

Over the past year we have analyzed Sezary leukemic T cells with retained functions with monoclonal hybridoma antisera that define T cell subsets. Some of these antisera obtained commercially define T cell subsets with different functions (e.g. OKT8 suppressor cytotoxic T cells, OKT4 cells with helper activity among other activities) whereas others define the state of T cell maturation (e.g. OKT10 both immature and activated cells, OKT9 the transferrin receptor on rapidly proliferating cells, OKT3 mature T cells and Ia activation antigens on T cells). We have also used a monoclonal antibody described below called anti-Tac that we prepared that defines the interleukin II receptor. The 10 Sezary leukemia cells studied bear the OKT3 and OKT4 antigens (e.g. mature helper phenotype) and are OK5, 8, Ia and Tac antigen negative. They bear the OKT9 antigen associated with rapidly proliferating cells. Thus the studies with monoclonal antibodies is in accord with our previous conclusions that Sezary cells are relatively mature T cells that are dedicated to helper interactions with B cells.

Over the past year we have performed extensive studies on a newly described adult T cell leukemia. This leukemia was first defined in patients in Japan but more recently has been found in patients in the Caribbean as well. This is the leukemia associated the human T cell leukemia lymphoma virus (HTLV) described by Gallo. Work in our laboratory helped to determine that retroviral genome is present in certain T cell subsets but not B cells from affected patients. Although these cells bear the surface phenotype OKT3+, 4+ they do not function as helper T cells but we have shown that they act as suppressors of in vitro immunoglobulin synthesis. Since they suppress systems involving B cells and irradiated T cells they appear to function as suppressors in their own right rather than acting as inducers of suppressors. Furthermore, they secrete suppressor molecules. Of interest these cells and these alone among cells obtained in vivo react with the anti-Tac antibody in all cases and thus have an active interleukin II or T cell growth factor receptor. These cells contrast with those with Sezary leukemia cells in terms of this reactivity with anti-Tac as well as with certain other monoclonals. For example they are T 10 positive whereas the Sezary leukemias are T 10 negative, they are T 9 negative whereas the Sezary cells are T 9 positive. In addition certain of these leukemias react with the antibody 3A1 whereas most Sezary T cells do not. Thus in terms of surface phenotype association with human T cell lymphoma leukemia virus and in terms of function, these cells appear to be distinct from the other mature T cell leukemia, the Sezary leukemia. These leukemic cells appear to be derived from a population of suppressor cells.

In other studies we have demonstrated two other cells within the suppressor T cell series. We have demonstrated neoplastic T cells of the surface phenotype OKT3, OKT8+, OKT4- that function as suppressors of T cell proliferation and in some cases of immunoglobulin synthesis as well. Furthermore as discussed previously we have demonstrated a leukemia of prosuppressor T cells. These cells are OKT10 positive but negative for all of the other antigens discussed above. We have shown that this leukemic T cell can mature in vitro in the presence of a radiosensitive inducer T cell or secreted products of this cell. Following such activation these cells become effectors of suppression and become OKT3 (mature phenotype), OKT8 (suppressor phenotype), and Tac (interleukin II receptor)

positive. Overall the analysis of leukemias with retained function are providing major new insights into the network of functioning cells that control immunoglobulin synthesis and into the phenotype and secreted products of these cells.

Purified human peripheral blood mononuclear cells stimulated with the mitogenic lectin Concanavalin A (Con A), continuous cultures of human T cells grown in interleukin II as well as human T-T cell hybridomas have been prepared that elaborate a variety of immunoregulatory molecules including suppressive factors. We find evidence for at least two different suppressor activities in the supernatants of these cells one of which negatively modulates in vitro immunoglobulin biosynthesis while the other inhibits T cell proliferation. Immunoglobulin production was studied using a pokeweed mitogen driven reverse hemolytic plaque assay. The humoral suppressor factor produced 40-80% inhibition of polyclonal antibody synthesis. This factor was: 1) of molecular weight 30-45,000 daltons, 2) noncytotoxic, 3) present as early as 8 hours after exposure to Con A, 4) reversed by the monosaccharide L-rhamnose but not by a variety of other simple sugars including alpha-methyl-D-mannoside, 5) produced by macrophage depleted T cell populations but not by B cells, 6) found in the supernatants of long term human T cell cultures. The second suppressor factor produced 40-85% inhibition of in vitro lymphocyte proliferation in response to the mitogenic lectins phytohemagglutinin and Con A and the antigens streptokinase-streptodornase and tetanus toxoid. This factor was also of molecular weight 30-45,000 daltons and noncytotoxic. Its effect, however, is blocked by N-acetyl-D-glucosamine but not by L-rhamnose. In contrast to the humoral factor, the production of this factor required the presence of macrophages. We conclude that activated suppressor cells elaborate different soluble factors which independently modulate humoral and cellular immune reactions.

As part of this broad investigation of biological modifiers of the human immune response, we have recently studied immunoregulatory properties of a murine monoclonal antibody produced on the Metabolism Branch termed anti-Tac. We showed that this monoclonal antibody selectively reacts with activated peripheral blood T cells, interleukin-2 (IL-2) dependent long term cultured T cell (CTC) lines and a subset of adult T cell leukemias including those recently associated with a human T cell leukemia virus. In contrast, this antibody does not bind to resting T or B cells, thymocytes, monocytes, or IL-2 independent T cell lines. This pattern of reactivity suggested the possibility that anti-Tac reacted with the human T cell surface receptor for IL-2. Consistent with this site of action, we showed that anti-Tac blocks: 1) IL-2 induced proliferation and amino acid incorporation in human CTC lines, 2) antigen and mitogen induced proliferation of peripheral blood T cells, 3) T cell division occuring in the autologous and allogeneic mixed lymphocyte reactions, 4) IL-2 dependent generation of human cytotoxic T cells and 5) T cell dependent B cell immunoglobulin production induced with pokeweed mitogen. In contrast this antibody does not interfere with B cell proliferation induced with the Epstein-Barr virus, a helper T cell independent polyclonal stimulant. These inhibitory effects are not secondary to cytotoxic effects and are reversed when this monoclonal antibody is eluted from the target cell surface.

Direct binding studies to define the relationship of anti-Tac and the human IL-2 receptor were also performed. In studies using purified radiolabeled IL-2, it was demonstrated that anti-Tac completely blocked binding of TCGF to its surface receptor on activated T cells. Further, using ^{125}I-anti-Tac, we demonstrated that activated T cells contain approximately 100,000 receptors per cell and that the K_d of this interaction is 2×10^{-9} moles/liter. These receptors were half saturated within 3.75 minutes at 4°C in both glutaraldehyde-fixed and untreated activated T cells. Competitive binding studies demonstrated that monoclonal anti-Ia, anti-Leu4, anti-Ly3.2, and anti-TCGF did not compete for binding sites with anti-Tac.

We have also purified and characterized the putative human IL-2 receptor. Employing internal labeling with ^{35}S-methionine, Triton-X-100 solubilization, immunoprecipitation with anti-Tac, and analysis on SDS-7.5% PAGE. A major protein with an M_r of 113,000 has been identified. This protein migrates identically under both reducing and non-reducing conditions suggesting that it is not composed of disulfied linked subunits. Further, this receptor does not appear to be a glycoprotein as indicated by lack of labeling with ^{3}H-glucosamine, ^{3}H-mannose or ^{3}H-galactose; an identical R_f in the presence and absence of tunicamycin; and nonretention of lectin affinity chromatographs. In addition, recent studies indicate that this receptor is phosphorylated suggesting that it is a transmembrane protein. Studies are presently underway to study whether TCGF induces this phosphorylation and additionally whether it involves tyrosine residues as has been described for other growth factors (epidermal growth factor, platelet derived growth factor, and insulin) as well as oncogene products for example the pp66 src protein of the Rous sarcoma virus.

GENETIC CONTROL OF THE IMMUNE RESPONSE

The critical importance of genetic factors in regulating the immune response has become increasingly apparent in recent years, and the number of diseases known to involve inadequate immune responses (malignancy as well as infectious diseases) or excessive immune responses (autoimmune diseases) has increased. Yet the mechanism of action of the antigen-specific immune response (Ir) genes, linked to the major transplantation antigens HLA in man and H-2 in mice, remains unknown. Dr. Berzofsky has studied the mechanism of action of these genes, and the structures and specificity of the T and B cell receptors involved, for the response to a set of well-defined natural protein antigens, the mammalian myoglobins.

His previous studies had shown that the serum antibody response and the cell proliferation response to sperm whale myoglobin were both controlled by the same two Ir genes mapping in different subregions (I-A and I-C) of the H-2 complex. Each gene controls the response to different parts of the antigen molecule. When T cells from (high responder x low responder) F_1 hybrid mice were tested with antigen and macrophage from high, low, or recombinant strain mice, the part of the antigen to which they responded depended on the source of the macrophage. This phenomenon has been called "determinant selection." These responses could also be blocked by antibodies to the Ia antigens of the macrophage, which map genetically in the same region as the Ir genes.

Dr. Berzofsky now developed an in vitro culture system in which he can measure the seondary antibody responses of cells in culture. He can measure antibody concentration in the culture supernatant by radioimmunoassay and can determine the fine specificity. This in vitro antibody response is T-cell and macrophage dependent, and is under the control of the same two determinant-specific Ir genes. Thus, this system can be used to study the cellular interactions in the mechanism of the Ir gene action. Low responder mice, immunized with myoglobin bound to a carrier molecule, fowl gamma globulin (FGG) was shown to have competent myoglobin-specific B cells capable of producing anti-myoglobin antibodies when cultured with FGG-specific helper T cells. However, they do not respond to help by syngeneic or (high responder x low responder) F_1 T cells. In fact, the F_1 cells can help high responder B cells but not low reponder B cells, even when supplemented with F_1 macrophages. Thus, the Ir gene control is manifested in a restriction on T cell-B cell interaction. We could not demonstrate a similar restriction on T cell macrophage interaction, but we cannot be sure that such does not also occur. The existence of a T cell-B cell restriction correlated with the predominant B cell subset in this response being the $Lyb5^-$ subset. In other studies not involving Ir gene control, it was this subset that required genetically restricted T cell help.

In order to examine the role of T cells while avoiding complicating allogeneic reactions, alloreactivity had be be eliminated. This was done by neonatal tolerization with F_1 spleen cells. The tolerized mice did not respond to the tolerated alloantigens in a mixed lymphocyte reaction or in cell mediated lympholysis. They contained 2-4% F_1 cells surviving in the spleen, but none detectable in the thymus. T cells from neonatally tolerized, myoglobin-immune, low responder mice were able to help F_1 or high responder B cells, but not syngeneic low responder B cells, and in this respect were phenotypically identical to T cells from H-2 tolerized high responder mice. This result could be shown not to be due to allogeneic effects or to help by contaminating F_1 cells. Thus, the low responder has competent myoglobin-specific helper T cells as well as competent myoglobin specific B cells, but the Ir gene defect is manifested in the inability of these two cells to collaborate with one another. Also, these experiments indicate that either the low responders have T cells capable of recognizing antigen on high responder B cells (and these are unmasked by removal of alloreactivity), or else the repertoire for antigen is altered (presumably outside the thymus) by neonatal injection of F_1 cells.

In examining the Ir genes control of the antibody response to other myoglobins, Dr. Berzofsky found that the response to equine myoglobin was controlled by genes in I-A and H-2D, different from those controlling the response to sperm whale myoglobin. The gene in H-2D is unusual in that no other Ir gene for the antibody response to a soluble protein antigen has mapped there.

To examine B cell receptors for myoglobin, antibodies were prepared for high affinity monoclonal anti-myoglobin antibodies. The antigeneic determinants (sites) on myoglobin recognized by three of these have been determined. Two of these antibodies recognize topographic sites which consist of amino acid residues far apart in the primary sequence but brought together by the folding of the native molecule. To understand

the structure of the complementary site on the antibody, we have separated heavy and light chains and begun to sequence them. Unfortunately, the heavy chain of the first antibody studied had a blocked amino-terminus. We have also prepared Fab fragments of two of the antibodies and are trying to crystallize them in a complex with myoglobin to study the structure by x-ray diffraction.

Finally, Dr. Berzofsky has been studying the specificity of T cell receptors of both cloned and uncloned T cells. The polyclonal population revealed the interesting result that there may be an antigenic site which is immunodominant for T cells. B10.S mice, high responders to both horse and sperm whale myoglobins, when immunized with either myoglobin, produce T cells which do not crossreact with the other myoglobin. Nevertheless, both sets of T cells recognize the same site on the myoglobin, centered on residue 109. However, they strongly distinguish 109 Glu of sperm whale myoglobin from 109 Asp of horse myoglobin. Whey should these two sets of T cells recognize the same position on the two noncrossreactive myoglobins? Both myoglobins are presented by the same macrophages using the same Ia antigen ($I\text{-}S^S$), as judged by anti-Ia blocking studies. One hypothesis consistent with "determinant selection" by macrophage Ia discussed above is that the same Ia antigen presents the same face of both myoglobins to the T cell. Thus, T cells which recognize this region are preferentially elicited, but the specificity of the T cell receptor distinguishes between Glu and Asp at this site. We have now produced myoglobin-specific continuously growing T cell lines, and are cloning these. We are studying the fine specificity of the lines and the clones, and also searching for a clone which recognizes a topographic determinant like those recognized by monoclonal antibodies. We are also testing the functional activity (help/suppression) of these T cell lines in the in vitro antibody response. We are trying to use the cloned T cells to purify and study biochemically homogeneous T cell receptors. all of these studies should ultimately shed light on the mechanisms of regulation of the immune response in disease.

CELLULAR MECHANISMS REGULATING NORMAL MUCOSAL IMMUNE RESPONSES AND DISORDERS OF IMMUNE REGULATION IN GASTROINTESTINAL DISEASE

One of the major unanswered questions in mucosal immunology is the question of why the B cells which develop in Peyer's patches and migrate to secretory surfaces are B cells that bear IgA on their surface In the studies performed during this period Dr. Strober has attempted to answer this question by developing cloned T cell lines from relevant lymphoid tissues and assessing the ability of these lines to regulate LPS-driven IgA immunoglobin synthesis and secretion.

First, Con A-induced cloned T cell lines from the Peyer's patches (PP) as well as from the spleens of mice were established; these cloned cell lines expressed Thy 1.2^+, Lyt 1^+2 antigens and thus belonged to helper/inducer T cell subsets. Next, the capacity of the cloned T cells to regulate Ig synthesis was determined by measuring their effect on LPS-induced polyclonal Ig synthesis by PP B cells. In initial studies Ig secreted by B cells was determined by double antibody radioimmunoassay. LPS in the absence of cloned T cells induced abundant amounts of IgM but little or no IgA.

The addition of PP cloned T cells to the cultures markedly supressed production of IgM but that of cloned T cells from the spleen suppressed only a little or not at all; IgG production was inhibited by both PP and spleen T cells clone (70% at the above T/B ratio), whereas both clones enhanced IgA synthesis, but only to a limited degree.

In subsequent studies the expression of class-specific surface and cytoplasmic immunoglobulin on/in unseparated as well as Ig class-specific PP B cells during culture with or without the cloned T cells was determined by immunofluorescence. The major findings were as follows: 1) as compared to unseparated B cell cultures and cultures of purified sIgM B cells containing LPS alone, cultures containing LPS and PP cloned T cells showed a marked decrease in cIgM-, sIgG- and cIgG- expressing cells which was accompanied by a striking increase (8-fold) in sIgA-bearing, but not cIgA-containing cells; in contrast, unseparated B cell cultures and cultures of purified sIgM B cells containing LPS and spleen cloned T cells did not show any increase in sIgA bearing cells; 2) as compared to purified sIgG-bearing B cell cultures containing LPS alone, purified sIgG-bearing B cell cultures containing both LPS and PP cloned T cells showed no substantial change in sIgG- or cIgG-expressing cells and no sIgA- or cIgA-expressing cells appeared; and 3) as compared to sIgA-bearing B cell cultures containing LPS alone, purified sIgA-bearing B cell cultures containing both LPS and PP cloned T cells showed no increase proliferation and cIgA cells did not occur.

From these results we conclude that PP cloned T cells induced class-specific switching from sIgM- to sIgA-bearing B cells, whereas cloned T cells lacked this property although they may have induced an IgM - IgG or intra-subclass IgG switch. Furthermore, the PP switch T cells appear to operate as true switch cells which govern the pathway of DNA recombination events rather than clasical helper cells which act to expand already differentiated cells. Finally, these data favor the concept that B cell development resulting in IgA expression involves preferential and class-specific differentiation from IgM - IgA, which is controlled by a new class of regulatory T cells, IgA-specific switch T cells. Peyer's patches are a source of IgA B cells because they are a repository of such switch T cells.

As reported previously, Crohn's disease is associated with the presence of a circulating T cell which have usually increased suppression activity. This is due to a T cell which is functionaly silent until it is fractionated on anti-Ig columns, hence it is called a "covert suppressor T cell." We have now determined that this covert suppressor T cell bears the OKT8 antigen and not the OKT4 antigen. More importantly, the suppressor T cell also bears the HNK-1 antigen, an antigen heretofore only associated width natural killer cells. Thus, anti-HNK-1 plus complement treatment of T cell populations obtained from Crohn's disease patients abrogates increased suppressor activity and, in FACS analysis, such T cell populations contain cells bearing both OKT8 and HNK-1. These studies indicate that HNK-1 is a marker found on activated suppressor T cells. In addition, they show that OKT8-positive cells in Crohn's disease are qualitatively abnormal. Finally, the presence of the HNK marker on suppressor cells in Crohn's disease will allow better distinction between suppressor and possible contrasuppressor T cell populations and therefore, elucidation of the covert statues of the suppressor activity.

In prior studies we have shown 1) the primary biliary cirrhosis (PBC) is associated with a defect in the capacity to generate an autologous MLR; 2) that the autologous MLR results primarily in suppressor T cells, rather than helper T cells; and 3) that activated B cells are far more efficient as stimulator cells in the autologous MLR than are resting B cells. These findings suggested that PBC is due, in part, to a defect in an antigen non-specific regulatory loop which results in decreased suppressor T cell activity. The defective cell appears to be an OKT4-positive inducer cell which is the central responding cell in the autologous MLR. In the present studies we have provided evidence to support this viewpoint. First, we have shown the cells from PBC patients fail to develop late-acting suppressor T cell activity in Epstein-Barr virus stimulated cultures of purified T and B cells. Such cultures represent in effect, autologous interactions between T cells and activated B cells and therefore failure of suppressor T cell generation represents a direct demonstration of a defect in the autologous suppressor T cell feedback loop. Second, we have corroborated our previous studies that indicated that PBC is associated with decreased natural killer cell activity (NK activity). This abnormality is not due to a decrease in cells bearing NK-associated markers, HNK-1 and OKM1; further, it is not due to defective elaboration of or response to interferon since the addition of the latter to NK cultures causes a normal increment in NK function. By exclusion, the NK defect is due to defective inducer cell function which is parallel to the suppressor T cell defect mentioned above.

MECHANISMS OF ACTION OF CYTOTOXIC MONONUCELAR CELLS AND THEIR CONTRIBUTION TO HOST DEFENSE

A major emphasis of Dr. Nelson's work continued to be on the production of human antigen-specific, "self" major histocompatibility complex (MHC) restricted cytototoxic T-cells (CTL) in vitro. Studies were continued to define the cellular requirements for the in vitro production of self-MHC restricted CTL immune to influenza viruses. In previous studies, Dr. Nelson demonstrated that the actual killer cells themselves and also their precursors were contained within the total T-cell population. Using a series of murine monoclonal antibodies to surface antigens expressed on subpopulations of human T-cells developed at Ortho (O) Pharmaceuticals by Patrick King (K) it was shown that the CTL precursors were $OKT4^{-}OKT8^{+}$. The actual CTL killers themselves possessed the same phenotype, i.e. $T4^{-}T8^{+}$, however, the maturation of such precursors into effectors required "help" or "amplification" by an additonal subset(s) of cells which could be $OKT4^{+}OKT8^{-}$ or $OKT4^{-}OKT8^{-}$. Moreover, he was able to show that the production of self-MHC restricted CTL required the presence of adherent, latex phagocytic cells which bore HLA D/DR MHC gene products on their surface.

With this knowledge at hand and the data from experimental animals suggesting a role for "self" MHC restricted CTL's being important in the recovery from viral infections and defense against neoplasia, he investigated immunodeficiency disease patients for the capacity to generate such CTL in vitro. Among patients with hypogammiaglobulinemia, 9 of 11 patients with common variable hypogammaglobulinemia (hypo) and 3 of 3 patients with x-linked hypo and isolated growth hormone deficiency produced influenza virus specific "self"-MHC restricted CTL normally. This observation

is consistent with: 1) the clinical findings that such patients are troubled primarily by highly pathogenic eucapsulated bacteria where antibody responses seem critical and they in general are not bothered by primary viral diseases, and that 2) many such patients have B-cell defects and that B-cells are not required for CTL generation. In contrast with these findings, 10 of 11 patients with ataxia telangiectasia (AT) and all seven patients studied with the Wiskott-Aldrich syndrome (WAS) failed to produce immune "self"-MHC restricted CTL in vitro. In all "non-responding" patients this inability to detect CTL was due to the lack of CTL generation as opposed to their cells being unable to become virus-infected and serve as usable target cells. The lack of immune "self"-MHC restricted CTL production in these latter two patient groups (AT and WAS) may be related to their recurrent infections and high incidence of neoplasia.

Dr. Muchmore has continued his clinical interest in assays of spontaneous monocyte mediated cytotoxicity and has developed a reliable in vitro assay which measures inhibition of monocyte cytotoxic function by very small numbers of tumor cells. This assay correlates well with his previous in vivo observations using peripheral blood from patients with malignant disease and more important activating agents such as x-irradiation reverse tumor induced suppression. These studies are of interest because they imply a unique mechanism resulting in actual activation of cytotoxic monocytes by agents which are normally thought of as toxic and will allow us to dissect the suppressive effects of tumor cells on monocyte cytotoxicity in vitro. Such activation may play an important in vivo role. He has also studied anti-inflammatory drugs used in the treatment of rheumatoid arthritis (ASA, Indocin, steroids and gold) and find that these two can enhance monocyte function.

In additional studies Dr. Muchmore has characterized the molecular requirements for the expression of synergistic cytotoxicity. This model which measures the ability of human serum factors to activate human monocytes and lymphocytes and to kill erythrocyte targets is now substantially characterized. Homogenous purified complement components obtained in collaboration with Dr. Harvey Colton of Boston have been used and we have shown that C5 and Factor B in the presence of activated human monocytes (which fail to kill by themselves) are necessary and sufficient to induce lysis. Lymphocytes also exhibit killing in this model and require at least C7 and C8 as well as C5 and factor B. This research is important not only because it represents a unique model of cell mediated cytotoxicity but also because it unites antigen idependent fluid phase lysis (alternate complement pathway) with antigen independent cell mediated lysis (NK and spontaneous cytotoxicity).

MECHANISM OF ACTION OF GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTORS

Several years ago Dr. Nissley reported that the concentration of MSA, an insulin-like growth factor was high in fetal rat serum and then declined following birth. This observation led to the proposal that MSA may be a fetal growth factor. He next showed that rat embryo fibroblasts have receptors for MSA and respond to MSA with increased DNA sythesis and cell multiplication. Rat embryo fibroblasts were then found to produce MSA providing a system to study the hormonal control of MSA production. Recently Dr. Nissley has shown that placental lactogen, a hormone homologous to growth hormone and produced

in large amounts by the placenta, stimulates the production of MSA by the rat embryo fibroblasts. Placental lactogen does not affect the half-life of MSA in the conditioned medium but does stimulate incorporation of radiolabeled cystine into MSA as determined by immunoprecipitation followed by polyacrylamide gel electrophoresis. Placental lactogen does not cause an increase in total protein in the medium nor does placental lactogen stimulate incorporation of radiolabeled cystine in total TCA placental precipitable protein. Thus it can be concluded that placental lactogen stimulates the biosynthesis of MSA by the rat embryo fibroblasts.

Several years ago Dr. Nissley found that insulin-like growth factors stimulate proteoglycan synthesis in rat chondrosarcoma chondrocytes in monolayer culture. He then demonstrated IGF receptors on these cells and by competitive binding studies and crosslinking studies showed that there was a predominance of the IGFII type receptor on these cells. He has now purified this IGF-II receptor from these cells using MSA-affinity chromatography. We have been able to show that the size for the binding subunit of the solubilized receptor (Mr 220,000) is the same as the size of the binding subunit determined by crosslinking studies on whole cells. Similarily, the binding characteristics of the purified receptor for insulin-like growth factors are the same as the binding characteristics for whole cells.

PROLINE METABOLISM

Dr. Phang has defined a metabolic cascade initiated by pyrroline-5-carboxylate which results in increased nucleotide formation. This cascade includes 1) generation of $NADP^+$ concomitant with the conversion of PC to PRO, 2) increased flux through the oxidative arm of the pentose phosphate pathway and 3) increased PRPP formation. These mechanisms were directly demonstrated in erythrocytes, the differential incorporation of C-1 and C-6 labeled glucose into the ribose moiety of nucleotides with PC treatment and the inability of PC to stimulate PRPP production or nucleotide formation in G6PD deficient cells are strong evidence supporting the cascade. Based on these findings Dr. Phang showed that PC markedly stimulates the conversion of 6-mercaptopurine to 6-mercaptopurine monophosphate. The latter is considered to be the active antitumor moiety. Obviously, the use of PC as a possible adjunct to chemotherapy will be an area of emphasis.

The demonstration that the proline cycle functions catalytically in the transfer of reducing equivalents is an important extension of the previous work of this laboratory. In a reconstituted system, the cycle transfers reducing equivalents into mitochondria, quantitated as the production of ^3H_2O from [5-^{3}H] proline, without altering the concentrations of the cycle intermediates, i.e. proline and PC. Dr. Phang is emphasizing the physiologic and/or pathophysiologic role of this cycle. "Stress states" e.g. higher temperature, anoxia, increased physical work, will be examined for proline cycling.

Finally, Dr. Phang showed that cell-cell interacions mediated by the interconversions of PRO and PC is a mechanism by which hepatocytes can alter the metabolism of fibroblasts. This demonstration provides a new conceptual framework in which to consider pathogenetic mechanisms in hepatic fibrosis.

PORPHYRIN METABOLISM

The kinetics of heme and hematoporphyrin uptake by malignant L1210 cells in culture have been studied in further detail by Dr. Donald Tschudy along with the porphyrin mediated killing of tumor cells by light. Quantitative comparisons of the efficiency for tumor cell killing by photons of various wavelengths in porphyrin treated cells showed a decreasing order of 503 mm > 397 > 531 > 621 > 636. The latter wavelength (636 mm) is outside the hematoporphyrin absorbtion spectrum and was only 10% as effective in killing porphyrin containing tumor cells as was 621 mm, the least effective wavelength in the hematoporphyrin spectrum. The above data apply to tumor cell killing efficiency in terms of photon exposure of cells. Quantitative data were also obtained for cell killing in terms of photons absorbed by tumor cells. Although the order of decreasing efficiency for killing by photons absorbed was somewhat different from that for photon exposure, 503 mm was the most efficient wavelength in both cases.

Dr. Tshcudy showed that hematoporphytin uptake by tumor cells can be increased by exposure of the cells to succinylacetone, dibucaine and chloroquine. While exposure to dibucaine and chloroquine requires only 30 minutes or less, exposure to succinylacetone required 2-3 days. The amount of light required to kill L1210 cells in the presence of hematoporphytin was decreased by addition of dibucaine or chloroquine to the medium, but was unaffected by vitamins A and C. The fact that beta-carotene, a known quencher of singlet oxygen, and the antioxidants, vitamins A and C, were ineffective in protecting tumor cells from photochemical damage by light in the presence of hematoporphyrin, suggests that photochemical destruction of tumor cells involves mechanisms other than generation of singlet oxygen. Tumor cells grown in the presence of succinylacetone were killed by smaller doses of light after addition of hematoporphyrin than those not exposed to succinylacetone.

The profound immunosuppressive activity of succinylacetone was further demonstrated by the fact that it inhibited the rejection of a mouse tumor by rats. Controls rejected the tumor rapidly, whereas the tumor grew to significant size in the treated rats before rejection.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04002-13-MET

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Defects in Immunoregulatory Cell Interactions in Patients with Immune Dysfunction.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
11	8	3

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were directed toward defining the role of disorders of immunoregulatory cell interactions in the pathogenesis of immune dysfunctions. Leukemias of pro-suppressors, suppressor effector and helper T cells have been identified. Excessive numbers of suppressor T cells have been demonstated in association with agammaglobulinemia, selective IgA deficiency, suppressor leukemias, infectious mnonucleosis, and post transplantation immunodeficiency states. Antigen nonspecific suppressor factors that inhibit B cell immunoglobulin synthesis have identified in the supernatants of cultured human T cell lines and T-T cell hybridomas. The structure and function of the IL-2 receptor has been elucidated using a monoclonal antibody to this receptor. Recombinant DNA technology has been applied to the study of the arrangement and rearrangement of immunoglobulin genes in lymphocytic leukemias. Unexpectedly the kappa gene was deleted or aberently rearranged in all lambda expressing B cells. A hierarchy of gene rearrangements was suggested with a rearrangement of heavy chain genes preceeding light chain genes and kappa gene rearrangements or deletions preceeding lambda.

Project Description:

Objectives: The objectives of the study were: to determine the sequential steps involved in the differentiation of stem cells into B cells and then into immunoglobulin synthesizing and secreting cells. Major efforts were directed toward defining the immunoglobulin gene rearrangements and deletions that occur as a stem cell matures into a pre B-cell and to determine the role of the regulatory network of suppressor T lymphocytes, helper T lymphocytes, and macrophages in the control of the maturation of B cells into plasma cells. Disorders of these suppressor and helper interactions have been defined in the primary immunodeficiency, allergic, autoimmune and malignant diseases of man. Human T cell leukemias were examined for surface phenotype, retained immunoregulatory function and for the capacity to undergo further maturation. Furthermore, studies were directed toward identifying, isolating and characterizing human factors that suppress immunoglobulin synthesis. In addition a monoclonal antibody to the interleukin II receptor was developed and used in the study of T cell interactions. Overall the studies were directed toward defining disorders of host immunoregulation that lead to immunodeficiency states that have a high incidence of malignancy or that lead to immunodeficiency and a high incidence of infection in patients who have malignancies of the T and B cell system.

Methods Employed: In vitro culture techniques of mitogen stimulated peripheral blood lymphocytes have been developed for the study of terminal differentiation of B lymphocytes into immunoglobulin secreting cells and for the analysis of helper and suppressor T cell and monocyte activity. A major accomplishment was the development of a method to study antigen specific antibody synthesis by human peripheral blood cells in vitro that were stimulated by antigen in the absence of polyclonal activators. Hybridoma procedures have been used to produce monoclonal antibodies to maturation antigen of T cells including the IL-2 receptor and to study biological modifiers of the immune response produced by T cells. Recombinant DNA technology and ^{32}P labeled probes to the human constant kappa, lambda light chain and the heavy chain genes were used to study the gene rearrangements in B cell maturation and to analyze disorders in lymphocytic leukemias and immunoglobulin deficiency states.

Major Findings: A major effort of the Metabolism Branch over the past few years has been directed toward defining the events of cellular differentiation, cellular interaction and cellular biosynthesis involved in the specific circulating immune response. These studies have placed special emphasis on defining the defects of B cell maturation and of regulatory T cell and macrophage interaction with B lymphocytes and plasma cells that occur in patients with immunodeficiency diseases associated with a high incidence of malignancy, in patients with autoimmune disorders as well as in patients with malignancies of the T or B lymphocyte systems. Overall these studies were directed at defining the factors in normal and abnormal states controlling the production of antibodies and the synthesis of the immunoglobulin molecules. Cells that ultimately produce antibodies undergo sequential maturation from stem cells in the marrow to B lymphocytes and then from B lymphocytes into immunoglobulin synthesizing plasma cells.

The human immunoglobulin light chain genes in their embryonic or germ line state are organized in a discontinuous system of multiple germ line variable (V_L) regions, alternative joining (J_L) segments and a single or even multiple constant (C_L) regions. Heavy chains are similarly organized, but have an additional diversity (D_H) segment incorporated between the V_H and J_H regions. During the process of differentiation of a stem cell into a mature B cell, there is a rearrangement of the cellular genome to combine a single V_H with a single D_H and a single J_H to form an active heavy chain gene. Subsequently, a single light chain variable region (V_K or V_L) combines with an appropriate J_K or J_L region to activate a light chain gene. ^{32}P-labelled clones of human constant, joining, diversity and variable region genes were used as probes to study the gene arrangements in T cell, B cell and non-T/non-B forms or human lymphocytic leukemia or cell lines. Genomic DNA was extracted from these cells, digested with the restriction endonucleases known to demonstrate both rearranged and germ line alleles and hybridized with the probes. A single germ line C_{kappa} region gene was found to be present on a 12.0 Kb Bam Hl fragment in all non-B cells. All kappa expressing B leukemic cells and lines demonstrated at least one kappa gene rearrangement when compared to the germ line configuration. The second C_{kappa} allele remained in the embryonic configuration in 5% of the cases, but showed a DNA rearrangement or deletion in the remaining cases. Therefore, three different patterns corresponded to the phenotypic phenomenon of allelic exclusion. The "excluded" allele may be germ line, may be deleted or may be rearranged presumably in an aberant fashion. Eight constant lambda genes and a process dispersed psuedo lambda gene exist in at least two germ line patterns due to polymorphism. EcoRl digests of all lambda expressing B cells showed a rearranged allele not seen in fibroblasts or T cells from the same individuals. Thirty percent of the individuals displayed a second aberent rearrangement as well.

The arrangement of the genes coding for the opposite isotype were then examined in kappa and lambda expressing B cells. In all kappa expressing B cells, the EcoRl lambda gene containing fragments remained in the germ line configuration. In contrast to the germ line arrangement of lambda genes in kappa expressing B cells, both C_{kappa} alleles were deleted or in rare cases rearranged in lambda expressing human leukemic B cells or lines. The J_{kappa} segment was also deleted in these lambda expressing clonal B cell populations. However, the particular J_{kappa} gene family examined was retained. The lambda genes in normal polyclonal human lambda expressing B cells showed a loss of kappa genes. The fact that lambda genes appear to remain in the germ line configuration in kappa producing cells, whereas kappa genes are rearranged or deleted in lambda producing cells, suggests a hierarchy of events involving light chain gene activation in which kappa gene rearrangements precede those of lambda.

In contrast to the immunoglobulin gene rearrangements in B lymphocytes, each of the T cell leukemias and lines studied displayed germ line patterns of both kappa and lambda light chain genes and in 90% of the cases had germ line heavy chain constant, joining and diversity chain genes as well.

Since all of the immunoglobulin gene reorganization and regulatory events appear to be initiated early in B lymphoid differentiation, we studied the gene rearrangements of the leukemic cells of patients with the most frequent form

of childhood acute, the non-T/non-B, lymphocytic leukemia, a leukemia that consists of cells at early stages of differentiation. A remarkable amount of heavy chain gene activity was present with 25 of 26 patients studied showing rearrangements for J_H. In addition a series of categories of immunoglobulin gene rearrangements were present in these cells that had not been seen in mature B cells or T cells. For example, some individuals had rearranged J_H genes but retained both kappa and lambda genes in the germ line configuration. In other individuals there was a rearrangement of mu genes and deletion of kappa genes with lambda genes still in the germ line configuration. In yet another group there were rearrangements or deletions of both mu constant region genes, deletions of both kappa constant region genes and rearrangements of both lambda region genes without the production of immunoglobulin chains. These studies suggest that in the majority of patients the leukemic cells of this form of childhood acute lymphocytic leukemia represent monoclonal expansions of early cells in the B cell/plasma cell series and that the previous requirement for cytoplasmic mu chain synthesis appears to be a far too stringent criteria for placing cells within this precursor series. In addition, certain cells may be "frozen" in their state of maturation as far as immunoglobulin syntheis is concerned since they show deletions and aberrant rearrangements of both heavy and light chain genes without producing an effective immunoglobulin molecule. Finally, the patterns of immunoglobulin gene rearrangements in pre-B cells as well as B cell leukemias and lines suggests an ordered hierarchy of gene rearrangements that occurs as a stem cell matures into a B cell with mu genes preceding light chains and kappa light chain genes generally preceding lambda.

Recently these techniques have been applied to other malignancies of controversial oreigin such as the hairy cell leukemia. All nine hairy cell leukemias examined showed rearrangements of both the heavy and light chain genes indicating that these cells are in the B cell series.

The maturation of B cells into antibody producing plasma cells is carefully regulated both positively and negatively by distinct subpopulations of lymphoid cells. Specifically many antigens and mitogens require the presence of both helper lymphocytes of thymic origin (helper T cells) and macrophages as well as the B cells to induce a full antibody response. More recently it has been recognized that a separate class of thymic derived cells, suppressor T cells, may act as negative regulators of B cell maturation inhibiting this process. We and others have shown that suppressor T cells emerge from the thymus as inactive prosuppressor cells that require an interaction with another T cell (termed a suppressor inducer or activator) in order to become a final effector of suppression. To study these events we have developed a series of techniques including techniques to study the terminal differentiation of B cells into immunoglobulin synthesizing and secreting cells, techniques to assess helper T cell function, and to detect both increased and decreased functional activities of suppressor effector T cells and their precursors and activators.

A major new accomplishment of the laboratory has been the development of a culture and assay system for the sensitization of human peripheral blood mononuclear cells with a T cell dependent antigen, sheep erythrocytes, in the

absence of nonspecific stimulatory agents and with the subsequent generation of macroscopic hemolytic plaques. We have shown that the antibody produced by the plaque forming cells generated in this culture system is genetically restricted and is specific for the sensitizing antigen. We have further shown that the antigen specific response measured by this system is dependent on adherent cells and two distinct helper T lymphocyte populations. One helper T cell population is nylon adherent and is sensitive to 2000R irradiation whereas the other is radioresistant and is not adherent to nylon columns. When an antigen is added at high concentrations radioresistant antigen specific suppressor cells are induced. This system is simple, sensitive, and should serve as an effective tool for the analysis of cellular interactions involved in the generation of human antigen specific PFC, the genetic control of the human immune response, and the pathophysiology of altered immunoregulation in disease. This system has been used to show that patients with ataxia-telangiectasia have a helper T cell defect as well as an intrinsic defect of their B cells.

Using the in vitro biosynthesis procedures with polyclonal activation we developed we have identified patients with disordered immunoglobulin synthesis due to a variety of mechanisms including disorders of intrinsic B cell activity, disorders of helper T cell function, disorders of the network of interacting T cells involved in immune suppression as well as disorders of monocyte function. Patients with hypogammaglobulinemia with primary B cell defects that we have defined include patients with x-linked agammaglobulinemia, patients with certain types of common variable immunodeficiency, with selective IgA deficiency or with the immunodeficiency characterized by elevated IgM levels and reduced IgG and IgA levels.

The technique for assessing helper T cell activity were applied to homogenous populations of T cells from patients with T cell leukemias. One of 20 acute T cell leukemias and seven of 12 patients with Sezary T cell leukemias retained the capacity to help normal B cells in pokeweed mitogen stimulated cultures. It is of interest that these patients had high IgA and IgE levels in 11 cases. Furthermore the Sezary cell leukemias were associated with a circulating monoclonal immunoglobulin in 11 cases.

Over the past year we have analyzed Sezary leukemic T cells with retained functions with monoclonal hybridoma antisera that define T cell subsets. Some of these antisera obtained commercially define T cell subsets with different functions (e.g. OKT8 suppressor cytotoxic T cells, OKT4 cells with helper activity among other activities) whereas others define the state of T cell maturation (e.g. OKT10 both immature and activated cells, OKT9 the transferrin receptor on rapidly proliferating cells, OKT3 mature T cells and Ia activation antigens on T cells). We have also used a monoclonal antibody described below called anti-Tac that we prepared that defines the interleukin II receptor. The 10 Sezary leukemia cells studied bear the OKT3 and OKT4 antigens (e.g. mature helper phenotype) and are OK5, 8, Ia and Tac antigen negative. They bear the OKT9 antigen associated with rapidly proliferating cells. Thus the studies with monoclonal antibodies is in accord with our previous conclusions that Sezary cells are relatively mature T cells that are dedicated to helper interactions with B cells.

Over the past year we have performed extensive studies on a newly described adult T cell leukemia. This leukemia was first defined in patients in Japan but more recently has been found in patients in the Caribbean as well. This is the leukemia associated the human T cell leukemia lymphoma virus (HTLV) described by Gallo. Although these cells bear the surface phenotype OKT3+, 4+ they do not function as helper T cells but we have shown that they act as suppressors of in vitro immunoglobulin synthesis. Since they suppress systems involving B cells and irradiated T cells they appear to they function as suppressors in their own right rather than acting as inducers of suppressors. Furthermore, they secrete suppressor molecules. Of interest these cells and these alone among cells obtained in vivo react with the anti-Tac antibody in all cases and thus have an active interleukin II or T cell growth factor receptor. These cells contrast with those with Sezary leukemia cells in terms of this reactivity with anti-Tac as well as with certain other monoclonals. For example they are T 10 positive whereas the Sezary leukemias are T 10 negative, they are T 9 negative whereas the Sezary cells are T 9 positive. In addition certain of these leukemias react with the antibody 3A1 whereas most Sezary T cells do not. Thus in terms of surface phenotype association with human T cell lymphoma leukemia virus and in terms of function, these cells appear to be distinct from the other mature T cell leukemia, the Sezary leukemia. These leukemic cells appear to be derived from a population of suppressor cells.

In other studies we have demonstrated two other cells within the suppressor T cell series. We have demonstrated neoplastic T cells of the surface phenotype OKT3, OKT8+, OKT4- that function as suppressors of T cell proliferation and in some cases of immunoglobulin synthesis as well. Furthermore as discussed previously we have demonstrated a leukemia of prosuppressor T cells. These cells are OKT10 positive but negative for all of the other antigens discussed above. We have shown that this leukemic T cell can mature in vitro in the presence of a radiosensitive inducer T cell or secreted products of this cell. Following such activation these cells become effectors of suppression and become OKT3 (mature phenotype), OKT8 (suppressor phenotype), and Tac (interleukin II receptor) positive. Overall the analysis of leukemias with retained function are providing major new insights into the network of functioning cells that control immunoglobulin synthesis and into the phenotype and secreted products of these cells.

Purified human peripheral blood mononuclear cells stimulated with the mitogenic lectin Concanavalin A (Con A), continuous cultures of human T cells grown in interleukin II as well as human T-T cell hybridomas have been prepared that elaborate a variety of immunoregulatory molecules including suppressive factors. We find evidence for at least two different suppressor activities in the supernatants of these cells one of which negatively modulates in vitro immunoglobulin biosynthesis while the other inhibits T cell proliferation. Immunoglobulin production was studied using a pokeweed mitogen driven reverse hemolytic plaque assay. The humoral suppressor factor produced 40-80% inhibition of polyclonal antibody synthesis. This factor was: 1) of molecular weight 30-45,000 daltons, 2) noncytotoxic, 3) present as early as 8 hours after exposure to Con A, 4) reversed by the monosaccharide L-rhamnose but not by a variety of other simple sugars including alpha-methyl-D-mannoside, 5) produced by macrophage depleted T cell populations but not by B cells,

6) found in the supernatants of long term human T cell cultures. The second suppressor factor produced 40-85% inhibition of in vitro lymphocyte proliferation in response to the mitogenic lectins phytohemagglutinin and Con A and the antigens streptokinase-streptodornase and tetanus toxoid. This factor was also of molecular weight 30-45,000 daltons and noncytotoxic. Its effect, however, is blocked by N-acetyl-D-glucosamine but not by L-rhamnose. In contrast to the humoral factor, the production of this factor required the presence of macrophages. We conclude that activated suppressor cells elaborate different soluble factors which independently modulate humoral and cellular immune reactions.

As part of this broad investigation of biological modifiers of the human immune response, we have recently studied immunoregulatory properties of a murine monoclonal antibody produced on the Metabolism Branch termed anti-Tac. We showed that this monoclonal antibody selectively reacts with activated peripheral blood T cells, interleukin-2 (IL-2) dependent long term cultured T cell (CTC) lines and a subset of adult T cell leukemias including those recently associated with a human T cell leukemia virus. In contrast, this antibody does not bind to resting T or B cells, thymocytes, monocytes, or IL-2 independent T cell lines. This pattern of reactivity suggested the possibility that anti-Tac reacted with the human T cell surface receptor for IL-2. Consistent with this site of action, we showed that anti-Tac blocks: 1) IL-2 induced proliferation and amino acid incorporation in human CTC lines, 2) antigen and mitogen induced proliferation of peripheral blood T cells, 3) T cell division occuring in the autologous and allogeneic mixed lymphocyte reactions, 4) IL-2 dependent generation of human cytotoxic T cells and 5) T cell dependent B cell immunoglobulin production induced with pokeweed mitogen. In contrast this antibody does not interfere with B cell proliferation induced with the Epstein-Barr virus, a helper T cell independent polyclonal stimulant. These inhibitory effects are not secondary to cytotoxic effects and are reversed when this monoclonal antibody is eluted from the target cell surface.

Direct binding studies to define the relationship of anti-Tac and the human IL-2 receptor were also performed. In studies using purified radiolabeled IL-2, it was demonstrated that anti-Tac completely blocked binding of TCGF to its surface receptor on activated T cells. Further, using ^{125}I-anti-Tac, we demonstrated that activated T cells contain approximately 100,000 receptors per cell and that the K_d of this interaction is 2×10^{-9} moles/liter. These receptors were half saturated within 3.75 minutes at 4°C in both glutaraldehyde-fixed and untreated activated T cells. Competitive binding studies demonstrated that monoclonal anti-Ia, anti-Leu4, anti-Ly3.2, and anti-TCGF did not compete for binding sites with anti-Tac.

We have also purified and characterized the putative human IL-2 receptor. Employing internal labeling with ^{35}S-methionine, Triton-X-100 solubilization, immunoprecipitation with anti-Tac, and analysis on SDS-7.5% PAGE. A major protein with an M_r of 113,000 has been identified. This protein migrates identically under both reducing and non-reducing conditions suggesting that it is not composed of disulfied linked subunits. Further, this receptor does not appear to be a glycoprotein as indicated by lack of labeling with ^{3}H-glucosamine, ^{3}H-mannose or ^{3}H-galactose; an identical R_f in the

presence and absence of tunicamycin; and nonretention of lectin affinity chromatographs. In addition, recent studies indicate that this receptor is phosphorylated suggesting that it is a transmembrane protein. Studies are presently underway to study whether TCGF induces this phosphorylation and additionally whether it involves tyrosine residues as has been described for other growth factors (epidermal growth factor, platelet derived growth factor, and insulin) as well as oncogene products for example the pp66 src protein of the Rous sarcoma virus.

Quantitative immunoprecipitation combined with total TCA precipitations indicates that this receptor forms roughly 0.1% of newly synthesized protein in one CTC line. Using immunoprecipitation and preparative PAGE with electroelution, this receptor has been purified to homogeneity in nanomolar amounts. Amino acid composition and primary sequence data are now being obtained.

Significance to Biomedical Research: The development of techniques for the study of the effect on helper and suppressor T cells on the maturation of B cells and their application to the study of patients with immunoglobulin disorders have been of great value in defining the critical stages of B cell maturation and of the network of immunoregulatory cells that control this maturation process. A series of new pathogenic mechanisms have been defined to explain the immunodefects in patients with immunodeficiency, autoimmunity, allergy and malignancy. Leukemias of immunoregulatory cells have been defined assisting the classification of leukemias. Furthermore, the studies of leukemias using recombinant DNA technology are providing insights into the earliest events of B cell maturation are aiding in the classification of malignancies and are providing insights into the causes that underlies the maturation failure of lymphoid malignancies. In general, these studies are providing a scientific basis for developing rational strategies for the therapy of immunodeficiency and malignant diseases.

Proposed Course: Studies directed toward understanding the pathways and regulatory mechanisms controlling the sequential development of stem cells into B cells and then into synthesizing plasma cells. Special emphasis will also be placed on the genes and their rearrangement that control immunoglobulins and into the role played by the network of immunoregulatory T cells in controlling immune responses. The studies of the nature and mode of action of biological modifiers of the immune response secreted by cloned T cell lines and T-T cell hybridomas will be extended.

Honors and Awards:

5th Philip McMaster Lecture Award, The Rockefeller University
6th Goldhamer Lecture Award, Mt. Sinai Medical Center
2nd Wellcome Special Lecture in Immunology, Johns Hopkins University Medical School
Presidents Lecture, American Society of Hematology

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04003-26-MET

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies of Porphyrin Metabolism in the Tumor-Bearing Host and Porphyria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main objectives are to study the mechanisms controlling heme biosynthesis and biochemical aspcts of porphyrin metabolism in experimental porphyria, tumors and tumor bearing hosts.

PHS-6040

Project Description:

Objectives: To study mechanisms controlling heme biosynthesis and biochemical aspects of porphyrin metabolism in experimental porphyria, tumors and tumor bearing hosts. To study the immunosuppressive and growth inhibitory mechanisms of succinylacetone.

Methods Employed: Quantitative enzyme determinations, chemical determination of porphyrins, porphyrin precursors and tissue heme; synthesis fumarylacetone and various pyrroles; cell culture methods; measurement of lymphocyte proliferation in response to mitogens and antigens; measurement of antibody levels.

Major Findings: The kinetics of heme and hematoporphyrin uptake by malignant L1210 cells in culture have been studied in further detail along with the porphyrin mediated killing of tumor cells by light. Quantitative comparisons of the efficiency for tumor cell killing by photons of various wavelengths in porphyrin treated cells showed a decreasing order of 503 mm > 397 > 531 > 621 > 636. The latter wavelength (636 mm) is outside the hematoporphyrin absorbtion spectrum and ws only 10% as effective in killing porphyrin-containing tumor cells as was 621 mm, the least effective wavelength in the hematoporphyrin spectrum. The above data apply to tumor cell killing efficiency in terms of photon exposure of cells. Quantittive data were also obtained for cell killing in terms of photons absorbed by tumor cells. Although the order of decreasing efficiency for killing by photons absorbed was somewhat different from that for photon exposure, 503 mm was the most efficient wavelength in both cases.

We have shown that hematoporphytin uptake by tumor cells can be increased by exposure of the cells to succinylacetone, dibucaine and chloroquine. While exposure to dibucaine and chloroquine requires only 30 minutes or less, exposure to succinylacetone required 2-3 days. The amount of light required to kill L1210 cells in the presence of hematoporphytin was decreased by addition of dibucaine or chloroquine to the medium, but was unaffected by vitamins A and C. The fact that β-carotene, a known quencher of singlet oxygen, and the antioxidants, vitamins A and C, were ineffective in protecting tumor cells from photochemical damage by light in the presence of hematoporphyrin, suggests that photochemical destruction of tumor cells involves mechanisms other than generation of singlet oxygen. Tumor cells grown in the presence of succinylacetone were killed by smaller doses of light after addition of hematoporphyrin than those not exposed to succinylacetone.

The profound immunosuppressive activity of succinylacetone was further demonstrated by the fact that it inhibited the rejection of a mouse tumor by rats. Controls rejected the tumor rapidly, whereas the tumor grew to significant size in the treated rats before rejection.

When sprayed on the leaves of plants, succinylacetone was shown to completely inhibit chlorophyl synthesis and profoundly inhibited plant growth.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04004-21-MET

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulatory Functions of Amino Acids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Endocrinology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	3	3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The enzymes catalyzing the interconversion of proline and pyrroline-5-carboxylate provide a mechanism for the intercompartmental, intercellular, and interorgan transfer of redox potential. Mediated by the transfer of redox potential, PC regulates PPRP and nucleotide production thereby linking amino acid and nucleotide metabolism. This linkage provides an approach to enhance the antitumor activity of purine antimetabolites e.g. 6-mercaptopurine.

Project Description:

Objectives: Recent reports have emphasized the primary role of metabolic regulation in malignant transformations of mammalian cells. The transport, biosynthesis and degradation of amino acids may play an important role in this metabolic regulation. More importantly amino acids are not only necessary building blocks for proteins but also are regulators in intermediary metabolism. This project emphasizes the biosynthesis and degradation of proline because the metabolism of proline has unusual, even unique, features. The metabolic intermediates of proline provide an important link of carbons between the TCA and urea cycles. In addition, these intermediates, functioning as redox couples, can regulate a number of redox-dependent metabolic pathways e.g., the production of nucleic acids. These studies may elucidate changes in growth rates, energy allocation and malignant transformations in tumors. The project is relevant to objective 3, Approaches 1 and 5 of the National Cancer Program.

Methods Employed: A variety of approaches are used to pursue the aforementioned objectives in animal tissues, freshly isolated human cells and cells maintained in long-term tissue culture. Cultured cell lines are especially useful since they can be cloned to insure genetically homogenous populations. Mutant cells are isolated by standard techniques or are obtained from patients with defined inborn errors of metabolism. Many biochemical techniques are utilized in these studies. Uptake of amino acids are assessed by using labeled amino acids and rates of protein and collagen synthesis are obtained by incubating cells with L-proline-^{14}C and separation of incorporated labeled amino acids by ion-exchange column chromatography. Metabolic conversions in intact cells can be determined by isolating labeled products from labeled precursors using high-pressure liquid chromatography. Methods have been developed to measure ornithine production from proline and glucose formation from lactate, proline and alanine. Nucleotides and their precursors, labeled and unlabeled, are quantitated using high-pressure liquid chromatography. The enzymes of biosynthesis and degradation of proline are assayed using specific radioisotopic methods. Biochemical studies on specific enzymes include the isolation of mitochondria, solubilization of enzymes from mitochondrial particles and purification. Physicochemical characteristics of these enzymes are studied by gel filtration and affinity chromatographic techniques.

Major Findings:

Clinical and Basic Research Goals: Proline, a nonessential amino acid, has a number of unique metabolic functions. Proline and its hydroxylated derivative, 4-hydroxy-L-proline, are abundant in collagen constituting 20% of the amino acid residues. More interestingly, our laboratory has shown that proline and its metabolism can regulate other major metabolic pathways thereby providing a mechanism for modulating the use of alternative energy sources. The two imino acids (PRO, HOP) are unusual in that they are the only naturally occuring amino acids without a free alpha-amino group. Instead, the alpha-amino group is incorporated within a pyrrolidine ring. Thus, proline and hydroxyproline cannot participate in the usual transamination and decarboxylation reactions

common to most other amino acids. We have shown that the specific enzyme system metabolizing proline is linked to general metabolic pathways via the redox state. Since the redox state is critical to a large number of metabolic systems (energy, gluconeogenesis, nucleotide metabolism, membrane function, ureogenesis, etc.) we propose that proline plays a role in regulating these metabolic systems and derangements of this regulation may produce disease.

Regulation of Purine Metabolism by Pyrroline-5-Carboxylate

The regulation of nucleotide metabolism plays an important role in cell energetics, cell proliferation and tissue differentiation. We have shown that pyrroline-5-carboxylate (PC), the central intermediate in the interconversions of proline, ornithine and glutamate, initiates a cascade of events resulting in markedly increased nucleotide formation and increased nucleotide pools. This cascade includes: (1) the generation of NADP+ concomitant with the conversion of PC to proline, (2) activation of the pentose phosphate pathway by NADP+, (3) increased production of PRPP by the pentose phosphate pathway and, (4) increased rates of incorporation of purines into their respective nucleotides. The mature human erythrocyte provided a useful model for these studies because in erythrocytes the de novo pathways for purine synthesis are absent and PRPPdependent salvage of purines is the sole mechanism for nucleotide formation in these cells. Furthermore, the dynamic nature of purine metabolism in erythrocytes relates to the delivery of purines to peripheral tissues. Inosine monophosphate appears to be the "mobile pool" i.e. the reservoir for this delivery process. We found that red cells incubated with PC showed markedly increased capacities to incorporate hypoxanthine into inosine monophosphate. The increase was dependent on duration of incubation and on the concentration of PC. The increased formation resulted in a net increase in IMP pools. Erythrocytes incubated with PC and hypoxanthine had IMP levels of 326 $\pm$ 28 nmol/ml cells as compared to 111 $\pm$ 10 nmol/ml cells in controls. The incorporation of C-1 versus C-6 labeled glucose into the ribose moiety of IMP confirmed the cascade described above as the mechanism for the PC-initiated increase in IMP formation.

Pyrroline-5-carboxylate has no effect on nucleotides in erythrocytes deficient in glucose-6-phosphate dehydrogenase

The increased generation of pentose by the oxidative arm of the pentose phosphate pathway is a central element in the proposed cascade of events initiated by PC. Erythrocytes deficient in glucose-6-phosphate dehydrogenase (G6PD) activity provided a system to test the putative cascade. From Sardinia we obtained erythrocytes form 4 subjects with Mediterranean G6PD deficiency as well as erythrocytes from 4 normal subjects to serve as controls. In G6PD deficient cells PC had no effect on pentose phosphate activity, PRPP formation or incorporation of hypoxanthine into IMP. In contrast, in the cells from normal Sardinian subjects, PC increased pentose phosphate activity by 6.45 X, PRPP formation by 3.0 X and IMP formation by 3.2 X. The maximum capacity of IMP formation in normal cells was 499 $\pm$ 75 nmol/h-ml cells compared to 114 $\pm$ 22 nmols/h-ml cells in G6PD deficient cells. These studies provided strong evidence that the effect of PC on nucleotides was mediated by the PC-initiated metabolic cascade.

The Effect of PC on the Activation of Purine Antimetabolites

The above findings documenting a PC-stimulated increase in nucleotide pools via a PRPP-dependent mechanism suggested that PC may affect the metabolism of purine antimetabolites. The activation of such antimetabolites (6-mercaptopurine, 6-thioguanine, azathiaprine) may be critical to the efficacy of these drugs as antitumor agents. The activation step is the PRPP-dependent conversion of these purine analogues to their respective monophosphates. In studies using 6-mercaptopurine as a model antimetabolite, we incubated erythrocytes with 6-mercaptopurine and quantitated the monophosphate derivative on HPLC. We found that PC-treated erythrocytes formed 6-MP monophosphate at ratess which were seven fold those of control. The magnitude of the effect was dependent on the concentration of PC and on the duration of incubation.

The Catalytic Nature of the Proline Cycle

We have previously shown that the interconversions of proline and pyrroline-5-carboxylate form a metabolic cycle by which cytosolic reducing potential is transferred into mitochondria. We now have demonstrated that the cycle functions catalytically, i.e. the transfer of reducing potential provided by the cycle greatly exceeds the net consumption of cycle intermediates. This demonstration was made possible by the finding that $^{3}H_2O$ is produced by mitochondria from the oxidation of [5-^{3}H] proline. A finding which allowed the monitoring of the transfer of reducing potential by proline into mitochondria. The disappearance of ^{14}Cproline was used to quantitate the utilization of cycle intermediates. The complete system included mitochondrial particles, PC reductase, NADP+ and a NADPHgenerating system. In the absence of NADP+, the cycle was nonfunctional and proline was rapidly converted to PC. About 15% of the proline was recovered as PC after 60 minutes of incubation. In contrast when NADPH was generated from NADP by the generating system, only 2% of the proline was recovered as PC. More importantly in the face of constant proline concentrations, $^{3}H_2O$ was formed from proline at a rate identical to that formed when proline was being consumed. These studies show that reducing potential was transferred from proline into mitochondria by the cycling of proline and PC and that the transfer occurred without consumption of the components of the cycle. Thus the catalytic nature of the cycle has been demonstrated for the first time.

The Transfer of PC as Oxidizing Potential from Hepatocyte to Fibroblast

Although abnormalities in proline and hydroxyproline metabolism have been reported in numerous studies of metabolically-induced hepatic fibrosis, the pathophysiologic relationship between these abnormalities and the fibrotic process remains undefined. We showed that the interconversions of proline and pyrroline5-carboxylate (also hydroxyproline and 3-OH-pyrroline-5-carboxylate) serve as metabolic signals between hepatocytes and fibroblasts. In isolated rat hepatocytes incubated with proline or hydroxyproline, PC or 3-OH-PC, respectively, are released into the incubation medium. Both pyrroline carboxylic acids are taken up by fibroblasts and are a source of oxidizing potential in these cells. The oxidation of glucose through the pentose phosphate pathway which is dependent on the availability of NADP is markedly increased by pyrroline carboxylic acids. From these studies we propose that these regulatory intermediates released by hepatocytes alter the metabolism of fibroblasts. Since the cascade

of events initiated by PC results in increased nucleotide synthesis, the pathophysiologic alteration of this cell-cell interaction may play a role in hepatic fibrosis.

Significance to Biomedical Research

The linkage of the proline metabolic pathway to glucose (energy) and nucleotide metabolism has far-reaching implications. Since the HMP pathway is the source of pentose for nucleic acids, the proline-PC interconversions provide a mechanism for adjusting the synthesis of macromolecules. Derangements in the linkage may be involved in the altered metabolism of malignant cells. Furthermore, the demonstrated proline-PC cycle provides not only a mechanism for ATP generation from the HMP pathway, but also serves as a model for small molecules acting as metabolic signals between cells. These studies provide a new approach in understanding pathophysiologic mechanisms in several diseases including: 1) metabolic derangements in sepsis, 2) increased collagen production in hepatic fibrosis, 3) retinal degeneration in gyrate atrophy and, 4) oncogenesis. The demonstrated mechanisms linking pyrroline-5-carboxylate and nucleotide metabolism may provide new adjunctive approaches in chemotherapy. The PC-initiated cascade can be used to augment the antitumor activity of purine and/or pyrimidine antimetabolites.

Proposed Course:

Recent studies on the mechanisms of oncogenesis (Src gene, etc.) have re-emphasized the importance of studies in the area of metabolic regulation. We think that the regulatory effects of the PC-proline axis is an important new facet of metabolic regulation. During the coming year we will emphasize the following specific areas:

1) We will focus on the physiologic and/or pathophysiologic implications of the catalytic proline cycle. It is likely that the proline cycle is important mainly under "stress conditions", e.g. with increases in temperature, increased physical work etc. We will use the differential metabolism of [5-^{3}H] proline and [U-^{14}C] proline to monitor cycling. The "stress conditions" may be produced in both cultured cells and in animals.

2) The PC-initiated stimulation of nucleotide formation. We will study the relationship between cell activation (mitogenesis) and the PC effect. The possibility that cell activation requires PC-initiated events will be examined physiologically and biochemically. Alterations in PC reductase e.g. kinetic properties, differential use of pyridine nucleotides (NADPH vs. NADH) and association of the enzyme with cell membranes will be examined. We will also focus on the activation of chemotherapeutic agents by PC. The conditions in which PC will activate 6-mercaptopurine, 6-thioguanine, azathiaprine and 5-fluorouracil in tumor cells and/or tumors in animals will be emphasized.

3) Pathophysiologic mechanisms in gyrate atrophy cells. We will continue our studies on the function of PC in these cells which have demonstrated deficiency in PC production. We hope to establish that the deficiency in PC results in abnormalities in PPRP synthesis and nucleotide metabolism.

4) Protein chemistry of PC reductase. We previously showed that PC reductase is present at high levels in erythrocytes and the enzyme in these

cells has kinetic characteristics and regulatory features distinct from the enzyme found in other tissues. Since these findings suggest the existence of isozymes of PC reductase, we hope to identify isozymes by physiochemical methods. Purification of the enzyme from erythrocytes may provide suitable substrate for studies directed at identifying possible post-translational modification (phosphorylation etc.).

5) A specific assay for PC levels in cells and tissue. Although the studies of the PRO-PC regulatory axis has been established, possible cellular fluctuations in PC have not been studied. We hope to develope an assay using derivatized PC and High-pressure liquid chromatography which would have enough sensitivity to measure PC concentrations in cultured and circulating cells.

Publications:

Lodato, R.F., Smith, R.J., Valle, D., Phang, J.M., and Aoki, T.T.: Regulation of proline biosynthesis: the inhibition of pyrroline-5-carboxylate synthase activity by ornithine. Metabolism 30: 908-913, 1981.

Lorans, G., and Phang, J.M.: Proline synthesis and redox regulation: Differential functions of pyrroline-5-carboxylate reductase in human lymphoblastoid cell lines. Biochem. Biophys. Res. Commun. 101: 1018-1025, 1981.

Yeh, G.C., and Phang, J.M.: The stimulation of purine nucleotide production by pyrroline-5-carboxylic acid in human erythrocytes. Biochem. Bioophys. Res. Commun. 103: 118-124, 1981.

Hagedorn, C.H., Yeh, G.C., and Phang, J.M. Transfer of l-pyrroline-5-carboxylate as oxidizing potential from hepatocytes to erythrocytes. Biochem. J. 202: 31-39, 1982.

Phang, J.M., Downing, S.J., Yeh, G.C., Smith, R.J., Williams, J.A., and Hagedorn, C.H.: Stimulation of the hexosemonophosphate-pentose pathway by pyrroline-5-carboxylate in cultured cells. J. Cell. Physiol. 110: 255-261, 1982.

Williams, J.A., and Phang, J.M.: Production of ornithine by intact human erythrocytes. Am. J. Physiol. 242: C393-397, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04014-12-MET

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies of the Immune Response in Normal and Pathological States

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

LAB/BRANCH Metabolism Branch

SECTION Immunophysiology Section

INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6	4	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were directed toward the analysis of immune response in normal humans and patients with a variety of immune disorders, as well as analysis of humoral and cellular immune responses of experimental animals.

PHS-6040

Project Description:

Objectives: Studies were directed toward the analysis of the humoral and cellular immune responses of experimental animals with particular emphasis on the study of experimental models of human disease. In addition, studies were directed toward the analysis of the immune responses of normal humans as well as individuals having a variety of immune disorders with concentration on immunodeficiency disorders as well as immunologic disorders of the gastrointestinal tract. These studies included the investigation of patient with glutensensitive enteropathy, dermatitis herpetiformis, inflammatory bowel disease, as well as patients with ataxia telangiectasia, common variable hypogammaglobulinemia, and intestinal lymphangiectasia. Each of these diseases are associated with a high incidence of reticuloendothelial malignancy and the mechanism underlying the association between malignancy and these diseases was sought.

Methods Employed: A host of techniques were used to assess immunologic function of experimental animals and patients with disorders of the immune system. Circulating B cells in peripheral blood and in tissue were enumerated with carefully prepared fluorescein-conjugated F(ab')2 antiserum with specificity for heavy chains of immunoglobulins. Circulating T cells were enumerated with sheep red blood cell rosette techniques and by staining with T cell specific monoclonal antibodies lymphoid cell in vitro proliferative responses were assessed with tritiated thymidine incorporation following exposure to specific and nonspecific mitogens. Mitogen-induced cytotoxic effector cells as well as antibody dependent and natural cytotoxic effector cells were assessed with ^{51}Cr-release assays using red cell and nucleated, metabolically active target cells. Homogenous cell populations were isolated with the use of Sephadex anti-$F(ab')_2$ columns, rosetting techniques and the use of colloidal iron followed by passage through a magnetic field. Short term organ culture was used in conjunction with solid phase immunoabsorption techniques to measure mucosal immunoglobulins; long term organ culture techniques were used to study in vitro models of intestinal disease. Cells obtained from various tissues as well as from peripheral blood were cultured in vitro under a variety of conditions. Supernatants from these cell cultures were assayed by radioimmunoassay in order to measure immunoglobulins produced in culture. Cells were adapted and maintained in long term culture with the use of T cell growth factors. Cells were cloned using limiting dilution techniques. Patients with immunologic disorders were studied with metabolic turnover techniques to measure immunoglobin synthesis and catabolism. Finally, a number of techniques specific to individual projects were employed and are mentioned below as appropriate.

Major Findings:

A. STUDIES OF THE REGULATION OF IgA IMMUNOGLOBULIN RESPONSES

Backgound: In recent years we have been concerned with the cellular mechanisms regulating mucosal immune responses in general and IgA immunoglobulin responses in particular. In this regard, we have demonstrated that IgA immunoglobulin synthesis are governed by regulatory T cells which are distinct from the regulatory T cells controlling IgM and IgG

synthesis. First we showed that lipopolysaccharide (LPS)-induced Ig synthesis by indicator mouse B cells was variably affected by Concanavalin A (Con A) treated Peyer's patch T cells: IgM and IgG synthesis was suppressed whereas IgA synthesis was enhanced. Next we showed that feeding a protein antigen (BSA) to mice resulted in the appearance in Peyer's patches of antigen-specific suppressor T cells for IgG anti-BSA responses concomitant with the appearance of antigen-specific helper T cells for IgA anti-BSA responses. This was shown in cell transfer studies using fed animals as donors of regulatory T cells and parenterally stimulated animals as recipients of the regulatory T cells. In this system it was also possible to show with monoclonal anti-LyT-2 (anti-suppressor T cells antibody) that antigen-specific suppression for the IgG response could be affected without affecting antigen-specific help for the IgA response and vice versa. Thus, these prior studies provided data from both in both polyclonal and antigen driven systems that the regulation of IgA responses was independent of the IgG/IgM response.

In the last annual report we discribed initial phases of a new line of investigation of immunoregulation of IgA. We reported that we had succeeded in establishing concanavalin A-induced continuous cell lines of Peyer's patch and spleen origin. These cell lines were Lyt $1^{+}2^{-}$, Ia^{+} cells capable of producing growth factor, IL-2. In addition, the PP-derived clones, but not the spleen-derived clone bore IgA-Fc receptors.

During the current period we perormed extensive studies of the properties of these cloned T cells. We found first of all that the addition of PP-derived cloned T cells lines (2 lines) to LPS-driven B cell cultures suppressed IgM in such coultures in a dose dependent fashion: at a 4:1 T/B cell ratio IgM synthesis was suppressed by 90%. In contrast, spleen-derived cloned T cells lines (2 lines) did not suppress IgM synthesis. In additional studies it was shown that 1) clones cell lines of both origins suppressed IgG synthesis and 2) cloned cell lines of both origins slightly enhanced IgA synthesis of LPS-induced B cell cultures.

These results were initially difficult to understand inasmuch as the cloned cells caused suppression and yet bore markers associated with inducer/helper T cell subsets. One way out of this dilemma was to assume that the apparent suppression of IgM and IgG synthesis represented isotype switching. Accordingly, we measured the effect of the cloned T cells on the expression of surface immunoglobulin B cells cultures with LPS. In these studies B cells bearing IgM, IgG and IgA were first purified on anti-Ig plates using and panning techniques; the adherent cells were then treated with appropriate anti-Ig and C in order to eliminate residual B cells bearing unwanted surface Ig. The B cells were then cultured with and without LPS and, in addition, the effect of adding cloned T cells to the cultures was determined.

It was found that as compared to sIgM B cells in cultures containing only LPS, sIgM B cells cocultured with Peyer's patch-derived cloned T cells under went no change in number of cells but did show a striking increase in the number of cells beaing s-IgA and a parallel decrease in cells bearing sIgM and sIgG (as well as cells containing these latter

Igs in their cytoplasm); nevertheless, cells with intracytoplasmic IgA did not appear in any substantial number. Thus, the PP-derived cloned T cells affected a sIgM -> sIgA "switch" but did not mediate differentiation of sIgA cells to IgA-producing plasma cells. If spleen-derived cloned T cells rather than PP-derived cloned T cells were cocultured with purified sIgM-positive B cells and LPS there was (compared to B cells cultured with LPS alone) a mild increase in cells bearing sIgG accompanied by a decrease in cells bearing sIgM and containing cIgM; however, this was unaccompanied by the appearance of sIgA cells. Thus, the spleen-derived T cell clones were in capable of mediating sIgM -> sIgA switches, but may have caused some sIgM -> sIgG or intra-subclass IgG switches.

Turning now to results with IgG B cell cultures, it was found that addition of PP-derived cloned T cell did not cause the appearance of IgA expressing cells as in cultures of sIgM B cells and did not significantly change the number of IgG-expressing cells. On the other hand, spleen-derived cloned T cells brought about a mild increase in sIgG-bearing cells and a decrease in cIgG-bearing cells. Finally, cutures of sIgA B cells cultured with either PP-derived or spleen-derived T cell clones (again when compared to sIgA B cells cultured with LPS alone) exhibited a change in number or state of differentiation. In particular, sIgA bearing B cells neither proliferated nor differentiated into cIgG expressing B cells under the influence of PP-derived cloned T cells.

These results can be summarized by stating that PP-derived cloned T cells but not spleen-derived cloned T cells cause IgM -> IgA isotype switches but not terminal differentiation into IgA secreting plasma cells. Such terminal differentiation requires additional T cell help in that we have shown, in preliminary studies, that uncloned T cells obtained from musenteric lymph node induces IgA synthesis and secretion in B cell cultures containing the T cell-dependent stimulus, staphylococcal protein A.

Significance: These studies indicate that Peyer's patches are a source of IgA B cells because they contain T cells specifically capable of inducing class-spcific switches of IgM -> IgA. This effect is not a mere proliferative influence that drives B cell differentiation in sequential and step wise 3' DNA heavy chain gene expression; this is shown quite clearly by the fact that sIgG cells are not induced to switch to sIgA cells. In addition, this effect is not simply terminal maturation of already differentiated sIgA cells, since, the PP-derived cloned T cells have no such effects on isolated sIgA B cells. In all, the PP-derived cloned T cells appear to function as true "switch" T cells which affect B cell development by guiding the course of intranuclear DNA recombination.

Proposed Course: Many new avenues of research are opened by the above studies in which we have identified what is, in effect, a new class of regulatory T cell.

1.) We plan to seek and characterize switch T cell factors by studying the supernatants of cultured of PP-derived cloned T cells. These factors will be investigated as to the nature of their target

cells. Ultimately, studies will be initiated on the nature of the intracellular events induced by the switch T cells, especially as they relate to control of DNA rearrangements;

2.) Switch T cells will be examined for histocompatibility and/or allotype restrictions. In addition the effects of switch T cells on B cells from various organ lymphoid tissues will be established. Finally, the effects of PP-derived cloned T cells on differentiatable cloned B cell populations will be determined;

3.) As a new approach, we will attempt to establish antigen-specific T cell clones from PP and to affect IgM -> IgA switches within an antigen-specific system. For this purpose, antigen-specific clones with a variety of antigen specificities will be produced and studied;

4.) Certain human disease states will be studied for the presence of defects in IgA specific switch T cells; these include selective IgA deficiency and ataxia telangiectasia.

B. STUDIES OF IMMUNOREGULATORY DEFECTS IN CROHN'S DISEASE

Background: Crohn's disease is an inflammatory disease of the small bowel and/or large bowel characterized by ulceration and granuloma formation. Patients develop bowel wall thickening, fistula formation as well as extra-intestinal manifestations including arthritis, iritis and skin lesions. In previous studies of immune funcion of patients with Crohn's disease, a number of abnormalitaies have been identified, but none which could lay claim as the primary cause of the disease.

In prior studies of Crohn's disease (CD) we have explored the possibility that this disease results from or is complicated by an abnormality in the regulation of the immune response. More specifically we have shown that many CD patients with relatively mild or inactive disease have in their peripheral blood a "covert" suppressor T cell exerts profound suppressor cell activity detectable in vitro by the use of indicator cultures consisting of allogenic purified B cells and irradiated T cells driven to produce Ig by the polyclonal stimulant pokeweed mitogen (PWM). Of great interest was the fact that the suppressor T cell was not manifest in unseparated cell populations, but only after purification of T cells on Sephadex-anti-$F(ab')_2$ columns; hence the term "covert" suppressor T cells.

During this period we performed studies directed at identifying the suppresor cell present in peripheral blood of Crohn's disease patients. First, using monoclonal antibodies having specificity for defined regulatory T cell subsets, the OKT and Leu 3 series of antibodies, we determined that Crohn's disese is characterized by a significant decrease in the fraction of T cells bearing OKT4 or Leu 2 markers, i.e., the markers usually identified with inducer/helper activity. Concomitantly, the proportion of cells bearing OKT8 or Leu 3 markers was normal despite the fact that the population of patients studied continued many individuals with enhance

suppressor activity. At the moment the reduction in OKT4, Leu 3 positive cells in the peripheral blood is not explained. It could result from sequestration of this population in inflamed intestinal tissue; alternatively, it could be the end result of extensive stimulation, followed by exhaustive depletion of an inducer T cell population.

In parallel studies we determined that patients' cells contained normal numbers of HNK-1-positive cells. This is a marker associated with natural killer function and indeed we found that patients with Crohn's disease have normal NK function as assessed against two separate target cells (K-562 & Chang) at a variety of effector to target cell ratios. This normalcy in NK function contrasts with previous studies of such function in this disease, a discrepancy probably explained by the fact that the patient population studied is far less seriously affected than was previous populations.

In parallel studies, we observed the depletion of patients T cell populations with anti-OKT8 and complement abrogated suppressor activity, indicating that covert suppressor activity was caused by OKT8-positive cells. Similar treatment with anti-OKT4 and complement had no such effect. Of somewhat greater interest was the fact that anti-HNK-1 + C' also destroyed suppressor activity. Thus, the suppressor cells in Crohn's disease bear both OKT8 and HNK-1 markers. Indeed, in extensive studies using FACS analysis we found that patients' T cell populations contained cells bearing both HNK-1 and OKT8 whereas in normals these markers were found in largely non-overlapping T cell populations. These studies indicate that suppressor T cell subsets are different from normal supressor T cell subsets in that they bear an additional surface antigen normally found only on natural killer cells. Thus, the suppressor T cell population in Crohn's disease can be distinguished from normal suppressor T cells even before fractionation on anti-$F(ab')_2$ columns.

Significance: These studies demonstrate for the first time that Crohn's disease is associated with T cells bearing both suppressor markers (OKT8) and natural killer cell markers (HNK-1). We believe that these cells represent activated suppressor cells (they are also radiation-resistant) and probably arise from the GI tract as a result of the underlying disease process. In this latter regard, our working hypothesis is that Crohn's disease is caused by an imbalance between regulatory cells normal generated in mucosal immune tissue, which leads to a prolonged and damaging inflammatory response.

Proposed Course: Several lines of research are suggested by the above studies.

1.) As indicated above, the suppressor cells in Crohn's disease are covert, i.e., only revealed after cell fractionation. We have done numerous preliminary studies which suggests that the suppressor T cells are opposed by "contrasuppressor" T cells in whole cell populations and this accounts for their functional silence in peripheral blood specimens. A problem encountered in our attempts to substantiate this possibility

is that it is difficult to separate suppressor and contrasuppressor T cell activity, since both probably bear OKT8 antigens. In that we have now established that suppressor T cells bear HNK-1 antigens it is now becomes feasible to eliminate suppressor actvity while retaining contrasuppressor activity. We therefore plan to treat T cell populations with anti-OKT4 (helper) and anti-HNK-1 to obtain cells rich in contrasuppressor cells but depleted of helper and suppressor cells;

2.) In collaboration with Dr. C. Elson of the Medical College of Virginia, we plan to study cells obtained from disease bowel segments of CD patients as well as diseased control patients to determine if increased or decreased suppressor T cell activity is present at this site and how such activity correlates with the presence or absence of circulating suppressor T cells. In this way we hope to gain insight into the question as to whether or not excessive or deficient suppressor T cell activity at mucosal sites can explain the disease on a primary basis.

3.) We plan to widen our study of covert suppressor T cells to include patients with related diseases, such as patients under various drug regimens and relatives of patients with Crohn's disease. In the latter regard, if covert suppressor T cell activity is found in related but unaffected individuals then this immunoregulatory defect may be regarded as a primary rather than secondary abnormality.

4.) Finally, we plan to establish long term T cell lines from inflammed tissue of Crohn's disease patients, in order to study the in vitro balance between various regulatory T cell populations at sites of disease. For this purpose, cells will be maintained in continuous culture with interleukin II (IL-2).

STUDIES ON IMMUNOLOGIC DEFECTS IN PRIMARY BILIARY CIRRHOSIS

Background: Primary biliary cirrhosis (PBC) is a progressive inflammatory disease of the liver which leads to intrahepatic cholestasis, cirrhosis and, eventually, hepatic failure. It occurs mainly in females and is not susceptible to treatment with corticosteroids.

Certain features of primary biliary cirrhosis suggest that immunological factors play an important role in the pathogenesis of the disease. These include pathologic changes in the liver such as lymphocytic infiltration of bile ducts, granulomas, lymphoid aggregates, and piecemeal necrosis of hepatocytes, the presence of elevated titers of non-organ specific auto-antibodies such as anti-mitochondrial antibody, the occurrence of high levels of circulating immune complexes, increased complement catabolism, and the frequent association of PBC with other diseases which are suspected of having an immunologic basis.

In previous studies of this condition we found that when cell cultures containing syngeneic mixtures of purified B cells and T cells obtained from PBC patients were set up, the T cells failed to manifest suppressor cell activity on PWM-stimulated Ig synthesis normally manifest at high T cell/B cell ratios. However, such suppressor activity was seen in cultures

containing purified patient T cells and allogeneic normal B cells. This finding suggested to us that patients are not capable of generating suppressor cells as a result of autologous interactions but are capable of generating such cells as a result of allogeneic interactions. In studies of PBC reported previously, we investigated this possibility by studying the ability of patients' T cells to mount proliferative responses when exposed to autologous and allogeneic non-T cells. In these studies we found that the patients had a marked deficiency in the ability to generate an autologous mixed lymphocyte reaction (MLR), but have a normal ability to generate an allogeneic MLR.

In studies reported last year we found that the autologous MLR was an immune reacion involving, in part, the stimulation of OKT4-positive T cells by lymphoblastoid B cells and resulted in the generation of suppressor T cells. Thus this reaction appears to be an important antigen-nonspecific negative feedback loop controlling immune responses. A key defect in PBC therefore appears to be a defect in the activation of an inducer cell necessary for suppressor T cell activation.

In the current period we further subtantiated this viewpoint in several ways:

1.) We demonstrated that cells from PBC patients do not generate suppressor cell activity after activation with the B cell mitogen, Epstein-Barr virus (EBV). In these studies we stimulated cultures composed of B cells and T cells with EBV and noted the production of Ig-producing cells at seven and 14 days using a reverse hemolytic plaque assay. In normal individuals (who are seropositive for EBV) 14 day plaques are reduced compared to seven day plaques because of the generation of suppressor T cells during the culture period. Such suppressor T cells are probably induced because the cultures represent an autologous MLR containing activated stimulator cells. In constrast, we found that in patients with PBC, suppressor cell generation was markedly decreased. Thus, these studies demonstrate in direct way that PBC is marked by decreased inducer T cell function and subsequent reduced suppressor T cell generation.

2.) In the past we have demonstrated that PBC is associated with reduced natural killer cell function (NK activity). It has been shown by others that natural killer cell function is dependent on inducer T cells which elaborate lymphokines necessary for NK function in PBC could be due to the same inducer T cell defect which also results in suppressor T cell abnormalities (as described above). In the current studies we corroborated the fact that PBC cells have low NK function using two different target cells. We also showed that this reduced activity was associated with the presence of normal number of circulating NK cells (defined by monoclonal antibodies specific for NK cells, OKM-1 and HNK-1). Finally we demonstrated that NK activity was boosted by the addition of interferon to a degree proportion to that found in normal individuals. Thus, we have established that the cellular elements necessary for normal NK function is present and therefore that reduced NK function is probably due to defective NK induction by lymphokines normally released by inducer cells.

Significance: These new observations improve our understanding of the underlying antigen-non-specific defect in PBC. In particular, it is now apparent that a major immune defect in this disease involves the function of a central OKT4-positive inducer cell which fails to generate suppressor T cells important in feedback regulation of activated B cells and in the generation of NK cells. This defect could consist of the failure to recognize autologous antigens in the autologous MLR or in the failure to generate lymphokines following such recognition and stimulation. Since patients with PBC also manifest defects in B cell activation, it is possible that the T cell regulatory abnormality described above operates in tandom with B cell abnormalities to result in autoimmune disease.

Proposed Course: The results obtained above suggest several additional studies of PBC and the autologous MLR.

1.) In the first place, the nature of the antigen on stimulator cells capable of activating autoregulatory T cells will be studied. In studies performed elsewhere it has already been determined that antigens controlled by histocompatibility genes (D-region antigens or I-region antigens) are important in the autologous MLR. We plan to investigate if other cell surface antigens also play a role in this reaction. For this purpose we have been producing monoclonal antibodies with specificity for activated B cells. Antibodies will be selected on the basis of their ability to inhibit the autologous MLR, but not the allogeneic MLR.

2.) The cells responding in the autologous MLR will be characterized more completely. In these studies we plan to establish long term cell lines of autoreactive cells with the use of T cell growth factors (IL-2) and to identify the surface phenotypes and functional characteristics of the cell line thus obtained. Using such cell lines we will be able to compare the characteristics of autoreactive cells with alloreactive cells and with antigen-reactive cells.

3.) In follow-on studies the capacity of patients with PBC to express NK activity, we plan to obtain autoreactive T cells from patients and normals (T cells activated by autologous B cells) and compare their ability to induce NK activity. In addition such cells will be compared as to their capacity to elaborate lymphokinesis possibly relevant to induction of NK cells.

4.) Finally, we plan to investigate the identity of suppressor cells induced during the autologous MLR. More spacifically, we will determine if such cells bear the OKT4 or OKT8 phenotype since cells in both of these T subsets have been shown to mediate suppressor functions. This study will be done in conjunction with studies of suppressor T cell induction by long term lines of autoreactive cells obtained from normal individuals (as mentioned above) and from patients with PBC.

Prizes and Awards:

Merrill Lecturer, Medical College of Virginia, April, 1982

Leo H. Criep Alumni Lectureship, 38th Annual Meeting, American Academy of Allergy

Publications:

James, S.P., Vierling, J.M., and Strober, W.: The role of the immune response in the pathogenesis of primary biliary cirrhosis. Seminars in Liver Disease 1:322-337, 1981.

James, S.P., Yenokida, G.G., Graeff, A.S., Elson, C.O., and Strober, W.: Immunoregulatory function of T cells activated in the autologous mixed lymphocyte reaction. J. Immunol. 127:2605-2609, 1981.

James, S.P., Yenokida, G.G., Graeff, A.S., and Strober, W.: Activation of suppressor T cells by autologou lymphoblastoid cells: a mechanisms for feedback regulation of immunoglobulin synthesis. J. Immunol. 128: 1149-1154, 1982.

Strober, W., Richman, L.K., and Elson, C.O.: The regulation of gastrointestinal immune responses. Immunology Today 2: 156-161, 1981.

Kawanishi, H., Saltzman, L.E., and Strober, W.: Characteristics and regulatory function of murine Con A-induced, cloned T cells obtained from Peyer's patches and spleen: mechanisms regulating isotype-specific immunoglobulin production by Peyer's patch B cells. J. Immunol. In press.

Strober, W., Elson, C.O., Graeff, A., and Richman, L.K.: Class-specific T cell regulation of mucosal immune responses. In Strober, W., Hanson, L.A., and Sell, K.W. (Eds.): Recent Advances in Mucosal Immunity. New York, Raven Press. In press.

Strober, W.: The regulation of the mucosal immune system. J. Aller. and Clin. Immunol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04015-12-MET

PERIOD COVERED October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Development and Function of Humoral and Cellular Immune Host Defense Mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

LAB/BRANCH Metabolism Branch, DCBD, NCI

SECTION Cellular Immunology Section

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TOTAL MANYEARS: 6	PROFESSIONAL: 4	OTHER: 2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study is directed towards determining mechanisms important to the development and function of humoral and cellular immune responses; the nature of deficiency in immune function in patients with primary immunodeficiency or immunodeficiency secondary to malignancy; and developing approaches to prophylaxis and/or therapy of infections and neoplastic disease associated with immune processes.

Project Description

Objectives: The objectives of this study were to determine mechanisms important to the development and function of both humoral and cellular immune responses in man and animals; to determine the nature of the deficiency in immune function characterizing such disease states as hypogammaglobulinemia, ataxia telangiectasia, the Wiskott-Aldrich syndrome, intestinal lymphangiectasia, and advanced malignancy; and to develop approaches to prophylaxis and/or therapy of the infections and neoplastic disease frequently associated with defective immune processes. This plan is directly related to Objective 2, Approach 1 of the National Cancer Plan.

Methods Employed: Antibody responses of experimental animals and man were determined after immunization with a variety of antigens and the antibodies were detected by standard techniques or methods developed in our laboratory. Cellular immune responses were measured by delayed hypersensitivity skin testing, testing for contact sensitivity to dinitrochlorobenzene and by skin allograft rejection. Proliferative responses in vitro were tested using nonspecific mitogens, specific antigens, and allogeneic cells in mixed lymphocyte culture. Immunoglobulin secretion by activated lymphocytes was measured by a newly developed reversed hemolytic plaque assay and this test was applied to cultured cells stimulated with a variety of mitogens, viruses, and chemical agents. Studies of the characteristics of various cell surface receptors on immunocompetent lymphocytes and monocytes-macrophages utilized autoradiography, immunofluorescence microscopy, and cellular rosette formation.

Major Findings: The studies of the interaction of the immune system of man with the Epstein-Barr virus (EBV) have been a major ongoing interest of the Cellular Immunology Section. EBV, a virus of the herpes group, is the etiologic agent of infectious mononucleosis and has been implicated in the pathogenesis of African Burkitts lymphoma, nasopharyngeal carcinoma, and in rare cases of immune deficiency disease and agammaglobulinemia. When EBV is added to resting human B lymphocytes in tissue culture, the virus transforms these cells into continuously growing long term cell lines, a probable reflection of the oncogeneic potential of this agent. Since this ubiquitous virus specifically attacks cells of the immune system, the B cells, and is capable of transforming these cells, normal host defense mechanisms responsible for the control of this agent are particularly interesting and important. In our previous studies we demonstrated that in addition to causing transformation, EBV infected resting normal B cells in culture are activated to secrete immunoglobulin. This in vitro response has proven to be particularly useful for studies of cellular immune phenomenon involved in defense against EBV. We have been able to define distinct T cell mediated regulatory mechanisms that occur in response to acute viral infection (i.e., in acute infectious mononucleosis) and after recovery in normal EBV seropositive subjects. In acute infectious mononucleosis exceptionally potent suppressor T cells are found in the patient's blood one to two weeks after viral infection. These suppressor T cells appear in response to the generalized B cell activation induced by the virus and function to inhibit or suppress any further B cell responses. Such suppressor cells block B cell activation

not only by EBV but also by all other activators as well. Because the EBV becomes integrated into the genome of the host B cells and may persist there for life, suppressor mechanisms which prevent virus induced B cell activation and transformation may be critical for survival. However, the suppressor T cell response in infectious mononucleosis would appear impractical for long-term control since it shuts down all forms of B cell activation and thus could result in agammaglobulinemia. In fact, we have studied patients with agammaglobulinemia following infectious mononucleosis and these patients were found to have retained the intense suppressor T cell activity in the peripheral blood T cells characteristic of patients with infectious mononucleosis.

We have found that the intense polyclonal and nonspecific T suppressor activity found in an acute EBV infection does disappear following clinical recovery and is replaced by another suppressor activity which is now restricted to the Epstein-Barr virus. In vitro this activity is revealed as a distinctive late appearing suppression of EBV induced immunoglobulin production. Following in vitro EBV infection of peripheral blood lymphocytes, the cultures begin producing an exponentially increasing number of immunoglobulin secreting cells after two to three days. In cultures established from EBV nonimmune subjects, this response continues to increase throughout a 14 day period of observation. In cultures from patients with infectious mononucleosis, no immunoglobulin production is observed during this period due to the presence of the suppressor T cells previously mentioned. In patients who have recovered from infectious mononucleosis, that is normal EBV immune subjects, the cultures begin to respond normally during the first week to 10 days of observation and then a striking late appearing suppression occurs so that by day 14 the response is inhibited by over 90%. This late suppression is seen only in EBV immune donors and is specific for EBV induced B cell activation. Thus such immune individuals now express a virus specific form of immunoregulatory cell which is capable of preventing endogenously EBV infected B cells from expressing uncontrolled B cell activation.

The capacity of the Epstein-Barr virus to activate these cells in culture has provided a unique tool for the study of various aspects of B cell activation and regulation in man. One of the areas of particular interest has been the effects of histocompatibility antigens on immune responses in vitro. A genetic restriction of the interaction between cells of the immune system has been repeatedly demonstrated in certain systems, while cellular cooperation in other immune systems occurs readily across allogeneic barriers. With pokeweed mitogen as a stimulant of B cell differentiation in culture, T cells, B cells, and macrophages from individuals differing in genetic makeup can cooperate with each other to produce a normal in vitro response. Thus T cells obtained from one subject can help B cells from another subject to produce immunoglobulin in vitro. Even though such a mixture of allogeneic cells may result in a mixed lymphocyte reaction occuring in such cultures, such a mixed lymphocyte reaction appears to have no significant consistent effect on the ultimate response of the B cells in such culture. Although Epstein-Barr virus does not require T cells for its ability to activate B cells to produce immunoglobulin, we have observed that the presence of allogeneic T cells in

such cultures has a profound effect on B cell differentiation when normal T cells from one individual are mixed with B cells from another and the co-culture is stimulated with Epstein-Barr virus. In a majority of cases such cultures fail to produce immunoglobulin. If the allogeneic T cells have been irradiated with 2000 R, the B cells are capable of responding and producing a normal immunoglobulin response suggesting that in the allogeneic co-culture an active process of suppression is occuring which prevents the EBV induction of B cell activation while at the same time having no significant effect on the capacity of pokeweed mitogen to induce B cell activation. In order to determine whether this suppressor effect observed in allogeneic cultures stimulated with EBV was related to antigens encoded by the major histocompatibility complex in man, family studies were undertaken. Such studies demonstrated that when family members shared all antigens of the major histocompatibility complex no suppressor effect was observed in coculture. When members of families differed at antigens in the major histocompatibility complex then the suppressor effect was observed, demonstrating that this genetic restriction was linked to HLA, the major histocompatibility complex of man. Further studies of co-cultures between B cells and T cells of individuals differing by single or multiple antigens in the HLA complex were then undertaken. It was shown that the suppressor reaction that occured in such allogeneic mixed cultures did not occur when the only differences in such culture were at the HLA-D locus. Thus, even though a significant mixed lymphocyte reaction might be occuring in culture no suppression was found when the only genetic difference was at the D locus. By contrast in allogeneic cell mixes in which D locus identity was present, but in which significant differences occurred at the A or B locus antigens, marked suppression of the EBV response was seen. Thus the activation of suppressor T cells effective in inhibiting EBV induced B cell activation by allogeneic interactions is tightly linked to recognition of A and B antigens of the HLA major histocompatibility complex in man. Studies are now in progress to use this observation of allogeneic suppression as a tool to study suppressor mechanisms in patients who may have disorders of immunoregulation.

EBV is the agent responsible for infectious mononucelosis and greater than 90% of normal adults have been infected with this virus. In spite of the ubiquitous nature of this infection, the malignancies associated with EBV are rare and restricted to very specific ethnic groups or geographic regions. However, increasing evidence is appearing that the virus may be associated with another disease common to all ethnic groups and geographic regions, rheumatoid arthritis. Patients with rheumatoid arthritis have elevated serum antibody titers to certain EBV associated antigens. EBV infected lymphocytes in culture will produce rheumatoid factor and EBV rapidly induces B cell lines from rheumatoid arthritis patients' peripheral blood lymphocytes. We have now studied over 50 patients with rheumatoid arthritis to determine whether this apparent association with the Epstein-Barr virus may be related to a defect in the cellular immune regulatory functions which we have described for normal individuals. We found that usual assays for helper and suppressor T cell functions and B cell activity in these patients were normal. Very strikingly, however, we found that the late acting suppressor T cell which inhibits EBV induced B cell activation in normal immune subjects

was markedly deficient in these rheumatoid arthritis patients even though they were immune to EBV as determined by serum antibodies to this virus. All of the adult patients with rheumatoid arthritis have revealed this defect in specific immunoregulatory T cell function. Children are also susceptible to rheumatoid arthritis, a disorder termed juvenile rheumatoid arthritis (JRA). This disease have been subcategorized into three groups. Those patients with single joint disease or monoarticular arthritis, those patients with multiple joint involvement and those patients with an acute febrile disease termed Stills disease. In our studies of children, we have found that all patients with muliple joint involvement have been immune to EBV as determined by the presence of antibodies to EBV related antigens. All of these pediatric patients shared with the adult patients with rheumatoid arthritis the striking defect in late suppressor T cell activity directed towards EBV. By contrast pediatric patients with a single joint involved with rheumatoid arthritis have either demonstrated no indication of EBV infection or if they had antibodies to the EBV demonstrated normal T suppressor activity similar to normal EBV immune subjects. Thus these data demonstrate that a major portion of the pediatric population with juvenile rheumatoid arthritis may also have this striking abnormality of immunoregulatory T cells function related to the EBV and that the clinical classification of disease severity based on clinical symptomatology and numbers of joints involved seems to closely correlate with presence of absence of an EBV related immune abnormality. We have not as yet had the opportunity to study children with acute Stills disease to determine if such patients have an abnormality in relating to this specific virus.

The importance of these T cell regulatory mechanisms for the EBV has been recently emphasized by the description of EBV containing lymphomas appearing in transplant recipients receiving intensive immunosuppressive drug treatment. A recently introduced agent with particularly profound immunosuppressive effects is the drug, cyclosporin A. Since several renal transplant patients receiving cyclosporin A immunosuppression have developed EBV containing lymphomas, we evaluated the effects of this drug on the in vitro T suppressor systems controlling EBV. We found that the drug has no effect on B cell responses to EBV permitting a normal stimulation of both proliferation and immunoglobulin production by the virus. However, the presence of the drug totally blocked the T suppressor function generated by exposure to alloantigen as described above as well as T helper cell activity needed for B cell activation by pokeweed mitogen. Interestingly the cyclosporin A was unable to block the suppressor T cell activity observed in infectious mononucleosis, a T suppressor cell which is activated in vivo before the in vitro assay is performed. Importantly, however, cyclosporin A did totally block the late T suppressor activity seen in normal EBV immune subjects and in fact the drug has proven to be an exceptionally useful agent for obtaining an endogenous EBV infected B cell lines in such subjects. Thus this unique immunosuppressive agent appears to inhibit this essential T cell function for the control of endogenous EBV infected B cells.

Another area of major activity in the cellular immunology section over the past few years has been an attempt to define the nature of the cellular structures involved in self-self and self-non-self recognition and in intracellular communication in immune and non-immune host defense processes. Dr. Muchmore's laboratory has identified a system of recognition of foreign targets by non-immune mononuclear phagocytes based on the interaction of effector cell surface lectin-like receptors with carbohydrate determinants on the target cell. This recognition system appears to be phylogenetically ancient in that it can be found in primative invertebrates such as the starfish as well as in mammals and man. We have exended these studies to explore the possibility that such lectin-carbohydrate interactions might also be involved in immunoregulatory signals between lymphoid cells. As described earlier, T cells from patients with infectious mononucleosis are profoundly inhibitory to the process of B cell activation in vitro by mitogens or by EBV. In order to test for a possible lectin-sugar interaction in this immunoregulatory process, suppressed lymphocyte cultures containing normal B cells, infectious mononucleosis T cells, and pokeweed mitogen were supplemented with high concentrations of a variety of monosaccharides, disacchardies and oligosaccharides and subsequent immunoglobulin production was measured. Over 30 tested sugars showed no activity in this system. However, two related monosaccharides were found which had a striking effect. D-mannose and alpha-methyl-D-mannose almost totally reversed the suppression caused by the infectious mononucleosis T cells permitting immunoglobulin production to occur in these cultures. At 25 millimolar these sugars reversed the suppression by an average of 85% and substantial activity could be detected with sugar concentrations as low as 2 millimolar. In preliminary studies a variety of synthetic mannose analogs have been tested and several compounds show activity at concentration as low as 50 micromolar. We have also studied two other situations in which similar suppressor phenomena are observed, the human newborn and in some patients with common variable hypogammaglobulinemia. The activity of suppressor T cells present in human cord blood is also removed by mannose and alphamethyl-mannoside. In suppressed indicator cultures containing cells from agammaglobulinemic patients, additional evidence for sugar-lectin mediated immunoregulation was found. Suppressor T cell activity from two patients was reversed by mannose or yeast mannan and suppressor monocyte activity in another patients was reversed by n-acetyl-d-glucosamine. These data appear to support the hypothesis that sugar lectin recognition system that evolved in primative invertebrate mononuclear phagocytes as a means of distinguishing foreigness has been preserved and expanded in higher vetebrates to form the basis of certain immunoregulatory cellular interactions as well.

Additional studies of patients with common variable hypogammaglobulinemia have led to an entirely new series of observations relating to the influence of certain steroid hormones on B cell differentiation in vitro. In studies of the suppressor T cells present in certain patients with hypogammaglobulinemia, it has been observed that these suppressor cells are sensitive to both irradiation and to hydrocortisone. Thus steroids or irradiation are able to block the suppressor activity of such cells. During the course of these experiments we observed that hydrocortisone

alone cultured with normal lymphocytes seemed to induce immunoglobulin production in culture. Human peripheral blood lymphocytes cultured with hydrocortisone or many of its analoges including dexamethasone, prednisolone, and methyprednisolone were found to produce in culture as many immunoglobulin secreting cells as did cultures stimulated with pokeweed mitogen. Kinetic studies showed that this response to steroid hormone in vitro began to appear at a about the 4th day and peaked somewhat later than pokeweed mitogen at the 8th to the 10th day of culture. Other steroid hormones such as dyethylstilbesterol and testosterone did not induce the activation of B cells in culture. Aldosterone, a steroid hormone with weak glucocorticoid activity, stimulated B cells to produce immunoglobulin only when used at very high concentrations in vitro. In order to determine the cellular basis for such stimulation, peripheral blood lymphocyte populations were separated into T cells, B cells and monocytes. The glucocorticoids were found to be unable to stimulate immunoglobulin production by purified B lymphocytes alone. Both monocytes and T lymphocytes were required in the cultures for glucocorticoids to have their stimulatory activity on B lymphocytes. Since glucocorticoids are able to reverse the suppressor T cell activity seen in certain pathologic conditions and also appear to be able to reverse normal suppressor T cell activity, a potetential mechanism for the action of steroid hormones in inducing B cell activation would be the inactivation of a suppressor cell maintaining such B lymphocytes in an inactive state. T cells irradiated with 2000 R to inactivate the radiosensitive suppressor T cell were mixed with B lymphocytes in culture. Such cell mixtures produced no more immunoglobulin than that produced by B lymphocytes alone. However, when corticosteroids were added to such mixtures a marked increase in immunoglobulin production was observed. Thus although we have not been able to directly demonstrate that corticosteroids are not simply inactivating a suppressor T lymphocytes, other procedures which inactivate supressor T cells do not result in the activation of immunoglobulin production by B cells without the addition of the glucocorticoid hormones.

To further examine the cellular sites of action of the steroid effect, soluble factors produced by cultured T cells were tested for their ability to substitute for intact T cells in this response. Supernatants from T cells cultured without stimulants for 24-48 hours were added to B cell cultures. Such supernatants were incapable of substituting for intact T cells when the cultures were stimulated with pokeweed mitogen, but they were able to replace T cells when corticosteroids were used as the stimulant. Thus, it appears that the principal site of action of steroid hormones in this system is upon the B lymphocytes. However, the B cell response to steroids is also clearly restricted to only a subpopulation of B cells. For example, human neonatal B cells do not respond with immunoglobulin production following corticosteroid stimulation. In addition, IgE production is only minimally enhanced in normal subjects with steroid stimulation while large amounts of IgE are produced by by lymphocytes from allergic subjects when stimulated with steroids. This lack of stimulation of cord blood lymphocytes, the preferential stimulation of IgE production in allergic subjects, and the lack of

detectable proliferation in steroid stimulated cultures actively producing immunoglobulin, have led us to propose that steroids are inducing a subset of memory B cells to produce immunglobulin in vitro.

Significance to Biomedical Research:

The present studies extend our understanding of the diverse, yet interrelated mechanisms contributing to the development and normal functioning of immune system in animals and man. They indicate that the normal expression of immune function is dependent on multiple processes and that defects in immunity may be the result of factors influencing a variety of these processes. For example, disfunction of the immune system can occur through such diverse factors as defects in differentiation on the one hand or through the mediation of exogenous viral pathogens on the other. Thus human disease may result from a deficiency of immune elements as in certain forms of agammaglobulinemia or from an excess of certain immune functions as in other types of agammaglobulinemia associated with excessive suppressive T cell activity. Suppressor T cell function may be part of a normal defense mechanism in such diseases as infectious mononucleosis and a deficiency of normal suppressor T cell activity may lead to the expression of certain autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus. Our new insights into the role of sugar-lectin interactions as immunoregulatory signals offers potential for precise definition of this communication process and for future therapeutic manipulation of these signals.

Proposed Courses:

Continue the studies directed toward the understanding of the pathways involved in the development and function of normal immune responses and the application of insights gained through such studies for the development of new approaches for the prevention and therapy of human disease.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04016-10-MET

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Mechanism of the Anabolic Action of Growth Hormone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION
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CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rat embryo fibroblasts have been shown to produce MSA (multiplication stimulating activity) an insulin-like growth factor. the MSA produced by the rat embryo fibroblast has been shown to be identical to the MSA species produced by the BRL-3A rat liver cell line on the basis of behavior in polyacrylamide gel electrophoresis and high pressure liquid chromatography. Placental lactogen has been shown to stimulate the biosynthesis of MSA by the rat embryo fibroblasts. An insulin-like growth factor receptor has been purified from chondrocytes isolated from a rat chondrosarcoma. The purified receptor has been shown to have a molecular weight of 220,000 and to display binding characteristics identical to the receptor on intact cells.

Project Description:

Objectives: The understanding of normal growth may be a prerequisite to understanding malignant growth. One experimental model for the study of normal growth is the growth hormone deficient animal following treatment with growth hormone. The anabolic effects of growth hormone are thought to be mediated by a second hormone called somatomedin. Somatomedin is a member of a family of peptides called insulin-like growth factors. The object of the project is to purify and characterize one of the insulin like growth factors called multiplication stimulating activity (MSA), study the regulation of the production of MSA, develop cell culture systems in which to study the biochemical events accompanying growth stimulated by MSA.

Methods Employed: MSA is being purified from serum-free medium conditioned by a rat liver cell line (BRL-3A) using ion exchange chromatography, gel filtration, and high pressure liquid chromatography (HPLC). Tertiary cultures of rat embryo fibroblasts derived from 16 day embryos are being used to study hormonal regulation of production of MSA. (autoradiography with [^{3}H]thymidine). The MSA produced by the rat embryo fibroblasts is characterized using a specific MSA radioimmunoassay, radioreceptor assays, competitive protein binding assay, and bioassay ([^{3}H]thymidine incorporation into DNA in chick embryo fibroblasts). Cell surface receptors for MSA are identified using radioiodinated MSA. An insulin-like growth factor receptor is being purified from chondrocytes isolated from a chondrosarcoma by homogenization, subcellular fractionation by differential centrifugation, solubilization of a membrane fraction with nonionic detergent followed by affinity chromatography using MSA-Sepharose. Receptor purification is followed by measurement of binding of radioiodinated MSA and separation of bound tracer from free tracer with albumin coated charocoal.

Major Findings:

We reported several years ago that MSA is found in high concentrations in fetal rat serum and declined to reach low levels by day 20 of extrauterine life. This observation led to the observation that MSA may be a fetal growth factor. We found that rat embryo fibroblasts have receptors for MSA and respond to MSA with increased DNA synthesis and increase in cell number. We also found that rat embryo fibroblasts produce MSA. MSA produced by the rat embryo fibroblasts had been shown to behave identically to MSA produced by the BRL 3A rat liver cell line in a radioreceptor assays, radioimmunoassays and bioassays. In addition we have now shown that the MSA produced by the rat embryo dfibroblasts is indistinguishable from BRL MSA by polyacrylamide gel electrophoresis and high pressure liquid chromatography. Thus the rat embryo fibroblast in culture constitutes a so-called autocrine or paracrine system (cells produce a growth factor which acts on the same or neighboring cells to produce a growth response.

The hormonal regulation of fetal growth is poorly understood. Placental lactogen is a polypeptide produced in large quantity by the placenta. Placental lactogen or chronic somatomamotrophin has a high degree of amino acid sequence homology with growth hormone and had been proposed as a candidate for a hormonal regulator of fetal growth. It was therefore of interest to test the effect of placental lactogen on MSA production by the rat embryo fibroblasts in culture. The addition of placental lactogen to the rat embryo fibroblasts caused a significant increase in MSA levels in the medium conditioned by the cells. By contrast, growth hormone, thyroxine, glucocorticoid, and platlet derived growth factor were without effect. Placental lactogen did not affect the half-life of MSA in medium conditioned by the cells but did stimulate the incorporation of radiolabeled amino acid into MSA as indentified by immunoprecipation with MSA antisera followed by polyacrylamide gel electrophoresis. Placental lactogen did not stimulate the incorporation of radiolabeled amino acid into total TCA precipitable material. We conclude that placental lactogen stimulates the synthesis of MSA by the rat embryo fibroblasts.

Several years ago, in collaboration with Richard Stevens and Vincent Hascall, we reported that insulin-like growth factors, including MSA, stimulate proteoglycan synthesis in chondrocytes derived from the Swarm rat chondrosarcoma. We next reported that receptors for the insulin-like growth factors could be identified in these cells. Competitive binding studies and analysis of receptor size by combining radiolabeled insulin-like growth factors to whole cells or membranes followed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SPS-PAGE) and autoradiography have indicated two types of insulin-like growth factor receptors. One type of receptor prefers IGF-I over IGF-II, recognizes insulin, and has a binding subunit size of Mr 130,000. A second type of receptor prefers IGF-II over IGF-I, is insulin insensitive, and has a binding subunit of Mr 220,000. Both crosslinking and competitive binding studies indicate that the chondrosarcoma chondrocytes have a predominance of the IF-II type receptors. In fact, Scatchard analysis of receptor number indicates that these receptors are present in very high numbers, as many as 2-5 x 10^5/cell. We have begun the purification of the IF-II type receptor from these cells. We purified the receptor by first preparing a 100,000 x g membrane fraction by differential centirfugation of a cell homogenate. The receptor was solubilized from the membrane preparation by nonionic detergent and further purified by MSA affinity chromatography and gel filtration on Sepharose 6B. The purified receptor was a simple major band on SDS-PAGE of Mr 220,000 in agreement with crosslinking studies on whole cells. By chemically crosslinking ^{125}I-MSA to the purified receptor we demonstrated that binding activity was associated with the Mr 220,000 band. Competitive binding studies on the purified receptor are identical to binding studies on intact cells, IGF-II >> IGF-I, and insulin does not compete for binding.

Significance to Biomedical Research:

The finding that rat embryo fibroblasts produce MSA, an insulin-like growth factor proposed as a regulator of fetal growth provides a system to study the hormonal regulation of MSA production. The observation that placental lactogen stimulates MSA biosynthesis by these cells supports the proposal that placental lactogen may regulate fetal growth by stimulating the production of MSA.

The ability to purify one of the IGF receptors from the rat chondrosarcoma cells open up a number of avenues of research including the examination of IGF receptor structure and the development of monoclonal antibodies to this receptor.

Proposed Course:

We plan to extend the observations on rat embryo fibroblasts regarding the control of MSA production to human fetal fibroblasts and examine whether the human counterpart of MSA (IFG-II) is made by these cells and if so whether or not placental lactogen regulates the biosynthesis.

We plan to develop monoclonal antibodies to the IGF-II type receptor and use these antibodies for a variety of studies including direct demonstration of importance of the receptor in the growth response and regulation of receptor biosynthesis.

We will continue to examine skin fibroblasts from patients with short stature who are candidates for an organ resistance to somatomedin.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04017-06-MET

PERIOD COVERED
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TITLE OF PROJECT (80 characters or less)

Biology of the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SUMMARY OF WORK (200 words or less - underline keywords)

Studies were undertaken to measure specific cytotoxic T-cell responses and humoral antibody responses by B-cells in vitro in normal individuals and patients with immunologic deficiency states. Patients were heterogeneous with regard to their ability to generate cytotoxic T-cells in vitro. Most patients with hypogammaglobulinemia produced cytotoxic T-cells normally, while patients with the Wiskott-Aldrich syndrome and ataxia-telangiectasia produced almost no cytotoxic T-cells. Cells from normal individuals produce specific antibody in vitro when stimulated with antigen. This was also true for 5 of 11 patients with hypogammaglobulinemia who produced no antibody in vivo. Thus a subset of hypogammaglobulinemic individuals have an immune deficiency which can be corrected in vitro. In contrast none of 7 ataxia-telangiectasia patients and none of 5 Wiskott-Aldrich syndrome patients produced antibody in vitro. Defects in the production of cytotoxic T-cells and specific antibodies may contribute to the increased incidence of neoplasia observed in immunodeficiency diseases.

Project Description:

Objectives: Exposure to infectious agents and other foreign antigens elicits biologic response phenomena in host tissues which may confer protection to the host organism upon subsequent exposure to the same antigen(s). These biologic phenomena are termed immune responses and have generally been divided into two major types 1) cell-mediated responses which require the immediate presence of immune cells for their effects to be manifest, and 2) humoral responses in which cells elaborate soluble molecules such as antibody which may exert their protective effects at some distance from the immune cells which produce them. Studies were undertaken to investigate the biology of cellular and humoral immune resopnsiveness in normal individuals and in patients with a variety of immunodeficiency diseases in which congenital and acquired defects in host reponsiveness are associated with an increased incidence of infection and malignant tumors. Particular emphasis was placed on studying the mechanisms underlying perturbations of immune function in these patients which might account for their increased incidence of neoplasia.

Methods Employed: Over the past year, special emphasis was placed on the study of antigen-specific cellular and humoral immune responses by human peripheral blood leukocytes in vitro. Two major classes of immune responses have been under investigation: 1) cell-mediated cytotoxicity responses by leukocytes whose development is thymus dependent (T-cells), and 2) humoral antibody responses produced by a second class of leukocytes, (B-cells) whose development is bone marrow dependent and requires T-cells for full maturation. Both T-cell and B-cell immune responses are highly dependent on monocytes which are a third type of mononuclear leukocyte. In vivo, these cellular and humoral responses normally occur concomitantly in a highly ordered fashion with multiple cellular interactions occurring among sub-populations of T-cells, subpopulations of T-cells with B-cells and macrophages, and B-cells with macrophages. These cellular interactions result in a finely regulated response which is promptly initiated and appropriately terminated following antigenic exposure.

A major component of host responsiveness to a variety of antigens may take the form of tissue destruction (cytolysis) whereby antigen bearing cells are lysed and eliminated. Cells capable of directly mediating such cytolytic events are termed cytotoxic effector cells. Although we have demonstrated that several types of leukocytes can function as cytotoxic killer cells, we have recently focused our attention on cytotoxic T-cells which possess specific cell surface receptors for antigens expressed on those cells which undergo lytic events (target cells). Cytotoxic immune T-cells are undetectable in the peripheral blood of non-immunized individuals. Human cytotoxic T-lymphocytes (CTL) with receptor specificities for surface antigens present on the cells of unrelated individuals may be generated during seven days of in vitro culture in which density gradient centrifugation prepared responder peripheral blood mononuclear leukocytes are immunized by coculture with mononuclear leukocytes from a non-related individual whose proliferative capacity has been inhibited by irradiation. In such cultures, responder CTL precursors proliferative and mature into cytotoxic effectors whose lytic activity is measured by a 6 hour radioisotopic release assay employing ^{51}Cr labelled target cells from the stimulating cell donor. These immune CTL

recognize a series of target cell surface molecules termed transplantation of histocompatibility antigens, which differ among individuals of the same species (alloantigens) and these cytotoxic effectors are therefore termed alloimmune CTL. Alloimmune CTL probably play a major role in the host rejection of histoincompatible allografts such as that occurring in kidney or heart transplantation and also in graft versus host disease in which an immunoincompetent host receives a graft of immunocompetent T-cells and the donor graft attacks host tissues.

Immune CTL have also been produced with receptor specificities for various chemical and viral antigens. Studies have demonstrated that individuals immunized either in vivo or in vitro with chemical haptens or viral antigens produce CTL which will lyse homologous antigen bearing autologous (self) target cells but will lyse antigen bearing target cells from other individuals only when the immune CTL and the target cell share genetically determined histocompatibility antigens. The relevant histocompatibility antigens which must be shared between target and immune CTL are those encoded by a genetic locus termed the major histocompatibility complex (MHC) which in humans codes for the human leukocyte antigens (HLA) A, B, C, DR, and D.

These cytotoxic T-cells with receptor specificities for foreign antigen plus autologous MHC antigens are termed self-MHC restricted CTL. Evidence from studies in experimental animals has suggested that self-MHC restricted CTL play a major in vivo role in the recovery from virus infections, immunologic surveillance against neoplasia, and deleterious autoimmune phenomena. In humans we have produced CTL with receptor specificities for chemical haptens (trinitrophenol-TNP) and autologous MHC antigens in vitro by seven days primary immunization of responder peripheral blood mononuclear leukocytes with hapten modified autologous luekocytes, followed by five additional days of secondary in vitro stimulation with fresh or cryopreserved autologous haptenated luekocyes. Lytic T-cell activity is detected by a 6 hour radioisotope release assay employing ^{51}Cr labelled TNP-modified target cells. Human CTL with specificities for viral antigens and self MHC gene products are produced in vitro by culturing responder peripheral blood mononuclear leukocytes with infectious allantoic fluid containing influenza A/Hong Kong virus or irradiated influenza virus infected autologous leukocytes for seven days. Such cultures proliferate and generate CTL whose lytic activity is tested on autologous or allogeneic virus infected targets in a short term ^{51}Cr release assay.

The second major area of emphasis centered on the assessment of humoral immune function by human peripheral leukocytes in vitro. In these studies we concentrated on measuring specific humoral antibody production by B-lymphocytes. The presence of specific antibody in biological fluids can be detected by a variety of methods including agglutination with particulate antigens, hemolysis employing antigen coated erythrocytes and radioimmunoassays. Each of these methods offers differing levels of sensitivity in detecting specific antibody. Recently, a very sensitive technique for measuring specific antibodies in the nanogram to picogram per milliter range has been described which involves the detection of antigen bound immunoglobulin thru an enzyme-conjugated second antibody directed against the bound first antibody. This technique has been termed the Enzyme Linked ImmunoSorbent Assay (ELISA). In a usual assay,

termed the Enzyme Linked ImmunoSorbent Assay (ELISA). In a usual assay, antigen (i.e. virus) is first allowed to bind nonspecifically in the wells of a mutliwell plastic plate and non-bound antigen is washed away. A source of putative antibody (human serum or in vitro human leukocyte culture supernatant) against the bound antigen is then added, incubated, and non-bound antibody is washed away. Next, an enzyme (alkaline phosphatase) conjugated heterologeous (rabbit) antibody directed against human antibody is added, incubated, and unbound conjugated antibody is washed away. Finally, enzyme substrate (paranitrophenolphosphate-PNPP) is added, incubated, and the conversion of colorless PNPP to yellow paranitrophenol (PNP) product is measured in a spectrophotometer. The amount of substrate PNPP converted to product PNP is related to the amount of enzyme conjugated antibody bound which in turn is proportional to the amount of human anti-virus antibody bound to the immobilized antigen. Such an ELISA employing purified whole influenza virus as the antigen was chosen for the measurement of human specific antibody production in vitro. The ELISA which has been developed is rapid, sensitive and antigen specific. To assess specific anti-virus antibody production in vitro by human B lymphocytes peripheral mononuclear leukocytes are first washed extensively with tissue culture media to remove residual traces of serum antibody adherent to the cells. Then, mononuclear leukocytes or purified subpopulation of these leukocytes (T-cells, B-cells) are cultured in vitro with infectious alloantoic fluid containing the antigenically distinct influenza viruses A/Hong Kong (A/Hong Kong/8/68-x-31 [H3N2]) or B/Hong Kong (B/Hong Kong/8/73/); or with formalin-inactivated, zonally purified A/Aichi (A2/Aichi/68 MN 25241 [H3N2]) and B/Hong Kong (B/HK/15/72 Rx 3560-1). After twelve days of in vitro culture, cumulative secretion of anti-influenza A or anti-influenza B virus antibody into culture supernatants is detected by ELISA.

Major Findings: Cytotoxic T-Cells Responses by Human Peripheral Blood Leukocytes In Vitro: Studies were continued to define the cellular requirements for the in vitro generation of alloimmune and self-MHC restricted CTL, and the potential of cells from patients with immunodeficiency diseases to produce self-MHC restricted CTL. Previously, we had demonstrated that highly purified human T-cells were necessary and sufficient as responders in the production of alloimmune and self-MHC restricted CTL when stimulated with irradiated allogeneic leukocytes and irradiated virus infected autologous leukocytes, respectively. Additional studies were undertaken to define the requirement for monocytes in both cytotoxic systems. Peripheral blood mononuclear leukocytes containing T-cells, B-cells and monocytes were rigorously depleted of monocytes by sequential removal of cells capable of ingesting particulate iron and then cells possessing receptors for the chrystalyzable fragment (Fc) of immunoglobulin G. Such monocyte-depleted lymphocytes did not generate self MHC restricted CTL when influenza virus itself was added to these cultures but could generate virus specific CTL when mixed with irradiated adherent monocytes which themselves did not contain CTL precursors. Thus the in vitro generation of virus-specific self MHC restricted CTL was shown to be dependent on both T-cells and monocytes. In additional studies, the presence of adherent monocytes bearing antigenic specifications recognized by the heteroantiserum anti-p, 23,30 which recognizes human antigens similar to murine Ia (immune-associated) antigens were shown to be required for the generation of influenza specific CTL. When responder cells for the generation of alloimmune CTL were equally rigorously monocyte-depleted, the generation of CTL was diminished

but not abolished. However, when both responder and the irradiated stimulator cells were monocyte-depleted no CTL were produced. The addition of irradiated adherent monocytes from either the responder or the stimulator individual restored the response even though neither population of monocytes contained CTL precursors. Thus the generation of human alloimmune CTL in mixed leukocyte cultures was dependent on monocytes function in the responder cells only when the irradiated sitmulator population was monocyte-depelted. Since mixed leukocyte cultures and the generation of alloimmune CTL are routinely used for the assessment of immunocompetence in many patient studies, this observation is critical to the interpretation of these results. Monocyte defects within the responder cell population may only be observed when a stimulator cell population of peripheral blood leukocytes is devoid of monocytes. On the other hand, when virus immune CTL are produced by the direct addition of virus to peripheral blood mononuclear leukocytes, the absence of CTL production can result from T-cell and/or monocyte defects in the responder cell population.

The remainder of our studies of cytotoxic T-cell function focused on examining the capacity of peripheral blood leukocytes from immunodeficiency disease patients to produce antigen-specific self-MHC restricted CTL. Given the observation that these CTL are restricted to recognize antigen and self-MHC gene products, one might ask how these receptor specificities are acquired? Studies of the cellular maturation processes and cell-cell interactions required for the production of virus-specific self-MHC restricted CTL in experimental animals have suggested the following scheme: 1) bone marrow stem cells differentiating into T-cells within the thymus develop receptor specificities for those MHC gene products expressed on the epithelium of the thymus prior to antigenic exposure, 2) these receptor specificities which then constitute the repertoire of MHC gene products considered as self are stable in the peripheral post thymic T-cell population, 3) T-cells expressing receptor specificities for foreign antigens plus self-MHC gene products are then selected from this peripheral T cell pool when antigen is encountered in association with irradiation resistant lymphoreticular cells (? monocytes), and 4) the maturation of such antigen-specific self restricted cells and may be positively and negatively regulated by other T-cell subsets termed helper-and suppressor-T cells; respectively.

Since results in experimental animals suggested that self-MHC restricted CTL play a major role in the recovery from viral infections and the lysis of autologous tumor cells, studies were undertaken to assess virus-specific self-HLA restricted reponses in patients with immunodeficiency diseases who have frequent infections and an increased incidence of neoplasia. One such immunodeficiency disease is termed common variable hypogammaglobulinemia. These patients have low levels of antibodies (gammaglobulin) in their serum and fail to make specific antibodies following in vivo antigenic challenge. In contrast to this observed functional B-cell defect, the patients exhibit a variable pattern of T-cell immunocompetence. Of eleven common variable hypogammaglobulinemic individuals studied thus far, the peripheral blood mononculear leukocytes of nine patients have produced influenza virus-immune CTL capable of lysing autologous virus-infected target cells. Since the assay system employs virus immune CTL tested on infected autologous target cells, negative results could be caused by either defective CTL generation and/or target cells which did not express the relevent antigenic structures

(HLA antigens, virus antigens) recognized by immune effectors. In the two patients in which virus-immune CTL for autologous targets were not detected, this lack of responsiveness was shown to be a defect in effector CTL generation since virus-infected target cells from both individuals were lysed by HLA compatible immune CTL from normal donors. Moreover, this deficiency within the effector cell population was not simply attributable to the lack of T-cells since both patients possessed normal proportions of T-cells within their cultured peripheral blood mononuclear leukocytes. Thus the majority of patients with common variable hypogammaglobulinemia possessed leukocytes capable of generating virus-immune, HLA restricted CTL; an experimental finding in accord with clinical observations that these patients are predominantly troubled by recurrent bacterial rather than viral infections.

Another human immunodeficiency disease studied for virus-specific CTL production in vitro was ataxia-telangiectasia. This disease is an autosomal recessive disorder characterized by progressive spinocerebellar degeneration leading to a clumsy gait (ataxia), oculocutaneous dilated blood vessels (telangiectasia), recurrent infections, and a high incidence of neoplasia. Histopathologic examination of the thymus gland from ataxia-telangiectasia patients reveals a thymus which is fetal in appearance and almost totally devoid of epithelial elements. Since the aforementioned studies of the development of virus-immune CTL in experimental animals suggested a critical role for the thymic epithelium in the maturation of self-MHC receptor specificties on CTL precursors, we were particularly interested in whether cells from ataxia telangiectasia patients could produce antigen-specific self-HLA restricted CTL. Of the 11 ataxia telangiectasia patients studied thus far, the mononuclear leukocytes of ten have failed to produce significant lysis on autologous virus infected target cells. In these ten patients, this lack of CTL function was shown to be a defect in effector CTL production since virus-infected patients' cells were lysed by normal HLA-related immune CTL. This defect was not readily attributable to lack of T-cells within the responder leukocytes since the proportion of T-cells among the patients' cultured leukocytes was not significantly different than normal. Additional studies were therefore undertaken to investigate the mechanism of this unresponsiveness in ataxia-telangiectasia patients. The serum of nearly all ataxia-telangiectasia patients contains elevated levels of alpha-fetoprotein, a fetal protein not normally found in adult serum. Since immunosuppressive effects have been ascribed to alpha-fetoprotein, we considered the possibility that the non-responsiveness of these patients might be due to serum inhibitory effects. Therefore, normal peripheral blood mononuclear leukocytes were cultured with virus in the presence of 5% normal pooled plasma (usual dulture conditions), or 5% pooled plasma from ataxia-telangiectasia patients with elevated alpha-fetoprotein levels.

We found that normal cells generated the same virus-immune CTL activity in plasma from ataxia-telangiectasia patients as in normal plasma. Thus ataxia-telangiectasia plasma did not appear to extend marked immunosuppressive effects on normal cells and the non-responsiveness of patient's leukocytes was not easily attributable to plasma inhibitory effects. An additional mechanism which might account for the non-responsiveness of ataxia-telangiectasia leukocytes is the presence of excessive cell-mediated immunosuppression. In order to evaluate the potential of excessive suppressor

cell activity, coculture experiments were undertaken in which cells from one ataxia-telangiectasia patient which did not produce virus immune CTL were mixed in an equal proportion with cells from an MHC identical normal sibling which were capable of producing virus-immune CTL. Such MHC-matched patient-sibling combinations are required for these experiments to avoid the generation of alloimmune CTL. If excessive cellular suppression was responsible for the non-responsiveness in the patient then this suppression might be expected to reduce the CTL activity generated by the sibling's cells. This experiment demonstrated that the MHC matched sibling's cells generated the same CTL: activity whether or not they were cocultured with the patient's cells. Thus in this one available patient-sibling combination, excessive cellular suppression did not appear to be the mechanism whereby leukocytes from the patient failed to generate self-MHC restricted CTL. The mechanism of this lack of responsiveness to viruses in association with self-HLA antigens in patients with ataxia-telangiectasia is still under investigation. Defective monocyte and/or T-helper cell functions remain to be teseted. However, this unresponiveness to viruses and self-MHC antigens may play a role in these patients undue susceptibility to recurrent infections and neoplasia.

The third immunodeficiency disease studied for virus-immune self-MHC restricted CTL production in vitro is the Wiskott-Aldrich syndrome. The Wiskott-Aldrich syndrome is a sex-linked recessive disorder characterized by low peripheral blood platelet counts (thrombocytopenia) recurrent infections, a skin conditions termed eczema, and an increased frequency of malignant neoplsms. Common viral infections such as chickenpox may be life-threatening in these patients and in one case neoplasia was associated with persistent urinary excretion of a papova virus and the same virus was isolated from the patient's tumor. In seven Wiskott-Aldrich syndrome patients studied thus far, the peripheral blood lekocytes of six failed to lyse influenza virus-infected, autologous target cells. The one patient who responded produced only minimal CTL activity which was greater than 16 fold less than simultaneously assayed normal controls. In all six patients not manifesting CTL activity, this defect was attributable to deficient production of immune CTL effectors since the virally infected patients' target cells were lysed by virus immune CTL from normal, HLA-related individuals. To investigate whether this defect in the production of immune CTL in the Wiskott-Aldrich syndrome is limited to viruses, experiments have been initiated to investigate the capacity of patient's cells to mediate TNP-specific cytotoxicity. One patient studied thus far also lacked peripheral blood leukocytes mediating TNP-specific cytotoxicity. This non-responsiveness was attributable to defective CTL effector production since the patient's TNP modified target cells were lysed by the TNP-immune, HLA-related normal CTL. Thus patients with the Wiskott-Aldrich syndrome are deficient in their ability to produce immune cytotxic T-lymphocytes in vitro.

Recently, immunohematologic normalization has been reported in patients with the Wiskott-Aldrich syndrome following bone marrow ablative therapy and transplantation of bone marrow from MHC identical siblings. Two patients whose cells lacked the capacity to produce virus-immune CTL prior to such therapy generated normal influenza immune CTL activity six months and 18 months following transplantation therapy which was performed by Drs. O'Reilly and Good at the Sloan-Kettering Institute. An additional Wiskott-Aldrich

patient who received immunosuppressive therapy (without bone marrow ablation) and bone marrow transplantation from a MHC indentical sibling, also possesses a normal potential to generate influenza immune CTL in vitro. While the precise mechanism(s) of non-responsiveness to viruses and self-MHC gene products in the Wiskott-Aldrich syndrome remains unresolved, this defective production of virus-immune CTL is correctable by bone marrow transplantation following either immunosuppression or bone-marrow ablative therapy.

These studies of virus-immune self-MHC restricted CTL production demonstrate a heterogeneity in responsiveness among immunodeficiency disease patients. Most hypogammaglobulinemic patients but not patients with ataxia-telangiectasia on the Wiskott-Aldrich syndrome are capable of generating immune CTL in vitro. These defects in the production of immune CTL may contribute to the pathogenesis of recurrent infections and the high incidence of neoplasia in some of these patients.

Humoral Immune Responses by Human Peripheral Blood Leukocytes In Vitro: Studies were initiated to develop methods for the assessment of specific humoral antibody production by human peripheral blood mononuclear leukocytes in vitro. Antibody molecules belong to a class of serum proteins termed the gammaglobulins. Since these gammaglobulins can confer immunity they are often termed immunoglobulins (Ig) and several distinct sub-classes of immunoglobulins (IgM, IgG, IgA, IgD and IgE) are known to exist. Immunoglobulin secreting cells are derived from a class of leukocytes called B-cells which possess cell surface immunoglobulin as antigen receptors. Studies in experimental animals have shown that transition of B-cells into immunoglobulin secreting cells is antigen-dependent, monocyte-dependent, and for most antigens requires the positive influence of T-helper cells and is negatively regulated by T-suppressor cells.

Much of our understanding of the maturation and immunoregulation of the human humoral immune response has derived from in vitro studies employing polyclonal activators wuch as pokeweed mitogen (PWM) and the Epstein-Barr virus which activate immunoglobulin secreting cells through receptors which are not antigen-specific. We have developed a method for studying antibody production by human PBMC in vitro which is antigen-induced and does not require the presence of polyclonal activators. For these studies, we have again employed influenza viruses as antigens. Cultures of peripheral blood mononuclear cells from >95% of normal adult individuals produce specific anti-influenza virus antibody in vitro in the presence of type A influenza viruses. Antibody secretion requires de novo protein synthesis, begins about day 5 of culture and reaches maximal rates between days 5 and 7 of culture. Antibody synthesis can be induced by both live influenza type A and B viruses as infectious allantoic fluid or formalin-inactivated, zonally purified type A and B viruses. Antibody generation was both antigen-dependent and virus-specific at the induction phase since: 1) cultures in media alone or stimulated with normal allantoic fluid produced no antibody and 2) cultures stimulated with type A viruses produced anti-influenza A antibody but not anti-influenza B antibody and vice versa. The production of antibody was shown to require the cooperative ineraction of T-cells, B-cells and monocytes in culture. This requirement for T helper cells capable of promoting the maturation of B-cells into antibody secreting cells was further investigated

using a series of hybridoma derived monoclonal antibodies directed at predominantly distinct subsets of human T-cells. Using this methodology the human T-helper cell for anti-influenza antibody responses was shown to bear the specificity recognized by the monoclonal antibody OKT4 but not the specificity recognized by the monoclonal OKT8 which recognizes virus immune CTL effectors. Thus a subset of human T-cells comprising 40-60% of the total T-cell population was identified as the helper cell subset for specific antibody responses by B-cells in vitro.

Studies were also undertaken to investigate the fine specificity of the human in vitro antibody response to influenza virus. Cultures of PBMC were stimulated with the following purified formalin-inactivated whole influenza viruses: A/Aichi/68(H3N2), A/Bankok/79/(H3N2), A/USSR/77/(H1N1) and B/Hong Kong. Antibody production was assayed by ELISA on plates coated with each of the viruses as well as plates coated with purified viral hemagglutinin (H3 and H1) molecules. Antibody production was influenza type specific in that cultures stimulated with B/Hong Kong made little or no antibody against any of the type A viruses and vice versa. There was also specificity among influenza virus subtypes - that is, cultures stimulated with A/USSR/(H1N1) made only 29% as much antibody directed against A/Aichi(H3N2) as was directed against the stimulating H1N1 virus and cultures stimulated with A/Aichi(H3N2) made only 19% as much antibody against A/USSR(H1N1) as against the homologous virus. Using the ELISA for antibody to purified virus hemagglutinin (one of the two major viral surface glycoproteins and the one responsible for virus attachment to cells), antibody to hemagglutinin was demonstrated in cultures stimulated with whole viruses of H#N2 and H1N1 subtypes. Moreover, cultures stimulated with H1N1 virus produced only 10% as much antibody to purified H3 as they did to purified H1 and cultures stimulatd with H3N2 virus produced only 2% as much antibody to purified H1 as they did to purified H3. And lastly, when early (A/Aichi/68[H3N2]) and late (A/Bankok/78/[H3N2]) strains of H3N2 virus were studied it was observed that 3 of 4 individuals made more antibody to A/Aichi than to A/Bangkok when stimulated with either virus - perhaps reflecting the phenomena of "original antigenic sin". Two major conclusions can be drawn from these in vitro studies of the B-cell repertoire to influenza virus: 1) the in vitro response faithfully recaptiulates the in vivo response and 2) unlike our previous studies demonstrating that influenza-immune cytotoxic T-cells were cross-reactive between subtypes (i.e. cells stimulated with H3N2 virus lyse H3N2, HON1 and H1N1) the B-cell repertoire for influenza is largely non-cross reactive among subtypes.

Since studies employing certain polyclonal activators such as pokeweed mitogen had demonstrated that allogeneic T and B cells could cooperate for the synthesis of immunoglobulin and a large body of evidence in experimental animals suggested that this might not be the case for specific antibody synthesis, studies were undertaken to examine the effects of allogeneic T-cell and B-cell interactions on the human in vitro specific antibody response to influenza virus. In eight allogeneic combinations studied, mixtures of 5×10^6 B-cells with 2×10^6 allogeneic T-cells produced only 25% as much specific antibody as the same B-cells with autologous T-cells ($p<.005$). Experiments to examine the mechanism of this effect demonstrated that irradiation of the T-cells reduced antibody response with autologous T-cells and resulted in increased antibody responses with allogeneic T-cells. These

studies with high T-cell density (2×10^6) suggested suppression as the mechanism for reduced antibody production with allogeneic T-cells. When cultures with low T-cell density were studied; however, a different pattern was observed. Cultures with $.5 \times 10^6$ B-cells and $.125 \times 10^6$ allogeneic T-cells made more antibody than cultures with autologous T-cells. Thus mixtures of B-cells and allogeneic T-cells can result in both positive and negative allogeneic effects, and depending on the culture condition, either can predominate.

Studies were also initiated to study the ontogeny of in vitro immunologic responsiveness to influenza viruses. These experiments were begun by examining the antibody resopnses of cord blood mononuclear cells (CBMC). CBMC from six neonates made no measurable in vitro antibody response to influenza virus. In addition, unlike adults, CBMC failed to proliferate to influenza virus demonstrating that the virus is not a non-specific mitogen. Moreover, CBMC made little or no antibody when stimulated with EBV. These results are consistent with lack of previous exposure to influenza and/or immaturity of the newborn immune system. However, cocultures of irradiated cord blood T-cells with allogeneic adult B-cells produced specific antibody to influenza virus. Thus, in spite of lack of previous exposure to influenza virus, cord blood T-cells are able to mediate positive allogeneic effects for specific in vitro antibody production.

Studies have been initiated to utilize this method to study maturational and immunoregulatory abnormalities in patients with immunodeficiency disease. Three patients studied with X-linked hypogammglobulinemia and isolated growth hormone deficiency failed to produce antibody in vitro in spite of having the capacity to mount a specific cytotoxic T-cell response to the same virus. In addition all three patients' cells could provide help to normal allogeneic B-cells and did not inhibit antibody production by cells from normal individuals. Thus these patients have an immune defect involving antibody secreting cells or their precursors. Of eleven hypogammaglobulinemic patients studied with a different disease termed common variable hypogammaglobulinemia (CVH), five made measurable antibody responese in vitro. Since CVH patients make specific antibody responses poorly in vivo, the finding of intact in vitro antibody responsiveness suggests that some patients have a host environmntal abnormality as the cause of their hypogammaglobulinemia which can be overcome in vitro.

Specific antibody response were also sought in patients with ataxia-telangiectasia and the Wiskott-Aldrich syndrome who lacked influenza specific CTL responses. Six of seven patients with the Wiskott-Aldrich syndrome failed to produce specific antibody as did four or five patients with ataxia-telangiectasia. Further studies were undertaken to define the cellular basis of this non-responsiveness in three ataxia-telangiectasia patients. One of the patients was shown to have intact monocyte function since his irradiated adherent cells reconstituted antibody resopnses in macrophage depleted cultures of his MHC matched sibling's cells. All three patients had immunocompetent B-cells since their cells produced antibody when: 1) stimulated with the polyclonal B-cell activator Epstein-Barr virus, and 2) provided T-cell help in the form of allogeneic irradiated T-cells. These results suggested a defect in T-helper cells as partial cause for the immunodeficiency in ataxia-

telangiectasia. However, when T-cells from two patients were added to purified allogeneic B-cells both were capable of "helping" antibody synthesis. These results suggest the existence of two subsets of T-cells, one which can help allogeneic B-cells and the second which helps autologous B-cells and that ataxia-telangiectasia patients are lacking the latter subset. These patient's T-cells behave as the previously mentioned cord blood mononuclear cells, again providing evidence for maturational arrests in ataxia-telangiectasia.

Thus, we developed a method to study specific antibody responses by human peripheral blood mononuclear cells in the absence of polyclonal activators. This assay in combination with assays of self-MHC restricted CTL function toward the same antigens will prove to be a powerful tool for investigating maturational and immunoregulatory events in humans.

Studies were also initiated to investigate the immunobiology of B-cell activation by polyclonal activators that were T-cell and monocyte independent (B95-8 strain of EBV) or T-cell and monocyte dependent (PWM). For these studies, total IgM and IgG immunoglobulin were measured in cell culture supernatants by ELISA. To investigate the EBV viral requirements for human B-cell activation, multiple microwells containing non-limiting numbers of B-cells (10^5/well) were cultured for 12 days with serially greater dilutions of the B95-8 strain of EBV. A plot of the number of wells producing either IgM or IgG versus the virus dilutions was consistent with a "single hit" model by Poisson analysis indicating that one infectious virus particle was sufficient to induce a B-cell to produce immunoglobulin. Additional studies using cultures containing limiting dilutions of B-cells, saturating concentrations of EBV, and "feeder" layers of irradiated T-cells showed that the mean frequency of EBV activatable B cells for IgM secretion was 1 in 146 (range 1:58 to 1:219) and for IgG secretion, 1 in 284 (range 1 152 to 1:420). When PWM was used instead as the polyclonal activator, the mean frequency of activatable B-cells for IgM was 1 in 1596 (range 1:222 to 1:3212) and for IgG was 1 in 1470 (range 1:172 to 1:3755). Thus the frequency of B-cells activatable by EBV is greater than that of B cells activatable by PWM. Moreover, when multiple replicate cultures containing limited numbers of B-cells and saturating amounts of EBV were tested for both IgM and IgG, the B-cell precursors secreted IgM or IgG, but not both isotypes. Similar results were obtained with PWM. Thus, human peripheral blood B-cell do not undergo isotype switching with either T-cell dependent (PWM) or T-cell independent (EBV) polylconal activators.

Proposed Course: Studies will be continued to assess T-cell mediated specific cytotoxic responses in normal individuals and in patients with immunodeficiency. Studies in experimental animals and man have suggested that the T-cell subsets involved in the production of immune CTL (i.e. CTL precursors, T-helper cells, T-suppressor cells) may be separable on the basis of cell surface antigens. Recently methods have been developed whereby antibodies recognizing only sub-populations of human peripheral blood T-cells can be produced (hybridoma antibodies). Such antibodies will be tested for their ability to recognize T-cell subsets in normal individuals which are required for the generation of immune CTL and also to identify immune CTL effectors. If antibodies useful in dissecting CTL responses in normal individuals are found, these reagents will be used to further probe the mechanism(s) or defective specific CTL responses in immunodeficiency diseases

(i.e. lack of CTL precursors, T-helper cells or monocytes, excessive T-suppressor cells). Attempts will be made to assess hapten-specific CTL responses generated in vivo by immunization with the cutaneous application of dinitrochlorobenzene. Additional studies of hapten-specific CTL generation in vitro will be undertaken in patients with the Wiskott-Aldrich syndrome and ataxia-telangiectasia to further asess the scope of defective CTL production in these individuals.

With regard to humoral antibody responses in vitro, studies will be expanded to further define the proportion of normal individuals capable of responding with antibody production to the influenza viruses and other antigens and the optimal in vitro culture conditions for generating such resopnses. The aforementioned studies employing hybridoma antibodies recognizing T-cells and additional antibodies possibly recognizing monocyes and B-cells will be expanded in attempts to further define the cellular interactions necessary to generate a response. Using purified virus antigens (i.e. virus hemagglutinin, virus neuraminidase), the specificity of the in vitro secreted antibody will be further defined. Attempts will be made to establish T-cell lines which may be cloned to proved purified subsets of T-helper and T-suppressor cells for further analysis. Studies of specific humoral immune responsiveness in vitro will be continued in patients with immunodeficiency diseases, particularly those patient groups already defined as having defective specific CTL responses (ataxia-telangiectasia, Wiskott-Aldrich syndrome) and those hypogammaglobulinemic patients with normal CTL responses. Specific CTL reponses and specific antibody responses will then be studied in parallel to further elucidate the mechanism(s) involved in these patients susceptibility to recurrent infection and cancer.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04018-06-MET

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Study of Human Immune Defense Mechanisms and Its Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Andrew V. Muchmore, M.D.	Senior Investigator	MET	NCI
OTHER:	Samuel Broder, M.D.	Senior Investigator	MET	NCI
	R. Michael Blaese, M.D.	Senior Investigator	MET	NCI
	Robert Hall, M.D., Ph.D.	Clinical Associate	MET	NCI
	Dean Mann, M.D.	Senior Invest.	IMM	NCI
	Bruce Littman, M.D.	Associate Professor		
		Medical College of Virginia, Richmond		

COOPERATING UNITS (if any)

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SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are aimed at understanding how the human immune system recognizes and destroys foreign cells. Special emphasis is placed on early antigen nonspecific cytotoxic responses (spontaneous cytotoxicity), which precede specific immune recognition. Both cell mediated and humoral mediated forms of spontaneous cytotoxicity have been examined. We have also purified to homogeneity as new class of immunosuppressive carbohydrate from human pregnancy urine. Finally in a collaborative study we have successfully made human human hybridomas from patients with systemic lupus erythematosus.

Project Description:

Objectives: The objectives of these studies were to 1) delineate the importance in man of a newly recognized system of non-specific cell mediated spontaneous cytotoxicity, 2) examine the effect of antisera which recognize gene products encoded by the human DRw locus on assays of cellular and human immunity, 3) characterize and produce human human hybridoma antibodies from patients with systemic lupus erythematosus, 4) examine the phylogeny of antigen non-specific cytotoxicity systems in vivo 5) assess the molecular mechanisms involved in some forms of cell-cell cooperation, 6) asses the effect of various chemotherapeutic anti-neoplastic drugs on spontaneous cytotoxicity, and 7) purify a new class of immunosuppressive molecules from human pregnancy urine.

Methods Employed: In vitro cellular cytotoxicity systems have been developed for antibody dependent cellular cytotoxicity, mitogen induced cellular cytotoxicity, cell mediated lympholysis, and spontaneous monocyte mediated cytotoxicity using a sensitivity micro ^{51}Cr release assay for various target cells. These results were correlated with standard in vitro and in vivo assays of cell mediated and humoral mediated immunity. For the characterization of immunoglobulin class specific Fc receptors on human T cells, purified IgG, IgM, and IgA chromatography, gel filtration and antigen specific absorption of certain monoclonal mouse myeloma proteins.

Major Findings: Our laboratory is continuing four major projects and is embarking on a fifth. Dr. Robert Hall has been working for the last year characterizing the molecular requirements for the expression of synergistic cytotoxicity. This model which measures the ability of human serum factors to activate human monocytes and lmyphocytes and to kill erythrocyte targets is now substantially characterized. Homogenous purified complement components obtained in collaboration with Dr. Harvey Colton of Boston have been used and we have shown that C5 and Factor B in the presence of activated human monocytes (which fail to kill by themselves) are necessary and sufficient to induce lysis. Lymphocytes also exhibit killing in this model and require at least C7 and C8 as well as C5 and factor B. This research is important not only because it represents a unique model of cell mediated cytotoxicity but also because it unites antigen idependent fluid phase lysis (alternate complement pathway) with antigen independent cell mediated lysis (NK and spontaneous cytotoxicity).

Dr. Bruce Littman has successfully formed human-human hybridomas from the peripheral B cells of patients with systemic lupus erythematosus (SLE). Not only were findings obtained using a classic HGPRT deficient drug marked human B cell line but more exciting is the successful fusion of SLE B cells onto a normal human B cell line using diethyl pyrocarbonate. This is the first example of successful B cell fusion using this very useful and general technique.

Our laboratory has also embarked on a major long term effort aimed at purifying immunoregulatory glycoproteins and glycolipids from human pregnancy urine. These efforts are just now coming to fruition. We have purified to homogeneity (by the criteria of high performance thin layer chromatography) a small molecular weight carbohydrate compound which is immunosuppressive at concentrations as low as 100 nanograms per ml. We are collaborating with Dr. Bo Willson in studies aimed at analyzing the biochemical properties of this compound.

We have continued our clinical interest in assays of spontaneous monocyte mediated cytotoxicity and have developed a reliable in vitro assay which measures inhibition of monocyte cytotoxic function by very small numbers of tumor cells. This assay correlates well with our previous in vivo observations using peripheral blood from patients with malignant disease and more important activating agents such as x-irradiation reverse tumor induced suppression. These studies are extremely important because they imply a unique mechanism resulting in actual activation of cytotoxic monocytes by agents which are normally thought of as toxic and will allow us to dissect the suppressive effects of tumor cells on monocyte cytotoxicity in vitro. Such activation may play an important in vivo role. We have also studied anti-inflammatory drugs used in the treatment of rheumatoid arthritis (ASA, Indocin, steroids and gold) and find that these two can enhance monocyte function.

We have continued a very basic thrust of our laboratory attempting to characterize sugar specific cell mediated recognition. We believe that certain sugars, L-rhamnose, D-mannose, and N acetyl glucosamine act by preventing different forms of cellular recognition phenomena. We have attempted to further characterize the mannose receptor in collaboration with Dr. Alan Rosenthal of Merck.

Significance to Biomedical Research: Our studies on both monocyte mediated cellular cytotoxicity and factor B dependent cell mediated cytotoxicity have several major implications. Our data suggest that a variety of "nonspecific" in vitro killing assays are in reality quite specific and represent target spceific cell surface sugar recognition. Our phylogenetic studies imply this phenomonen is ubiquitous. We believe this type of cytotoxicity represents an important aspect of host immune defense which is independent of prior antigen exposure. From a clinical perspective, we have evidence that both therapeutic levels of x-ray and the radiometric drug Cis PPD enhance in vitro killing. This enhancement occurs via different mechanisms ... x-ray inactivates a suppressor cell while Cis PDD appears to directly activate monocytes. We propose that this phenomena may be an important mechanism by which these agents exert their anti-neoplastic effects in vivo as well as their well described direct toxicity towards tumor cells. We have recently analyzed a large group of patients with malignancy and have shown them to have defective in vitro

monocyte mediated killing. Our evidence suggests that successful Cis PDD treatment results in restoration of monocyte cytotoxicity in vitro and in vivo. Our data concerning the effect of various sugars on in vitro assays of antigen specific proliferation have enormous implications concerning how cells communicate with each other. At this juncture we are postulating that carbohydrate recognition plays a central role in self-self and self-non-self recognition phenomena.

Our studies with SLE B cell fusions have led to the production of monoclonal human hybridoma antibody. These antibodies not only promise to be invaluable tools for the study of this poorly understood human disease but also the technique promises to offer enormous potential for the production of other human antibodies. Finally our studies on human pregnant urine suggest that we have discovered a whole new class of immunoregulatory carbohydrates which may be very useful in dissecting further the control of immunologic reactivity during pregnancy.

Proposed Course: Our laboratory will continue to characterize the nature of carbohydrate receptors and the effect of blocking these receptors on in vitro and in vivo immune reactivity. We will extend our studies to an in vivo model in efforts to control immunologic responses in an intact animal in order to gain insight into possible approaches and therapy of diseases which are the result of over active immune responses. We are embarking on a major effort to screen chemotherapeutic anti neoplastic agents both in vivo and in vitro to asses their capacity to activate monocyte mediated killing.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04019-09-MET

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immunoregulatory Cells in Human Neoplasia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Dean Mann, M.D.	Senior Investigator	IMM	NCI
	David Poplack, M.D.	Senior Investigator	POB	NCI
	Zeev Lando, Ph.D.	Visiting Fellow	MET	NCI
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COOPERATING UNITS (if any)

LAB/BRANCH

Metabolism Branch, DCBD, NCI

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	3	2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to analyze the cellular control of immune function in normal individuals and in certain individuals with neoplastic diseases. We are currently determining the helper and suppressor activity of neoplastic cells from patients with a variety of T-cell lymphomas. We are also currently attempting to develop human (and subhuman primate) monoclonal antibodies directed against human T-cell surface membrane antigens and against the antigens encoded by a retrovirus which is associated with T-cell lymhpomas in certain geographic clusters. Finally, we are analyzing cultured lines of B cells derived from patients with T-cell lymhpomas to determine the isotype preference, susceptibility to normal immunoregulatory influences, and heavy chain switching events in these populations.

Project Description:

Objectives: T cells play a critical role in a number of immune systems. These cells are involved in cell-mediated immunity which includes such phenomena as delayed hypersensitivity, allograft rejections, graft vs. host reaction, tumor cell killing in some systems, and lysis of virally-infected target cells. Normal T cells proliferate in vitro in the presence of plant mitogens, such as phytohemagglutinin and concanavalin A. They may mediate the mixed-lymphocyte-reaction, act as killer cells in certain models of lymphocyte-induced cytotoxicity, and generate soluble effector proteins such as lymphokines. In addition, T cells play an indispensable role in regulating humoral immune response by acting as potentiators (helper cells) or inhibitors (suppressor cells) of the transition of B cells into immunoglobulin producing plasma cells. Both antigen-specific and nonspecific helper and suppressor functions have been identified.

In this project, we have examined the regulatory capacity of neoplastic T-cells derived from patient's with several kinds of lymphoproflierative disorders. We have also examined the immunoglobulin-secreting activity of certain B-cell clones derived from such patients. We have also examined the feasibility of developing monoclonal human antibodies (or closely related Gibbon monoclonal antibodies) directed against neoplastic T-cells or retroviral antigens associated with a human T-cell neoplasm.

Methods Employed: To study the transition of circulating human lymphocytes into immunoglobulin secreting plasma cells, peripheral lymphocytes were cultured in the presence of pokeweed mitogen, a water soluble mitogen derived from a Nocardia opaca, and Epstein-Barr virus. The amount of IgM, IgA and IgG synthesized and secreted into the media was determined by double-antibody radioimmunoassays, by the reverse hemolytic plaque assay or by enzyme-linked solid-phase assays (ELISA). The presence of circulating suppressor cells was assayed by a co-culture technique. Circulating neoplastic T cells (and T-lines) from various patients and peripheral blood indicator lymphocytes from normal control individuals were co-cultured. The synthesis of immunoglobulin by cells of the two subjects in the co-culture was related to the sum of the expected contribution of each population. In order to assay helper activity purified B cell populations, freed of T cells, were used as indicator cells in the presence of pokeweed mitogen. It should be noted that pokeweed mitogen is a highly T-cell dependent activator, whereas Epstein-Barr virus (EBV) and Nocardia opaca water soluble mitogen are relatively thymic independent polyclonal activators.

We have established and are now carrying both cloned and uncloned T-cell lines, which are T-cell growth factor dependent. Certain cell-surface-membrane markers were analyzed using monoclonal antibodies in an indirect fluorescence activated cell sorter (FACS) system.

Monoclonal Antibodies: In our attempt to develop monoclonal antibodies directed against either core proteins or envelope proteins encoded by a retrovirus (HTLV) associated with certain human T-cell tumors, we are following two pathways. First, we have identified several patients with T-cell lymphomas who have serologic evidence of prior exposrue to HTLV. The peripheral blood lymphocytes (and when available,splenic lymphocytes) from such patients were fused to several human drug-marked lymphoid cell lines using polyethylene glycol. In certain experiments, the fusion partner used was NS-1, a drug-marked plasma-cell line in wide use. Following fusion, hybridomas were grown in appropriate selection media. Hybrid lines secreting antibodies potentially reactive with retroviral or related tumor products were screened using one of several binding assays involving disrupted retorvirus or malignant T-cell lines that are known to shed retroviral particles as targets. In certain experiments, a subhuman primate (gibbon) was immunized with purified retroviral core proteins and cells known to shed the retrovirus. Peripheral blood lymphocytes were obtained sequentially, and various hybrids were generated and screened as described above.

NEOPLASMS OF REGULATORY CELLS

Background: A number of essential insights into the nature of the humoral immune responses resulted from the study of neoplasms of the B cell/plasma cell series. The recognition that so-called paraproteins derived from patients and certain animals with multiple myeloma (a malignant proliferation of plasma cells) represented extremely homogeneous immunoglobulins has affected essentially every phase of immunologic research. An understanding of antibody structure, function, metabolism and genetics depends heavily on the use of such homogeneous immunoglobins. Indeed, certain immunoglobulin classes (such as IgD and IgE) would have been difficult or impossible to evaluate without the availability of myeloma-related proteins. Recently recognized membrane and functional differences between B cells and T cells provided an important new basis for classifying neoplastic lymphocytes. Such a classification has already proved to have therapeutic and prognostic relevance in characterizing lymphomas and certain leukemias. The study of malignant T cells and their products may prove to be as important in answering questions regarding cellular immunity, and especially, the T-cell regulation of humoral immune responses, as myeloma cells and their protein products have proved to be in answering questions concerning the immunoglobulin synthesizing system. We have focused a considerable degree of research and clinical effort in studying patients with acute and chronic leukemias thought to be of T-cell origin. During the past year we have extended our analysis of the immunoregulatory properties of leukemic T cells from patients with Sezary syndrome. The Sezary syndrome is a cutaneous T-cell lymphoma in adults characterized by severe erythroderma, diffuse lymphadenopathy, and circulating neoplastic cells with cerebriform nuclei. Mycosis fungoides is a related aleukemic cutaneous T-cell lymphoma. We have given special attention to the leukemic T cells of some patients with a kind of T-cell leukemia lymphoma which may be associated with a human retrovirus. Much of the early work in this field was directed by Dr. Robert Gallo. This virus (called HTLV) can be detected

in the genome of certain T-cell leukemias, particularly those which appear to be clustered in southern Japan, the West Indies, and southern regions of the United States. HTLV is not an endogenous virus ubiquitous in human germ lines, and it is readily distinguishable from the known animal retroviruses, including those from subhuman primates. Antibodies to two HTLV proteins, p 24 and p 19, have been found in sera with T-cell malignancies in the above geographic regions.

We have previously shown that certain patients with the Sezary syndrome may have leukemic cells which act as so-called helper T cells. We have observed a helper-like activity in leukemic cells from one patient with acute T-cell leukemia, and in one clone of cells from a patient with mycosis fungoides. By refining our analysis, we have learned that approximately one-half of patients studied had helper activity in a system where any contaminating residual normal T-cell effects were minimized by dilution studies. We have discovered one patient with acute lymphoblastic leukemia whose neoplastic cells acted as T cells that could perform a helper function. Also, we have analyzed the neoplastic cells from an unusual child with a cutaneous T-lymphoma, and found them to function as potent suppressor cells. The neoplastic T cells from patients with HTLV-associated disease frequently function as suppressor cells.

Major Findings: The neoplastic lymphocytes of essentially all patients with Sezary type T-cell leukemias failed to produce immunoglobulin under any of the conditions assayed. None of the Sezary patients studied had neoplastic T cells which exhibited suppressor cell activity using pokeweed mitogen. Indeed, on a number of occasions, normal lymphocytes showed a greatly amplified immunoglobulin synthesizing and secreting capacity in the presence of Sezary cells.

We have data supporting the view that some (but not all) patients with the Sezary syndrome variant of cutaneous T-cell lymphoma have a disease that represents a homogeneous expansion of polyclonally active helper-like T cells. Highly purified normal B cells do not undergo a transition into immunoglobulin-secreting cells in vitro after stimulation with certain lectins, such as pokweeed mitogen, unless a source of helper T cells is provided. The neoplastic T cells from certain patients with the Sezary syndrome provide such a helper effect by promoting the maturation of normal indicator B cells (rigorously depleted of T cells) into immunoglobulin-secreting cells following stimulation with pokeweed mitogen in vitro.

We have examined the surface membrane phenotype of Sezary cells using certain monoclonal reagents. Essentially all Sezary T cells studied express the OKT4 antigen (thought to be present on normal helper T cells), but did not express the OKT8 antigen (thought to be present on normal suppressor T cells). We consider these membrane-marker studies as supplements to functional studies. At this time, we do not believe that membrane-marker studies alone can be used to ascribe immunoregulatory characteristics to neoplastic cells. We have succeeded in establishing both uncloned and cloned T-cell lines from peripheral blood samples. The surface antigenic phenotypes did not correlate with regulatory function in certain cases. In addition, further research is needed to define the stability of these markers.

The neoplastic cells from patients with Japanese (retrovirus-associated) T-cell adult leukemias resemble Sezary cells in terms of surface membrane phenotype, although they may express certain markers (e.g., OKT10- an antigen found on immature cells or many lineages) not found on typical Sezary cells. However, they generally have a suppressor-cell function. Interestingly, such cells often express an antigen referred to as Tac which is now thought to be a receptor for an important T-cell growth factor.

At the present time, most investigators rely on murine monoclonal antibodies to classify, and in some cases treat, human neoplastic cells of T-cell origin. A great deal of our effort in our lab has been spent in trying to establish monoclonal human antibodies reactive to human T-cell tumor cells or HTLV-associated antigens. We have used UC729 (an azaguanine-resistant human line) and NS-1 (an azaguanine-resistant murine line) as fusion partners in an attempt to generate human-human or human-mouse hybridomas from peripheral blood (and spleen) of patients with T-cell neoplasms. We studied patients who were felt to have been exposed to HTLV as well as those who had not had such exposure. The results of these experiments are still being evaluated, but as yet none of the hybridomas appear to secrete relevant antibodies.

More recently we immunized a gibbon with HUT 102 (a neoplastic HTLV-positive T-cell line) and purified disrupted HTLV retrovirus. We have been able to obtain a number of IgM and a lesser number of IgG-secreting hybridomas. One of these hybridomas (uncloned) reacts with HUT 102, Sezary cells, and viral-core proteins. At the present time, this hybridoma is being cloned and expanded. Further immunizations and hybridomas are being undertaken. Our data would suggest that gibbon immunoglobulins are exceedingly similar to human immunoglobulins. We feel this approach promises the development of mononclonal antibodies that are virtually identical to human monclonal antibodies and that can react with important antigens on the surface of neoplastic T cells.

Turning to the problem of B-cells in patients with neoplasms of T-cell origin, we have attempted to grow cloned B-cells from lymph nodes of patients with cutaneous T-cell lymphomas. Such patients may have significant elevations of IgA and IgG in their serum. At the present time, we have several clones of B cells derived from a patient with Sezary syndrome. The Epstein-Barr virus status of these cells is still being determined.

Several of these clones secrete IgA exclusively, while others secrete IgG and IgA together. Of interest, it was possible to induce IgG production in clones which constitutively produce IgA alone when normal T-cells were added in culture.

We will continue to study these cloned B-cells as models of IgA regulatory events and the genetic events in isotype switching.

SIGNIFICANCE TO BIOMEDICAL RESEARCH

The recognition that some T-cell leukemias (or lymphomas) may represent a homogenous proliferation of T cells which are programmed for either helper or suppressor function is important for a number of reasons. First, certain forms of humoral immune deficiency may be due to a helper cell deficit, and not totally due to an intrinsic defect in B cells alone or excessive number of suppressor cells. It is possible that certain classes of neoplastic cells or soluble products produced by such cells could prove useful in the treatment of immune deficient states due to thymic dysfunction. Similarly, if such helper cell factors could be isolated (or synthesized), they might be useful in those situations where it is desirable to amplify a normal immune response, for example, following immunizations or in overwhelming infections. In mice, it has been possible to show that there exists a genetically controlled Ly-system of membrane markers which may correlate with helper or suppressor function. Three groups of T cells have been identified in mice. One population bears all three of the so-called Ly determinants ($Ly\text{-}1^+$, 2^+, 3^+). Another population bears only Ly-1 determinants and a third population bears Ly-2,3 determinants. It has been demonstrated that those cells which are Ly 1^+ act as helper cells under some conditions. In addition, as already dicussed, in mice there is a subregion of the I portion of the major histocompatability complex (designated I-J) which may encode suppressor cell function and certain suppressor cell factors. Furthermore, antisera raised against I-J encoded products may potentiate certain responses. Such anti-I-J antisera may apparently promote tumor rejection in certain systems presumably by eliminating suppressor cells which interfere with host immune responses directed against lethal tumors. The use of neoplastic T cells as starting reagents for the development of appropriate antisera or the isolation of various factors may make it easier to establish and verify serological marker systems for human helper and suppressor function, and may make it possible to serologically manipulate immune responses in certain cancer patients to potentiate tumor rejection or to restore various other elements of the immune system (vide infra). Finding it is possible to develop hybridomas that secrete gibbon antibodies against determinants on human T-cell tumors or an asociated retrovirus might expedite certain antibody-based therapies against human T-cell Moreover, it is possible that certain subtle antigenic components on lymphomas, or antibody-based manipulation of regulatory cells in various immunologic abnormalities. Subhuman primate hybridoma antibodies will likely be less antigenic and, therefore, more practical for long-term administration in human beings. Moreover it is possible that certain subtle antigenic components on human tumor cells would not elicit a murine antibody response, thus precluding the development of monoclonal antibodies to such antigens using mice. It is conceivable that subhuman primate antibodies could nevertheless be raised, thereby making it possible to produce suitable hybridoma reagents.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-CB-04020-06-MET

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Genetic Control of the Immune Response to Natural Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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4.4	3.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms of determinant-specific Ir gene control and of antigen recognition by T and B lymphocytes were explored at several levels in the response to myoglobin. In a secondary in vitro antibody response, Ir gene control was manifested by a failure of (high responder x low responder) F_1 T cells to help low responder B cells, even though the latter could be helped by carrier-specific T cells and so must have been present and competent. This result was independent of macrophage source. Low responder T cells, from mice neonatally tolerant to high responder Ia antigens, were competent to help high but not low responder B cells, and so were phenotypically identical to F_1 or high responder T cells. B cell restriction correlated with Lyb5$^-$ phenotype. The response to horse myoglobin was controlled by an unusual gene in H-2D. Monoclonal antibodies specific for topographic determinants of myoglobin are being sequenced and crystallization of their Fab fragments with myoglobin is being attempted. B cell presentation of antigen is under study. T cell lines specific for myoglobin have been prepared and are being cloned.

Project Description:

Objectives: The critical importance of genetic factors in regulating the immune response, and especially of genes which appear to be specific for particular antigens (so-called immune response or Ir genes), has become increasingly apparent in the last 15 years. However, the mechanism of action of these genes and the nature of the product encoded by them remain unknown. Most of the Ir genes so far described are linked to the major transplantation or histocompatibility gene complex. The primary objective of this project has been to define the mechanism by which Ir genes linked to these major transplantation antigens (HLA in man and H-2 in the mouse) control the immune response to natural protein antigens, with the hope of understanding the modes of recognition involved at this regulatory level, the cells which participate and the cell membrane receptors which mediate the regulation.

Methods Employed: Myoglobins from various species were purified by the method of Hapner et al., (J. Biol. Chem. 243:683 (1968)). Fragments of myoglobin were prepared by CNBr cleavage and chromatographic purification. For antibody responses, mice were immunized i.p. with 200 micrograms in complete Freund's adjuvant and boosted twice with 100 micrograms in saline, as described previously (Berzofsky, J. Immunol. 120:360 (1978). Myoglobin and its fragments were radiolabeled at the N-terminal alpha-amino group with $K^{14}CNO$ or N-succinimidyl-[2,3-^{3}H] propionate. Antibodies to these were measured using the polyethylene glycol precipitation direct binding assay described previously (Berzofsky, J.A., J.Immunol., 120:360 (1978)). In competition studies, other unlabeled myoglobins were added at various concentrations.

T-lymphocyte proliferation was studied by a method based on that of Corradin, Etlinger and Chiller (J. Immunol. 119, 1048 (1977). Briefly, nylon-wool passed T-cells from draining lymph nodes of mice immunized s.c. in the tail 8 days previously with myoglobin in Freund's adjuvant were cultured for 5 days in the presence of varying concentrations of antigen (myoglobin or fragments). Proliferation was assessed from the amount of methyl-^{3}H-thymidine incorporated into DNA during the final 4 hours of culture. For experiments assessing macrophage reconstitution of response, such T cells were passed twice over nylon wool to deplete of macrophages. For a source of macrophages, a method was developed for purifying liver macrophages (Kupffer cells) by perfusion in situ, triteration, treatment with collagenase and DNase to free the macrophages, density gradient centrifugation over metrizamide, glass adherence, and irradiation with 1500 R from ^{137}Cs before use. Alternatively, anti-Thyl-plus-complement treated irradiated, splenic glass adherent cells were used. These were cultured with macrophage depleted T cells and antigen as described above, or were pretreated with antigen in culture medium at 37^{o} in polypropylene tubes at 10^{6} cells/ 0.2 ml for one to 24 hours, washed and then added to macrophage-depleted T cells without soluble antigen present during the 5-day culture, to assess stimulation of the T cells. In experiments to determine the effect of anti-Ia antibodies, dilutions of appropriate antisera or monoclonal hybridoma culture fluids were added during the pretreatment of the liver macrophages with antigen, and then washed out before these cells were mixed with T cells.

A culture system for assessment of in vitro antibody responses to myoglobin and its fragments was developed by a modification of that of Mishell and Dutton (J. Exp. Med. 126: 423 (1967)). Optimum conditions were determined to be as follows: 4 X 10^6 spleen cells from immunized mice were cultured with 0.1 to 1.0 ug myoglobin in 1.5 ml of supplemented RPMI-1640 medium with 10% fetal calf serum in flat-bottom wells for 9 days at 37°, 6% CO_2, on a rocking platform. On the fourth day one ml of supernatant was exchanged for fresh medium, and cultures were fed daily thereafter. On the 9th day, culture supernatants were taken to test for the presence of secreted antibody, or else cells were harvested on day 6 to count antibody-secreting cells by the hemolytic plaque method. Besides whole spleen, various cell mixtures could be cultured. For instance, spleen cells depleted of macrophages by passage over Sephadex G10 (Hodes and Singer, Eur. J. Immunol. 7: 892 (1977)) were cultured alone or with macrophages from syngeneic or semisyngeneic sources. Macrophages were either liver Kupffer cells (described above), or irradiated, glass-adherent, splenic macrophages. T cells were eliminated, where necessary, by treatment with rabbit anti-mouse brain antiserum plus complement. T cells were purified, where necessary, by removal of B cells and macrophages on nylon wool columns.

Concentrations of antibodies in culture supernatants were measured by a solid phase radiobinding assay. Wells of polyvinyl chloride flexible microtiter plates were coated by incubation with 50 ul myoglobin, 100 ug/ml, for 1.5 hrs. Unoccupied sites on the well were saturated by a 15 minute incubation with 10% bovine serum albumin. Then 50 ul aliquots of culture supernatants were incubated in the wells for 1.5 hours and unbound material washed out. Finally, ^{3}H-labeled, affinity purified, goat antibody to the Fab fragment of mouse IgG was incubated in the wells for 1.5 hrs to bind to any antibody attached to the myoglobin on the plastic. All incubations were carried out at room temperature, and were followed by 3 washes with 1% bovine serum albumin in saline. Individual wells of the plate were the cut apart and radioactivity determined by scintillation counting. Controls for nonspecific binding were all close to machine background. A standard curve with known antimyoglobin antibody was used for quantitation.

Hybridoma monoclonal antibodies specific for sperm whale myoglobin were described in a previous annual report (1980-81). These had high affinities, between 0.2 X 10^9 and 2.2 X 10^9 M^{-1}. Their relative affinities for myoglobins of other species were determined by competitive radioimmunoassays, in which increasing concentrations of competitor were added to a constant concentration of labeled sperm whale myoglobin (7 nM) and of monoclonal antibody (on the order of 4-6 nM), and polyethylene glycol (MW 6000, final concentration 10%) used to precipitate all immunoglobulin plus bound antigen. The concentration of competitor resulting in a 50% decrease in bound/free ratio for labeled myoglobin was taken as a measure of relative dissociation constant.

Antiidiotypic antibodies were raised in guinea pigs, tolerized one day previously with 5 mg of deaggregated mouse Ig given intravenously, by immunizing subcutaneously in the footpads with 20 micrograms of affinity purified monoclonal antibody emulsified in complete Freunds adjuvant. The animals were

boosted intramuscularly three and five weeks later, first with 20 micrograms in complete adjuvant, and bled repeatedly from 10-18 days after the last immunization. The sera were absorbed extensively by repeated passage through columns of normal mouse Ig-Sepharose to remove antibodies to constant region determinants. They were studied using an enzyme-linked immunoassay (Elisa) as follows: Myoglobin was bound to polyvinyl chloride microtiter plates. Alkaline-phosphatase conjugated monoclonal antimyoglobin antibodies were preincubated with a putative antidiotypic antisera, and then incubated on the myoglobin-coated plastic. After washing to remove unbound material, the amount of enzyme bound was determined by optical absorbance change at 405 nm after addition of p-nitrophenyl phosphate as substrate.

Major Findings:

A) Ir genes and cellular interactions.

Background: We have previously studied the in vivo serum antibody response and in vitro T-lymphocyte proliferative response to a natural protein antigen, sperm whale myoglobin (Mb), and shown that both of these responses are controlled by the same Ir genes (Berzofsky, J. A., J. Immunol. 120: 360 (1978)). Using inbred strains of mice which differ only in the H-2 complex (known as congenic strains), we demonstrated that two different genes, mapping in distinct subregions (I-A and I-C) of the I region of H-2, controlled the responses to different antigenic determinants (distinct chemical sites) on the same antigen molecule - the first example of such independent control of responses to different chemical moieties on the same protein molecule. Moreover, the gene which controlled the T-cell proliferative response to a given determinant of myoglobin also controlled the production of antibodies specific for that same region of the molecule - an indication of parallel control of T-lymphocyte recognition and activation of B lymphocytes with similar specificity (Berzofsky et al.: Proc. Natl. Acad. Sci 76:4046 (1979).

One level at which this Ir-gene-controlled choice of antigenic determinants recognizable is mediated is the recognition by T-lymphocytes of antigen in association with cell surface structures on macrophages, which serve as antigen presenting cells. These surface structures, which differ in different inbred strains of mice, are encoded by genes also mapping in the I region of H-2 , and are therefore designated Ia (for I-associated) antigens. In our experiments, myoglobin-immune lymph node T cells, depleted of macrophages, from (high responder X low responder) F_1 hybrid mice were held constant as the source of responding (proliferating) T cells, and the cultures were reconstituted with liver macrophages from high responder parental mice with both Ir genes, low responder parental with neither gene, or recombinant mice with only one gene or the other. We found that the magnitude of response, and the selection of which fragments of myoglobin could stimulate these F_1 T cells in vitro was determined by the source of the macrophages, and corresponded exactly to the response pattern of the strain of mice from which the macrophages were obtained (Richman et al.: J. Immunol. 124:619 (1980). Macrophage pretreated with antigen could also stimulate immune T cells in the absence of soluble antigen. This stimulation manifested the same Ir genetic

restriction for macrophage source, and could be inhibited by inclusion of appropriate monoclonal or conventional anti-Ia antibodies, without complement, during the pretreatment with antigen (Berzofsky and Richman, J. Immunol. 126: 1898 (1981).

The limitation of T cell proliferative responses is that they involve only T cells and macrophages, not B lymphocytes which produce antibody. In order to study the mechanisms of determinant-specific Ir gene control of the antibody response to myoglobin in vitro, we have now developed a modified Mishell-Dutton culture system in which secreted antibody specific for myoglobin can be measured in the culture supernatants by a solid phase radioimmunoassay. By this method, we can also measure the fine specificity of the supernatant antibodies for different fragments of myoglobin. The in vitro spleen cell antibody response to sperm whale myoglobin was found to be controlled by the same two Ir genes in I-A and I-C which controlled the in vivo response, at the level of individual determinants on different fragments of myoglobin. In addition, we extended the mapping to recombinant H-2 haplotypes not previously studied. Also, we found this in vitro antibody response to be macrophage and helper T-cell dependent, as shown by experiments depleting these cells, and reconstituting with purified T cell or macrophage populations.

Current results:

In order to determine which cells and cell interactions are involved in mediating this determinant-specific Ir gene control of the antibody response to myoglobins, we selectively mixed T cells, B cells, and macrophages from different sources. In most cases, two of the cell types derived from (high responder x low responder) F_1 hybrid mice, and the origin of the third cell was varied. When the T cells were of F_1 origin, no allogeneic effects were to be expected. However, when the T cells were the cell being varied, T cells from neonatally tolerized mice had to be used to avoid allogeneic effects. First, myoglobin-immune helper T cells from (high responder x low responder) F_1 hybrid mice would help T cell-depleted populations of B cells plus macrophages from myoglobin immune high responder mice but not myoglobin immune low responder mice. To be sure that the low responder B cells were adequately primed in vivo, we used B cells from mice immunized with myoglobin coupled to an immunogenic carrier, fowl gamma globulin. We demonstrated that low responders immunized with myoglobin-fowl gamma globulin (FGG) responded as well as high responders, and that their B cells would make antimyoglobin when cultured with myoglobin-FGG if provided with FGG-specific syngeneic helper T cells. Thus, low responder mice contained competent, myoglobin-specific B cells which could be primed by immunizing with myoglobin-FGG. However, even these B cells were not helped by myoglobin-specific T cells of syngeneic or F_1 origin. To determine whether this Ir-gene restricted failure to help was due to the low responder B cells or macrophages, we performed the same experiment in the presence of varying numbers of F_1 macrophages. However, F_1 T cells in the presence of F_1 macrohages still could not help competent, primed, myoglobin-specific low responder B cells to make antimyoglobin. The presence of suppressor cells in the B cell population was ruled out by showing that the low responder B cells did not suppress the response of unseparated F_1 spleen cells. Also, allogeneic effects were further excluded by using low responder - F_1 radiation bone-marrow chimeras, immunized with myoglobin in the presence of F_1 macrophages. T cells from the

chimeras could help F_1 B cells plus macrophages, but failed to help the syngeneic chimeric low responder B cells even in the presence of F_1 macrophages.

We were forced to conclude that one site of Ir gene function was the failure of competent, myoglobin-specific B cells to be helped by myoglobin-specific T cells, even though they could be helped by FGG-specific T cells.

Since this type of T cell-B cell genetic restriction, as opposed to a T-cell macrophage restriction, had been seen previously in non-Ir gene controlled systems in cases in which $Lyb5^-$ but not $Lyb5^+$ B cells functioned (Singer et al., J. Exp. Med. 154: 501 (1981); Asano et al., J. Exp. Med. 154: 1100 (1981), we examined the Lyb phenotype of the B cells functioning in our in vitro secondary response by comparing the responses of $Lyb5^-$ populations from (CBA/N x DBA/2) F_1 male mice, bearing the x-linked CBA/N defect, with those of $Lyb5^+$ and $^-$ B cells from male progeny of the reciprocal cross carrying normal x chromosomes, but otherwise genetically identical. The response of the $Lyb5^-$ B cells was actually slightly higher than that of the mixed B cell population, an indication that the response in our culture system may involve primarily $Lyb5^-$ B cells. This result supports the hypothesis that Ir gene control of T cell-B cell interaction, as opposed to T cell-macrophage interaction, will be seen when the B cells participating are primarily $Lyb5^-$.

Second, when we did the reciprocal experiment of depleting macrophages alone from the F_1 spleen cell populations of T and B cells, macrophages of either the high responder parent or low responder parent could equally well reconstitute the response. This result was also found when the macrophages were pretreated with myoglobin and then no additional soluble antigen added. Thus, while we cannot exclude the possibility that residual F_1 macrophages are presenting antigen, we have been unable to demonstrate an Ir genetic restriction on macrophage function in this in vitro secondary antibody response, despite multiple attempts. Therefore, since we did observe a Ir restriction for B cells plus macrophages, it is possible that in the in vitro secondary antibody response to myoglobin, Ir gene function is involved in T cell-B cell interactions but not in macrophage-lymphocyte interactions. Further tests of this possibility are in progress.

Third, we wanted to explore the ability of low responder T cells to function if provided with F_1 or high responder B cells, but to do so without allogeneic effects and without intentionally altering the T cell repertoire by use of chimeras, we used T cells from mice neonatally tolerized to the high responder H-2 antigens. Newborn mice, within 24 hours of birth, were injected i.v. with 2×10^7 unirradiated spleen cells from adult F_1 mice. They were immunized with myoglobin at 8-12 weeks of age, and at the time of the experiment were confirmed to be tolerant by the absence of a mixed lymphocyte reaction (MLR) or cell-mediated cytotoxicity (CML) against the tolerated H-2 hyplotype. However, the spleen cells from these mice were shown by fluorescence activated cell sorting (kindly performed by S.O. Sharrow of the Immunology Branch, NCI) to contain 2-4% residual F_1 cells, whereas the thymuses of these mice contained no detectable F_1 cells. The presence of these F_1 cells will be important later.

When T cells from tolerized low responsder mice were cultured with (high responder x low responder) F_1 B cells (plus macrophages), they provided almost as much help as tolerized high responder T cells. Thus, the low responder mouse had myoglobin-specific T cells competent to help F_1 B cells. However, we had to be sure that the help we were observing was not a subtle allogeneic effect, despite the lack of MLR or CML reactivity. Therefore, we cultured T cells from tolerized mice which had not been immunized in parallel with ones from immunized mice and found that immunization with myoglobin was necessary for the help. Therefore, the help was not a nonspecific allogeneic effect. Secondly, we had to test for the possibility that the help was due to the 2-4% contaminating F_1 cells, rather than homozygous low responder T cells, despite the fact that such low numbers of F_1 cells should not have been adequate to provide the help observed. Therefore, we treated the low responder, myoglobin-immune T cells from neonatally tolerized mice with antibodies to the H-2 antigens borne by the F_1 but not the low responder cells. Such treatment with antibody and complement could eliminate all help by F_1 cells, but failed to decrease the help by the neonatally tolerant low responder T cells. Therefore, the help was due to homozygous low responder cells not contaminating F_1 cells.

Were these low responder T cells which helped F_1 B cells restricted to react with high or low responder Ia antigens on the B cells? When we used low responder or high responder B cells instead of F_1 B cells, we found that both high responder and low responder T cells (from mice tolerized with F_1 spleen cells) could help high but not low responder B cells. The failure to help low responder B cells was not due to suppressors in the low responder population. Thus, high and low responder T cells are phenotypically alike in their ability to help high but not low responder B cells and macrophages in a myoglobin-specific response. The Ir gene defect is manifested in the failure of low responder B cells to receive help. As noted above, this is at least a B cell restriction, although we cannot exclude a possible macrophage restriction as well. However, since the myoglobin-specific low responder B cells are competent and able to respond to carrier (fowl gamma globulin)-specific help, the Ir gene defect represents a failure of myoglobin-specific helper T cells of any strain to collaborate with low responder myoglobin-specific B cells.

These studies also reflect on the development of the T cell repertoire. Since low responder T cells, neonatally tolerant to high responder alloantigens, could cooperate with high responder B cells and macrophages in a genetically restricted response, either the repertoire for myoglobin in the context of alloantigens was always present but masked by alloreactivity, or else the process of neonatal tolerization altered the repertoire. Since we could detect F_1 cells only in the spleen and lymph nodes not the thymus, we would have to postulate that the repertoire can be altered by recirculating cells in the periphery, not just in the thymus.

In order to identify the structual features of sperm whale myoglobin that make it immunogenic in high responders and nonimmunogenic in low responders, we studied the Ir gene control of the response to other myoglobins. The antibody response to equine myoglobin, which shares the three-dimensional structure and 87% of the sequence of sperm whale

myoglobin, was found to be under completely different Ir gene control from that to sperm whale myoglobin. Mice (such as B10.BR) that were low responders to sperm whale myoglobin were high responders to equine myoglobin. Moreover, the Ir genes mapped in different loci. The antibody response to equine myoglobin was controlled by complementing Ir genes genes in I-A and H-2D. We were surprised to find an Ir gene for an antibody response to a soluble protein antigen mapping in H-2D, rather than the I region, since none had mapped there before. Thus, either the mechanism of action of this Ir gene is different from those in the I region, or equine myoglobin is the first soluble protein antigen recognized in association with a class I (H-2K/D) rather than Ia antigen, or there is a class II (Ia) antigen mapping close to the H-2D locus. One plausible hypothesis is that the H-2D region is a restriction element for an Ly2+ suppressor T cell, but so far we have been unable to detect suppressor cells in this response. Experiments to test the role of the H-2D antigen in presentation of equine myoglobin are in progress, but so far the results are equivocal.

B) Monoclonal T cells, B cells, and their receptors.

Background:

In order to understand the mechanism of action and specificity of these Ir genes, we have also been studying the myoglobin-specific receptors of the T cells and B cells involved in the response. The previous annual report described six monoclonal antibodies to myoglobin, all of high affinity (10^8 to 10^9 M^{-1}). We were able to identify the antigenic determinants of myoglobin recognized by 3 of these. At least two of the antibodies recognize topographic antigenic determinents consisting of amino acid residues from distant parts of the primary sequence, brought together by the folding of the molecule. These determinants are distinct from those reported by others. In the previous report we also described the idiotypes of these monoclonal antibodies. Interestingly, some which have distinct antigenic specificity nevertheless share idiotypes, some in the combining site.

Current results:

Three of the monoclonal antimyoglobin antibodies have been purified in large quantity by affinity chromatography of the ascites of mice injected with the monoclonal hybridoma. We would like to determine the primary and tertiary structure of the combining sites of these in order to understand the complementarity of a globular protein antibody for a globular protein antigen with topographic determinants, and also to understand the nature of the idiotopes which are shared. The heavy and light chains of one of these (HAL38-ID5) were separated by reduction and alkylation and gel filtration on Sephadex G-100 in 6M urea in 1M acetic acid. The heavy chain was pure by SDS-polyacrylamide gel electrophoresis, but the light chain was contaminated by some MOPC-21 light chain from the NS-1 myeloma parent cell line. Unfortunately, the amino terminal of the heavy chain was blocked, so sequencing will be delayed as we try to deblock it. The heavy and light chains of the others are currently being separated for sequencing.

In order to study the tertiary structure, we prepared Fab fragments of two of the monoclonal antibodies (HAL38-ID5 and HAL 43) by papain digestion. (Pepsin did not work at any pH on these proteins, both IgG_1 K). The Fab fragments were purified by passage over a Protein A-Sepharose column to remove Fc fragment and any intact IgG. They were then separated from Fab containing the MOPC-21 light chain by reacting with excess myoglobin and gel filtration on Sephadex G100 superfine. We were able to separate the complex of Fab containing the correct light chain, bound to myoglobin (total MW approximately equal to 63,000) from the free Fab containing the MOPC 21 light chain (MW approximately equal to 45,000). This procedure also provided us with a stoichiometric myoglobin - Fab complex. We are currently trying various conditions to crystallize this complex, in collaboration with Drs. Enid Silverton and David Davies of NIADDK, in order to examine the interaction of the two molecules by x-ray diffraction.

Another set of experiments was performed to ask whether the hybridoma cells, of B cell lineage, could present antigen, and if so, whether the surface immunoglobulin played a role. Preliminary experiments suggest that they can present myoglobin, and their ability to present other myoglobins depends on the specificity of the monoclonal antibody on the cell surface.

To study the antigenic determinants of myoglobin recognized by T lymphocytes, we used an approach similar to that used for the monoclonal antibodies. Mice were immunized subcutaneously with myoglobin of one species, and their draining lymph node cells were tested for a proliferative response in vitro to myoglobins of other species. Assuming that those myoglobins which stimulated shared one or more determinants with the immunizing myoglobin, while those which failed to stimulate shared none, we were able to localize antigenic residues in several cases. B10.S T cells appear to be highly sensitive to the difference between glutamic acid (Glu) and aspartic acid (Asp) at position 109. If immunized with sperm whale myoglobin (Glu 109), they responded only to myoglobins bearing Glu, not Asp, at 109. In contrast, when immunized with equine myoglobin (Asp 109), the reverse was true. Thus, noncrossreacting populations of T cells could be elicited in the same mouse, each specific for a carboxylic acid residue at position 109, but able to distinguish between the Glu and the Asp, which differ by 1 carbon atom in side chain length. Since the same macrophages can present both myoglobins (and since the B10.S has only an I-A molecule but appears to lack an I-E molecule), we believe this discriminatory ability is a function of the T cell receptor, not the macrophage. In another strain (B10.GD), the Glu 109 of sperm whale myoglobin also appears to be critical, but, in addition, a change at position 116 (histidine ---> glutamine) also interferes with T cell stimulation. Thus, this strain appears to recognize a determinant overlapping with but distinct from that recognized by the B10.S strain. Moreover, the two residues involved are 7 residues apart in the primary sequence, but are brought together by the folding of the native molecule. While these residues are close enough to be on the same fragment of myoglobin, they may still represent a topographic determinant recognized by T cells.

Finally, to extend this sort of analysis in ways that can be done only with monoclonal populations, and to isolate the T cell receptors, we have produced a B10.D2 continuous T cell line which proliferates in response to sperm whale myoglobin but not equine myoglobin. We are currently cloning the line, and testing its fine specificity and H-2 genetic restriction. Also, experiments are in progress to test its ability to provide help in an in vitro secondary antibody response.

Significance to Biomedical Research: The results of the in vitro antibody response studies demonstrate that Ir-gene defects may not represent the lack of either antigen-specific T cells or antigen-specific B cells or antigen presenting cells. Rather, they represent a failure of competent, antigen-specific T cells to help syngeneic low-responder antigen-specific B cells, which are also competent when provided with carrier-specific help. This restriction cannot be overcome by F_1 antigen-presenting cells. This Ir gene defect at the level of T-B cell interaction may depend on the type of B cell which participates in the response. We have seen it in this secondary in vitro response in which $Lyb5^-$ B cells predominate, whereas others have not seen it in responses dominated by Lyb5+ B cells. The same work bears on the development of the T cell repertoire. The T cells from neonatally tolerized mice help antigen-specific allogeneic B cells in this genetically restricted response. Thus, either the repertoire is present for recognition of antigen in association with foreign Ia, but masked by alloreactivity, or it can be induced by injection of F_1 spleen cells which seed the periphery, not the thymus. Either way, it is not limited to the Ia antigens of the thymic epithelium. The Ir gene controlling the response to equine myoglobin which maps in H-2D is unprecedented. It suggests either a new type of Ir gene, or a new type of genetic restriction element for presentation of soluble antigen.

The studies of the sequence and 3-dimensional structure of the monoclonal antibodies should extend the knowledge gained from hapten-binding myeloma proteins by showing us how antibodies form combining sites complementary to topographic sites on globular protein antigens. The B cell presentation studies imply not only that B cells can present antigen, but that they may use antigen-specific immunoglobulin receptors in doing so. Finally, the studies of T cell receptors and specificity should help elucidate the relationship between the specificity of T cells, of B cells, and of the Ir genes themselves. Moreover, if topographic determinants requiring the native structure can be demonstrated to be recognized by T cells, this would imply that macrophage processing involving cleavage of antigen into fragments is not an obligatory step in T cell recognition. If T cell clones can be expanded, they should provide a source of homogeneous T cell receptors to study biochemically. All of these studies are hoped to ultimately shed light on the mechanisms and role of genetic controls on immune responses which play a role in human disease, whether they be normal host defenses, excessive responses which escape control (in autoimmune disease), or deficient responses, as in malignancy or immunodeficiency diseases.

Proposed Course: In the myoglobin-specific in vitro secondary antibody response, we are using neonatally tolerized recombinant strain T cells, T cells from mice immunized with fragments, and T cell lines and clones to test the hypothesis that the fine specificity of the helper T cell limits the possible antibody specificity by helping only those B cells which bind antigenic determinants somehow sterically related to the site recognized by the T cell. We are exploring the mechanism of tolerization and the development of the T cell repertoire in neonatally tolerized mice, in collaboration with Drs. Singer, Hodes, Morrissey, Gress, and Sharrow of the Immunology Branch, NCI. We are examining the mechanism of the unusual Ir gene for horse myoglobin which maps in H-2D, by studying antigen presentation and T cell-B cell restriction and anti-Ia blocking in these strains, as well as attempting in other ways to look for suppressor mechanisms. We are attempting to sequence the monoclonal antibodies with the help fo Dr. Stuart Rudikoff (NCI), and to crystallize their Fab fragments in collaboration with Drs. Enid Silverton and Davia Davies (NIADDK). We are continuing the study of antigen presentation by antigen-specific B cells. We are studying the fine specificity of the T cell clones, and searching for clones specific for topographic determinants. Finally, we are trying to isolate purified T cell receptors from the clones.

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SUMMARY STATEMENT
ANNUAL REPORT
DERMATOLOGY REPORT
DCBD, NCI
October 1, 1981 through September 30, 1982

The Dermatology Branch conducts both clinical and basic research studying the etiology, diagnosis and treatment of inflammatory and malignant diseases involving the skin and the host's response to these diseases. The basic research involves biochemical as well as biological studies of skin and is subdivided into eight separate, though frequently interacting, areas. The Branch also serves as Dermatology Consultant to all other services of the Clinical Center (approximately 1500 patients are seen in consultation each year). The main research achievements of the Dermatology Branch for the past year are as follows:

Immunopathologic Mechanisms Involved in Inflammatory and Neoplastic Skin Diseases:

During the past year we have continued our studies of the immunopathology of skin diseases in two major areas. The first is in cell mediated immunity and the role of epidermal Langerhans cells in the induction and expression of cell mediated immune responses. Using hapten - modified epidermal cells (containing Langerhans cells) as sensitizers we have shown that Langerhans cells are critical for the generation of contact sensitivity in mice. As well, we have shown that Langerhans cells are essential for allogeneic T cell stimulation in vitro. Ultraviolet light radiation alters this allogeneic T cell stimulation capacity by either affecting the cell surface of the Langerhans cell or by altering the production and/or release of mediators from the keratinocytes. In this regard we have shown that keratinocytes produce a substance which can activate T cells in vitro. This substance, called ETAF (epidermal thymocyte activating factor) is not elaborated by keratinocytes after UV radiation. ETAF can partially restore the above-cited UV abrogated allogeneic T cell stimulation. Thus within the epidermis the are two types of immunologically relevant cells, the Langerhans cells and the keratinocytes. In other studies relating to cell mediated immunity we have shown that a topically applied chemical, DNTB (dinitrothiocyanobenzene) can induce suppressor T cells to specifically suppress allergic contact dermatitis. The mechanism by which this chemical interacts with cells is currently under study.

The second major area of study is the identification and characterization of antigens and antibodies involved in the pathophysiology of some of the so-called auto-immune blistering skin diseases. We are currently concentrating on cell surface antigens involved in the disease called pemphigus and on the basement membrane zone constiuents involved in normal physiology and in the disease called pemphigoid. Using immunoprecipitation techniques and fluorography we had reported (last year) that pemphigoid antigen was a disulfide-linked glycoprotein of molecular weight of approximately 220K. We have more recently shown that pemphigus antigen is also a glycoprotein of m.w. 130 kd. We are also currently characterizing skin specific antigens which are identified by monoclonal antibodies produced in our laboratories. One of these is a unique stratified squamous epithelia specific lamina densa antigen which is identified by KF-1 antibody. Cultures of cells which produce these antigens are currently being sought.

Detection and Analysis of Circulating Immune Complexes:

Increasing evidence indicates that circulating antigen-antibody complexes play a role in the pathogenesis of a variety of dermatologic, rheumatologic, neoplastic and infectious disease states. We have identified and partially characterized the immune complexes which exist in several diseases i.e. Sjogren's syndrome, mixed cryoglobulinemia, mixed connective tissue disease, acute and chronic schistosomiasis, hepatitis and various types of cutneous and systemic vasculitis, utilizing two highly sensitive radioimmunoassays for detecting immune complexes i.e. ^{125}I-Clq binding assay and the Raji cell radioimmunoassay. We have recently developed a new, sensitive radioimmunoassay for the detection of IgA containing immune complexes and have demonstrated that patients with dermatitis herpetiformis, gluten-sensitive enteropathy and IgA nephropathy, among others, have circulating IgA containing immune complexes. Studies addressing the role of dietary protein in the induction of soluble immune complexes are currently underway in patients with dermatitis herpetiformis and in gluten-sensitive enteropathy. We have also examined the influence that certain genes of the major histocompatibility complex exert on immune function in vivo and in vitro in humans. In this regard we have demonstrated an Fc receptor reticuloendothelial system clearance defect in 50% of patients with dermatitis herpetiformis and have found that a high percentage of HLA-B8/DRw3 positive normal individuals also have delayed splenic clearance of IgG coated autologous erythrocytes indicating abnormal FcIgG receptor function of splenic macrophages. In attempts to determine the extent of this FcIgG receptor defect we studied lymphocyte receptors and found that normal HLA-B8/DRw3 positive individuals and HLA-B8/DRw3 positive dermatitis herpetiformis patients also have decreased numbers peripheral blood lymphocytes bearing receptors for the Fc portion of IgG. These HLA-B8/DRw3 patients and controls were also shown to have increased numbers of spontaneous immunoglobulin secreting cells in their peripheral blood as measured by a plaque forming assay.

Therapy of Skin Cancer and Disorders of Keratinization:

We are continuing to evaluate safety and effectiveness of new oral and topical agents particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne. Oral 13-cis-retinoic acid was effective in the treatment of skin cancer, and a variety of disorders of keratinization (lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris), and cystic acne. An oral synthetic aromatic derivative of retinoic acid (RO-10-9359) was similarly tested and found to be more effective and less toxic than 13-cis-retinoic acid in the treatment of the disorders of keratinization. A high initial, low maintenance dosage of 13-cis-retinoic acid was found to be a comparably effective schedule but less productive of toxicity than previously used continuous high-dosage schedules in the treatment of cystic acne. With regard to safety, we are continuing to closely monitor long term and short term side effects of the retinoids. Patients treated with 13-cis-retinoic acid showed small but significant elevations in plasma lipids and changes in lipoproteins. RO-10-9359 produced similar changes which were dose dependent and responsive to dietary management. Absorption studies showed that the RO-10-9359 is better absorbed with milk than with water.

The profound beneficial effect of 13-cis-retinoic acid in the treatment of acne and both retinoids in the treatment of cutaneous keratinizing diseases

indicates that other keratinizing disorders of man, for instance, preneoplastic squamous metaplasia of tracheo-bronchial and urinary bladder epithelial origin, could be successfully treated with the synthetic retinoids. Treatment of the keratinizing dermatoses and acne may also provide useful information in the evaluation of newer and potentially more potent and less toxic synthetic retinoids.

In conjunction with these clinical studies we are continuing to evaluate the morphologic and biochemical effects of Vitamin A and its analogs on skin. Freeze fracture studies have indicated that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms. In addition, a specific cytosol retinol binding protein has been identified in mouse skin and human skin from patients with Darier°s disease, psoriasis and basal cell carcinomas. A retinoic acid binding protein has also been identified in normal skin.

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

UV-radiation is believed to be the major cause of the most common type of human cancer, cancer of the skin. Patients with xeroderma pigmentosum (XP) are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor development in XP patients would, therefore, elucidate the role of DNA repair in preventing, in normal humans, those cancers which may be due to certain chemical and physical carcinogens. Extension of this work to include study of other diseases in which DNA repair may be defective, especially those associated with an usually high incidence of cancer such as ataxia telangiectasia, will further increase our understanding of the relationships between DNA repair processes and carcinogenesis. Some XP patients develop neurological abnormalities which are due to the early death of neurons. Our studies of these disorders and of some neurodegenerative disorders are designed to elucidate their pathogenesis as well as to develop presymptomatic diagnostic tests. We have used a trypan blue exclusion test to determine the number of lymphoblasts surviving after irradation with x-rays. Under conditions of maximum sensitivity of the assay, that is, when ataxia telangiectasia homozygote and heterozygote lines could be distinguished from normal lines, we detected hypersensitivity to x-rays in lymphocyte lines from patients with the following diseases: Huntington disease, familial dysautonomia, olivopentocerebellar atrophy, tuberous sclerosis, muscular dystrophy and retinitis pigmentosa. Lymphoblast lines from 11 other inherited diseases tested were not hypersensitive to x-rays. Although this assay had lost its sensitivity we have now obtained restored sensitivity.

We have studied cells from a patient who has both Cockayne syndrome and xeroderma pigmentosum. This combination of diseases has been seen in only one previous patient (our patient XP11BE in complementation group B). We have conducted UVinduced unscheduled DNA synthesis complementation group studies with the new patient°s fibroblasts. His cells complemented those from the previous patient as well as those from XP patients in complementation groups

A, C, D, E, and G. Studies are underway to determine whether his cells complement those of group F patients. It is likely that this new patient will represent a new XP complementation group.

With Dr. Dominic A. Scudiero we have detected hypersensitivity to the lethal effects of N-methy-N-nitrosoguanidine (MNNG), a mutagenic and carcinogenic DNA-damaging chemical, in fibroblasts from 6 patients with Huntington disease, 4 with familial dysautonomia, 5 with muscular dystrophy and with Usher syndrome. We have increased the number of normal lines studied from 13 to 21, and we have studied 3 additional Usher syndrome and 4 additional muscular dystrophy lines. Our results show that Usher syndrome and muscular dystrophy are diseases in which the patients° fibroblasts are significantly hypersensitive to the lethal effects of MNNG. Fibroblasts from patients with demyelination (e.g., multiple sclerosis, Cockayne syndrome, Charcot-Marie-Tooth disease) were not hypersensitive to MNNG. All our results show that hypersensitivity to MNNG is present in cells from patients with primary degeneration of post-mitotic excitable tissue (e.g., nerves, photoreceptors, muscle).

Biochemical Characterization of Mammalian Melanosomes:

We are continuing our studies of the abnormal proteins produced in malignant melanoma. These studies are aimed at elucidating the mechanism of formation of these atypical proteins, as well as their importance to the immunology of melanoma and possible immunotherapy potential. The melanosome in normal pigment cells is composed of multiple proteins, many of which are loosely bound and easily extracted. These probably constitute the proteins of the limiting membrane of the organelle. Other proteins are of lower molecular weight and are tightly bound to the granule, and probably constitute the structural, fibrillar protein; they appear to be the proteins which complexes with the melanin polymer. Melanosomal proteins from melanoma tissues vary in structure from those of normal tissues. Several of the proteins seem to be missing completely from these granules, which are also structurally distinguishable from normal granules by electron microscopy. Perhaps more importantly, many of the proteins in melanoma melanosomes are unique and are not found in normal melanin granules. This has been found to be the case in human melanoma as well. In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules, resolved a slight, but significant, difference of isoelectric points (4.8 and 4.5, respectively). There also is a difference of 10,000 MW between the two proteins; both have amino acid sequences contents which are identical with respect to 13 amino acids, but differ significantly with regards to four amino acids (asp, glu, val, arg). The carboxy and amino terminals however are identical. Peptide mapping has revealed that amino acid sequences are deleted in 3 or more regions of the abnormal protein. Other proteins in these tissues seem to differ in a similar manner. It has been found that tumor-specific proteins similar to these can be found in the serologic fluids of melanoma patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro.

We are also studying the role of tyrosinase in melanogenesis in normal tissues and the importance of the altered enzyme found in melanoma. Since this enzyme is essential for melanin biosynthesis, it is a unique system for the study of enzymatic control mechanisms in normal and malignant tissues. We have examined the status of enzymatic control of melanogenesis and have found that tyrosinase is the enzyme responsible for melanin synthesis in many different pigmentary

systems. It has been found in this study that tyrosinase is under allosteric control and that phospholipids may play a part in the expression of the enzyme°s activity. It has also been found that this allosteric regulation in murine melanomas is altered; this perhaps explains the atypical melanosome formation in these tissues. Recent evidence has been found which supports the theory that the enzyme is additionally controlled by enzyme-associated factors which can either inhibit or stimulate the production of pigment. It is hoped that further study of the cause of enzymatic differences in the malignant tissues will provide insights into the nature of neoplastic transformation.

Applications of Scanning Electron Microscopy to Soft Biological Tissues:

Our goals with the scanning electron microscope (SEM) have been to develop new methods and interpretive criteria for the study of a wide variety of soft biological tissues, and thereby capitalize on the unique capabilities of this instrument. Repeated attempts to achieve reproducibly high yields of fixed leukocytes from suspension on poly-L-lysine coated coverslips have shown erratic results and frequent low yields (below 10%), despite variations in many parameters. It now appears that our practice of including 20 units heparin per milliliter in the rinse solutions (to avoid clumping of the cells) has contributed to these poor results. Omission of heparin, however, did not ensure consistently high yields through routine critical point drying procedures with the three cell types examined. However, brief treatment with 0.005M sodium periodate substantially improved cell retention on poly-L-lysine coated glass coverslips. Periodate treated cells processed for SEM by freeze drying showed 95% retention in duplicate samples, and this value compared favorably with the yield of our more cumbersome filtration/freeze dry procedure. SEM examination of these specimens revealed no topographic changes caused by this procedure. These findings represent the first evidence that this method employing poly-L-lysine "glue" almost universally used for retaining fixed cells from suspension for SEM, can be easily modified to ensure reproducibly high yields.

Tumor Virus Expression In Vitro and In Vivo:

Papilloma viruses are a common cause of epidermal tumors in man. Some lesions induced by these viruses may undergo malignant conversion. Little has been known about the functional organization of the genomes of these viruses or how lesions progress from a benign to a malignant state. The determination of the transforming sequences of bovine papilloma virus (BPV) DNA represents a first step towards understanding how these tumors are formed. Our results indicate that the transforming protein(s) is (are) encoded in a short region of the viral genome and that transformation can occur independently of viral replication. The finding that interferon treatment can cure some tissue culture cells of BPV sequences and revert them to the normal phenotype provides an experimental basis for attempts to treat papillomavirus induced disease with this drug.

The studies of viral and cellular oncogenes have also made considerable progress. In collaboration with the laboratory of Dr. Edward Scolnick, we have previously defined the molecular organization of the Harvey and Kirsten murine scarcoma viruses. A normal rat gene homologous to the oncogene of the Harvey virus has also been shown to have oncogenic potential when it is expressed at high levels. We have now determined that although Harvey and Kirsten viruses both encode crossreacting 21kd transforming proteins (p21), the Harvey and Kirsten virus p21 coding genes are derived from different cellular genes. Thus Harvey and Kirsten p21 genes are part of a multigene gene family. Both

of these genes are conserved in evolution and are readily detected in human DNA. We have therefore molecularly cloned from normal human DNA four different p21 genes; two are closely related to the Harvey viral gene and two are more closely related to the Kirsten viral gene. The structure of one human Harvey type gene is very similar to that of a rat Harvey type gene. This normal human gene also has the capacity to induce oncogenic transformation of mouse cells when the p21 protein encoded by this gene is expressed at high levels by virtue of attaching a retroviral LTR in vitro. In contrast the Harvey and Kirsten viral p21 proteins, which are both phosphorylated at a threonine residue, the human and rat cellular Harvey 21 proteins are not phosphorylated, except for a very low level of serine phosphorylation. This result indicates that threonine phosphorylation is not required for p21 to be capable of inducing cellular transformation.

Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments:

One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. We have continued our studies of the keratin filaments and of a basic protein called "filaggrin", which are the most prominent intracellular components of all epidermal cells. A study of their chemistry, structure and biosynthesis in both normal and abnormal epidermis will be of profound importance in studying tumors in this tissue.

The polypeptide chains which comprise the subunits of the keratin filaments of normal bovine and murine epidermis have been isolated and individually characterized by standard protein chemical techniques. The unfractionated mixture of polypeptides or combinations of two of the purified polypeptides spontaneously polymerize in vitro in dilute salt solution into filaments that are uniformally 80 A wide and up to 40 μm long. The polypeptide composition of these filaments, and their structure, based on electron microscopy and X-ray diffraction, are the same as the in situ keratin filaments. The stoichiometry of the recombination experiments reveals that the polypeptides are present in the filaments in the precise molar ratios of 1:2. This suggests that the epidermal keratin filament has a three-chained unit structure and this is supported by the x-ray diffraction analyses.

Comparisons of the polyacrylamide-gel electrophoretic profiles of the keratin subunits obtained from abnormal human epidermis such as Darier°s disease and lamellar ichthyosis with those of normal epidermis show prominent differences in numbers and mobilities of bands. Also, the abnormal polypeptides show limited facility for polymerization in vitro. Therefore, there may be differences in the chemical structures of the proteins. Filaments assembled in vitro from psoriatic epidermis are also abnormal, and interestingly, form °paracrystalline° structures consisting of several filaments associated side-by-side in an apparently ordered manner. This may be due to the presence of an additional protein that perhaps functions like the basic protein or may be a feature characteristic of the filament proteins of psoriatic epidermis.

Normal mouse epidermal cells grown in monolayer culture can be made to synthesize the normal complement of keratin polypeptides. These proteins have been isolated and characterized by standard protein chemical techniques as done earlier with the bovine proteins. The reason why the mouse keratins are also being studied is that this cell culture system is currently being used for studies of in vitro carcinogenisis. Since the keratins are the principal synthetic products of the cells, they will be used as specific markers for the

studies on carcinogenesis. To this end, a specific radioimmune assay has been developed to follow the changes occurring during carcinogenesis. Preliminary experiments have revealed marked alterations in the synthesis of the keratin proteins during treatment with carcinogens such as TPA and other growth effectors such as vitamin A.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03657-08 D

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Immunopathologic Mechanisms Involved in Inflammatory Skin Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.I. Katz	Senior Investigator	Derm NCI
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	J. Stanley	IPA	Derm NCI
	S. Breathnach	Visiting Scientist	Derm NCI
	J.D. Fine	Medical Staff Fellow	Derm NCI
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COOPERATING UNITS (if any)
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Dermatology Department, USUHS, Bethesda

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.0	5	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pemphigus, herpes gestationis, bullous pemphigoid and dermatitis herpetiformis are blistering skin diseases all of which are associated with so-called auto-immune phenomena. We have have identified and characterized serum and in vivo bound antibodies in these diseases. Our efforts have centered around the mechanisms of tissue destruction, ultrastructure, and ultra-structural localization of antibodies. Immunogenetic considerations with regard to HLA associations and the identification of specific B-lymphocyte antigens have provided tools to study the triggering mechanisms involved in the early stages of an abnormal immune response. Another major effort is the study of Langerhans cells in inflammatory and immunologically mediated reactions. We have identified the origin of this cell which probably represents the peripheral most limb of the immune response in the skin and have identified its immunological function both in vivo and in vitro. Identification and functional characterization of alloantigens on epidermal cells and of basement membrane constituents are other integral parts of the overall program. We have identified a unique basement membrane zone antigen in human skin, the bullous pemphigoid antigen, and are currently producing monoclonal antibodies to human skin components.

Project Description

Objectives:

1) To investigate the mechanisms involved in the expression of certain immunologic skin diseases, namely pemphigus, bullous pemphigoid, dermatitis herpetiformis and herpes gestationis.

2) To determine the cell surface characteristics of the lymphocytes and epidermal cells involved in these diseases.

3) To determine whether or not genetic factors are important in the developments of these diseases.

4) To determine the ultrastructure and ultrastructural localization of antibodies in blistering skin diseases.

5) To better understand the distribution of collagen types in human skin and to characterize the chemical constituents of the basement membrane zone.

6) To determine the functional capabilities of Ia-bearing epidermal cells (Langerhans cells) in mice, guinea pigs, and humans as these cells probably play an integral role in antigen presentation and in allogeneic T cell activation.

7) To identify and characterize normal cell surface, basement membrane and cytoplasmic structures in human skin and to determine their possible role in skin cancer, wound healing, and in blistering diseases.

Material:

Skin biopsies, either punch of Castroviejo keratome slices are used. Also the small intestine of patients with dermatitis herpetiformis are studied in order to determine their antigen binding characteristics. Blister fluid studies for inflammatory mediators are also under investigation. The roofs of suction blisters are also assessed for the distribution of Langerhans Cells. Guinea pigs, mice and rabbits are used for the identification of lymphocyte and epidermal cell surface antigens as well as for the production of antibodies. Mouse skin is used to prepare epidermal cell suspensions which are used for sensitization.

Methods Employed:

Direct and Indirect Immunofluorescence, Cell Mediated Cytotoxicity, Immunochemical methods for identifying immunoglobulins and immune complexes. Radioimmunoassays. Mixed leukocyte cultures and *in vitro* antigen priming studies. Delayed type hypersensitivity reactions including contact hypersensitivity are generated by skin painting and by the injection of haplenated cells into syngeneic mice. Radioimmunoprecipitation techniques are

also employed as are standard techniques for the production of monoclonal antibodies.

Major Findings:

1) By cell culture and immunoprecipitation techniques we have demonstrated that the bullous pemphigoid antigen, a unique glycoprotein of stratified squamous epithelia, is produced by epidermal cells and has a molecular weight of 220Kd. Using the same types of culture systems we have also demonstrated that the pemphigus antigen is a 130Kd glycoprotein.

2) In vivo studies indicate that Langerhans cells play an integral role in the induction of contact hypersensitivity. Epidermal cells devoid of Langerhans cells cannot perform this function.

3) Ultraviolet light irradiation affects the antigen presenting function of Langerhans cells in such a way so that when UV irradiated Langerhans cells are irradiated and then injected subcutaneously, they induce a state of specific immunological tolerance.

4) Keratinocytes devoid of Langerhans cells produce a factor which has Interleukin (IL) I activity; that is , it enhances PHA induced thymocyte proliferation. It has many of the same physicochemical characteristics of IL 1. We have termed this factor ETAF. ETAF serves to reconstitute UV induced abrogation of allogeneic T cell stimulation.

5) We have produced monoclonal antibodies against normal epidermal cell constituents and are currently characterizing the antigens and studying their role in various pathologic states, such as wound healing and skin tumor formation.

6) We have demonstrated that dinitrothiocyanobenzene applied to skin induces a state of unresponsiveness to the allergic contactant DNCB.

Significance to Cancer Research:

A basic understanding of immunologic injury in various autoimmune disease states is important in interpreting and furthering current concepts in self-recognition. Pemphigus is associated with thymoma and myasthenia gravis and its study may provide a clue as to the association between pemphigus and other malignancies. Our in depth studies of herpes gestationis, an immunologic blistering disease of pregnancy, may provide important clues to maternal-fetal interactions. Studies are underway to determine whether there is an association between dermatitis herpetiformis, gluten sensitive enteropathy and cancer. The role of Langerhans cells in immune reactions in in the skin and their possible function or dysfunction after ultraviolet light exposure should provide some insight into their role in skin tumor formation. The study of the basement membrane and its disruption by cancer cells is essential to the study of tumor invasion.

Proposed Course of Project:

Outlined above.

Publications:

1. Stanley, J.R., Alvarez, D., Bere, E.W., Eaglstein, W.H., Katz, S.I.: Detection of basement membrane zone antigens during epidermal wound healing in pigs. J. Invest. Dermatol. 77: 240-243, 1981.

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4. Sauder, D.N., Tamaki, K., Moshell, A.N., Fujiwara, H., Katz, S.I.: Induction of tolerance to topically applied TNCB, using TNP-conjugated ultraviolet light irradiated epidermal cells. J. Immunol. 127: 261-265, 1981.

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6. Sauder, D.N., Carter, C.S., Katz, S.I., Oppenheim. J.J.: Epidermal cell derived thymocyte activating factor (ETAF). J. Invest. Derm. (in press).

7. Stanley, J.R., Hawley-Nelson, P., Yaar, M., Martin, G.R., Katz, S.I.: Laminin and bullous pemphigoid antigen are distinct basement membrane proteins synthesized by epidermal cells. J. Invest. Derm. (in press).

8. Stanley, J.R., Beckwith, J.B., Fuller, R.P., Katz, S.I.: A specific antigenic defect of the basement membrane is found in basal cell carcinoma but not in other epidermal tumors. Cancer. (in press).

9. Hall, R.P., Lawley, T.J., Smith, H.R., Katz, S.I.: Bullous Eruption of Systemic Lupus Erythematosus: Dramatic response to Dapsone therapy. Ann. Int. Med. (in press).

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12. Katz, S.I.: Erythema elevatum diutinum in Dermatology in General Medicine. Fitzpatrick TB et al (Eds.) McGraw Hill Co. New York (in press).

13. Katz, S.I: Immunogenetics of Dermatitis Herpetiformis in Proceedings of the XVI International Congress of Dermatology, University of Tokyo Press, Japan (in press).

14. Katz, S.I. and Lawley, T.J.: Advances in Dermatology: Dermatitis Herpetiformis in Proceedings of the XVI International Congress of Dermatology, University of Tokyo Press, Tokyo, Japan (in press).

15. Stanley, J.R., Yaar, M., Hawley-Nelson, P., Katz, S.I.: Pemphigus Antibodies Identify a Cell Surface Glycoprotein Synthesized by Human and Mouse Keratinocytes. J. Clin. Invest. (in press).

16. Pehamberger, H., Tamaki, K., Henkart, P., Katz, S.I.: Langerhans Cell Containing Epidermal Cells are not Cytotoxic in Antibody Dependent - and Mitogen Induced - Cellular Cytotoxicity. Immunobiology (in press).

17. Sauder, D.N. and Katz, S.I.: Immune Modulation by Epidermal Cell Products: Possible Role of ETAF in Inflammatory and Neoplastic Skin Diseases. J. Amer. Acad. Derm. (in press).

18. Sauder, D.N. and Katz, S.I.: Strain Variation in the Induction of Tolerance by Epicutaneous Application of Trinitrochlorobenzene. J. Invest. Derm. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03666-04 D

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Detection and Analysis of Circulating Immune Complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T.J. Lawley	Senior Investigator	Derm NCI
Other:	K.B. Yancey	Clinical Associate	Derm NCI
	R.P. Hall	Guest Worker	
	J. Cason	Technician	Derm NCI

COOPERATING UNITS (if any)
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LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.9	1.4	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Increasing evidence indicates that circulating antigen-antibody complexes play a role in the pathogenesis of a variety of dermatologic, rheumatologic, neoplastic and infectious disease states. We have identified and partially characterized the immune complexes which exist in several diseases i.e. Sjogren°s syndrome, mixed cryoglobulinemia mixed connective tissue disease, acute and chronic schistosomiasis, hepatitis and various types of cutaneous and systemic vasculitis, utilizing highly sensitive radioimmunoassays. We have developed a new, sensitive radioimmunoassay for the detection of IgA containing immune complexes. We have determined the antibody classes present in the immune complexes and examined the physiochemical characteristics of these complexes, as well as the reaction of these complexes with mediators of inflammation such as the complement system. We have examined the correlation between absolute levels of circulating immune complexes, the extent and severity of clinical disease, and reticuloendothelial system function. We have also examined the influence that certain genes of the major histocompatibility complex exert on immune function in vivo and in vitro in humans. We have studied the role of dietary protein in the induction of soluble immune complexes.

Project Description:

Objectives:

1) To further characterize circulating immune complexes in human diseases with regard to size and nature of the antigen, and subclass of antibody.

2) To develop reliable methods for specifically purifying immune complexes.

3) To continue clinical studies of immune complex diseases and to further assess the effects of therapy on levels of immune complexes and reticuloendothelial system clearance function.

4) To examine the relationships between immunologic function and HLA antigen expression in patients and normal volunteers.

5) To continue to assess the relation of specific genetic phenotypes with the presence and function of immunologically relevant cell surface receptors in humans.

6) To characterize the role of intermediate filaments in the activation of the complement system.

7) To evaluate in vivo the role of human C5a in the inflammatory response of normals and individuals with immunologically mediated diseases.

Material:

Serum, red blood cells, white blood cells and skin biopsies from patients and controls will be used. Purified human Clq, Raji cells, and ^{125}I (Bolton-Hunter reagent) and immunospecific antisera are used in the immune complex assays. Purified IgG fraction of anti Rh(D) human antiserum and ^{51}Cr.

Methods Employed:

^{125}I-Clq binding assay, Raji cell IgG radioassay, Raji cell IgA radioassay, direct and indirect immunofluorescence, Fc specific reticuloendothelial system clearance assay, column chromatography, sucrose density gradient ultracentrifugation, monoclonal antibodies, fluorescein activated cell sorter.

Major Findings:

1) We have found that approximately 50% of patients with gluten sensitive enteropathy (GSE) have IgA containing circulating immune complexes as well as IgG immune complexes.

2) The presence and level of immune complexes in GSE patients does not

correlate with disease activity and serial studies during dietary gluten challenge showed no consistent changes in immune complex levels.

3) We have demonstrated that a high percentage of patients with Berger°s disease (IgA nephropathy) have IgA containing circulating immune complexes, and that renal deposits consist of IgA_1.

4) These IgA immune complexes are more common early in the disease process and sediment as 9S-13S molecules.

5) We have demonstrated that high percentage of patients with psoriasis have IgA containing circulating immune complexes. The amount of IgA immune complexes correlates with pretreatment disease severity scores.

6) We have found an HLA-B8/DRw3 associated functional Fc receptor defect in vivo and in vitro in humans.

7) HLA-B8/DRw3 positive individuals have normal in vitro expression of Fc receptors on peripheral blood monocytes despite having defective reticuloendothelial system Fc mediated clearance.

8) HLA-B8/DRw3 positive normals, dermatitis herptiformis patients (HLA-B8/DRw3 positive), and non-HLA-B8/DRw3 normal controls have nearly identical cell surface marker profiles as assessed by monoclonal antibodies and fluorescein activated cell sorter analysis.

9) Patients with onchocerciasis have a high incidence of circulating immune complexes.

10) These levels are essentially unchanged by treatment with diethylcarbamazine over a two month treatment course.

11) Individuals with onchocerciasis who initially have high levels of immune complexes are at much higher risk to develop occular and systemic complications of diethylcarbamazine therapy.

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6. Lawley, T.J.: Methods of detection of circulating immune complexes.

7. Hall, R.P., Lawley, T.J.: Circulating immune complexes. Their role in dermatological disease. In Progress in Diseases of the Skin. Fleischmajer, R. (ed) Grune and Stratton. pp. 79-101, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03659-08 D

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Therapy of Skin Cancer, Disorders of Keratinization, and Cystic Acne

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	G.L. Peck	Senior Investigator	Derm NCI
	E.G. Gross	Expert Consultant, Dermatopathologist	Derm NCI
	J.J. DiGiovanna	IPA, Univ. of Miami	Derm NCI
	G. Gantt	Registered Nurse	Derm NCI
	S. McClean	Senior Clinical Chemist	Clin. Chem. Serv., NIH
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2) Molecular Disease Branch, NHLBI, NIH, Bethesda, Maryland 20205

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	3.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Oral 13-cis-retinoic acid was effective in the treatment of skin cancer, and a variety of disorders of keratinization (lamellar ichthyosis, Darier's disease, pityriasis rubra pilaris), and cystic acne. An oral synthetic aromatic derivative of retinoic acid (RO-10-9359) was more effective and less toxic than 13-cis-retinoic acid in the treatment of the disorders of keratinization. A topical synthetic aromatic retinoid (RO-11-1430) was ineffective in the treatment of disorders of keratinization. A high initial followed by a low maintenance dosage of 13-cis-retinoic acid was comparably effective but less toxic than previously used continuous high-dosage schedules in the treatment of cystic acne. The high-low dosage schedule was superior to the high initial dose schedule used alone and to a continuous low dose schedule. 13-cis-retinoic acid led to small but significant elevations in plasma lipids and changes in lipoproteins during therapy. RO-10-9359 produced similar changes which were dose dependent and responsive to dietary management. Absorption of RO-10-9359 is greater with milk than with water. Etretinate is bound in plasma to beta-liproproteins.

Project Description:

Objectives:

To evaluate safety and effectiveness of new oral and topical agents particularly the synthetic retinoids, in the treatment of skin cancer, disorders of keratinization and cystic acne.

Agents:

1) 13-cis retinoic acid, 10, 20 and 40 mg capsules
2) An ethyl ester of a trimethylmethoxyphenyl derivative of retinoic acid (RO-10-9359) 10, 25 and 50 mg capsules.
3) An ethylamide of a tremethylmethoxyphenyl derivative of retinoic acid (RO-11-1430) in 0.1% cream and 0.3% gel.

Materials:

1) Keratinizing Dermatoses

   A Ichthyosis
      a) lamellar ichthyosis
      b) X-linked ichthyosis
      c) ichthyosis vulgaris
      d) epidermolytic hyperkeratosis
      e) non-bullous congenital ichthyosiform erythroderma
   B Darier°s disease
   C Psoriasis
   D Keratoderma palmaris et plantaris
   E Pityriasis rubra pilaris
   F Nevus comedonicus
   G Erythrokeratodermia variabilis
   H Netherton°s syndrome
   I Hailey-Hailey disease
   J Lichen planus
   K Pachyonychia congenita
   L Dyskeratosis congenita

2) Basal cell carcinoma

   A Nevoid basal cell carcinoma syndrome
   B Sunlight induced basal cell carcinomas
   C Arsenical induced basal cell carcinomas
   D X-ray induced carcinomas

3) Cystic and Conglobate acne and hidradenitis suppuritiva

Methods Employed:

1) Disorders of Keratinization

a) Oral 13-cis retinoic acid was given for 6 month courses of therapy at 1 mg/kg/day. The dose was gradually increased (up to 8 mg/kg/day) to tolerence.

b) Oral RO-10-9359 was also given for 6 month courses of therapy beginning at 0.5 mg/kg/day. The dose was increased up to 1.5 mg/kg day or to tolerence.

c) Topical RO-11-1430 cream, 0.1%, was given in a double-blind manner versus placebo to 9 patients with disorders of keratinization. Topical RO-11-1430 gel, 0.3% was similarly tested in 3 patients. Therapy was given twice daily for 4 to 8 weeks without occlusion.

2) Basal Cell Carcinoma

Oral 13-cis retinoic acid was given for 6 month courses of therapy at 1 mg/kg/day. The dose was gradually increased (up to 8 mg/kg/day) to tolerance.

3) Cystic acne

a) Patients, 16 years and older, with at least 10 cystic lesions were treated with oral 13-cis-retinoic acid in an initial pilot study (14 patients) or in a double-blind, randomized study against placebo (33 patients) or in a third study testing a high initial and low maintenance dosage schedule (40 patients) or in a fourth study testing the need for the low maintenance dose.

b) In the pilot study Oral 13-cis retinoic acid was given for 4 month courses of therapy beginning at 1 mg/kg/day. The dose was gradually increased to tolerance.

c) In the double-blind designed protocol, treatment was begun at a dosage of 0.5 mg/kg/day and was only increased if there was a significant worsening of the disease.

d) In the "high-low" study, 20 patients with predominantly facial acne, they were given a high initial dose of 1.0 mg/kg/day for either 2 weeks or 4 weeks and then were given a low maintenance dose of 0.25 mg/kg/day for the remainder of a 16 week treatment period. Similarly, 20 patients with predominantly trunk acne received a high initial dose of 2.0 mg/kg/day for either 2 or 4 weeks followed by a low maintenance dose of 0.5 mg/kg/day for the remainder of the 16 week course of therapy.

e) In the fourth study 3 groups of 24 patients received either a) a high-low dosage schedule consisting of 2.0 mg/kg/day for 2 weeks and 0.5 mg/kg/day for 14 weeks, b) a high-dosage schedule alone in which patients received 2.0 mg/kg/day for 2 weeks followed by placebo for 14 weeks, or c) a continuous low dosage schedule consisting of 0.5 mg/kg/day for 16 weeks.

Major Findings:

1. 14 patients with treatment-resistant cystic and conglobate acne was treated for 4 months with oral 13-cis retinoic acid, a synthetic isomer of naturally occurring all-trans-retinoic acid. The average maximum dose re-

ceived was 2.0 mg/kg/day. 13 patients experienced complete clearing of their disease; the other had 89% improvement, as determined by the number of acne nodules and cysts present before and after therapy. Prolonged remissions, currently lasting an average of 46 months after discontinuation of therapy, have been observed in all 14 patients. One patient developed 2 lesions 12 months after discontinuation of therapy. Therapy was resumed in his case and he is once again free of disease. No other patient has required or been given additional therapy. The mechanism of action of 13-cis-RA in the therapy of acne probably involves a direct inhibitory effect of the drug on the sebaceous gland. Evidence for this inhibitory effect comes from 3 skin biopsies which revealed a marked decrease in size of the sebaceous gland, and from forehead skin surface lipid film analyses, which indicated sebaceous gland inhibition by significantly lower levels of squalene and wax esters during therapy. Additionally, there was an average 84% decrease in mean forehead sebum production as compared to pretreatment values. There was a complete return to pre-treatment values after discontinuation of therapy in the sebum composition studies but only a partial return to normal of sebum production.

2. A study comparing 13-cis-RA at initial dose of 0.5 mg/kg/day versus placebo in a double-blind format in the treatment of cystic and conglobate acne has been completed. 17 patients who initially received placebo worsened to the point where the double-blind code was broken and treatment with 13-cis-RA was begun. There was an overall 57% increase in the number of cystic lesions in this group. 16 of these 17 patients then received 13-cis-RA with a resultant 97% improvement. An additional 17 patients who had been randomly assigned to receive initial therapy with 13-cis-RA had an overall 96% reduction in number of nodules and cysts. There were an average of 46 nodules and cysts before treatment, and 2 afterwards. The average maximum dose of 13-cis-RA received by all 33 patients was 1.2 mg/kg/day, or 90 mg/day. 22 of the 33 patients have become completely free of lesions and only 3 patients have more than 3 lesions remaining. Patients who typically responded rapidly to a low dose were female, with facial lesions, and with an average of 27 cysts pretreatment. Slower responders requiring higher doses were typically male, with chest and back lesions, and with an average of 67 cysts pretreatment. 21 patients received one 4-month course of therapy; 12 received 2 courses after a 2-month treatment-free interval. 4 patients who had cleared completely after one course of therapy with 13-cis-RA had mild relapses after 4 to 12 months post-discontinuation. 3 of these 4 patients had received only 0.5 mg/kg/day during their initial course of therapy, and all cleared completely with their second course. All other patients continue to have prolonged remissions ranging from 43 to 50 months, average of 46. These results indicate that the beneficial therapeutic response is not a placebo effect, that 0.5 mg/kg/day is an effective dose particularly for facial lesions and is also productive of the common side effects. Higher doses are frequently required for control of chest and back lesions. Continued healing after discontinuation of therapy indicates that lower doses or alternate dosage schedules may also be effective.

3. At the first post-treatment follow-up visit: 1.0 mg/kg/day for 2 weeks group there was an overall 70% reduction in cysts with an 85% reduction of

facial cysts, in particular. In the 1.0 mg/kg/day for 4 weeks group there was an overall 82% improvement with an 89% response on the face. In the 2.0 mg/kg/day for 2 weeks group there was an overall 86% improvement with a 96% improvement on the face, 81% on chest, 83% on the back. In the 2.0 mg/kg/day for 4 weeks group there was an over all 77% improvement with 95% improvement on the face, 77% on the chest, and 63% on the back. Both 1.0 mg/kg/day groups had temporary, slight increases in numbers of observed cysts during the first 2 weeks of therapy, whereas the 2.0 mg/kg/day groups did not. We conclude that for most patients, especially for facial lesions, 2.0 mg/kg/day for 2 weeks is the optimal high dosage. In some patients with back acne, the high initial dose of 2.0 mg/kg/day may have to be prolonged beyond 2 or even 4 weeks. The observed toxicity during the initial high dose periods was similar to our other studies. However, once the low maintenance dose was begun there was a marked reduction initally in severity of toxicity and later in incidence. We conclude that except for a few patients with comparatively resistant back acne, the high-low dosage schedule is comparable in effectiveness to the continuous high dosage schedule of previous studies but is superior in minimizing the incidence and severity of observed toxicities.

4. In the fourth acne study, it was found that the high-low dosage schedule was superior to the initial high dose when used alone in the treatment of cystic acne. The high-low dosage schedule was also superior to the constant low dosage schedule in the treatment of cystic acne of the trunk. Cystic acne of the face responded well to both the high-low and the constant low dosage schedules. It was found that 12 of 21 patients receiving the high-low schedule, and 7 of 22 patients receiving only the high dose schedule, and 6 of 21 patients receiving the constant low dosage schedule had a 75% or greater reduction in total number of acne cysts at the end of the 16 week treatment period.

5. 12 patients with multiple basal cell carcinomal induced by sunlight, X-ray, arsenic, or the nevoid basal cell carcinoma syndrome were treated with oral 13-cis-retinoic acid. Of 270 tumors, 43 (16%) underwent complete clinical regression. Twenty-one of 35 of these tumors when biopsied after treatment were found to be gone microscopically as well. Correlation of therapeutic response with tumor size revealed that 19 of 83 (23%) tumors 3-5 mm in diameter and 18 of 99 (18%) tumors 6 to 10 mm in diameter underwent complete clinicl regression, whereas only 6 of 88 (7%) tumors 11 mm or greater in diameter responded completely. Of the remaining tumors, 173 (64%) decreased in size and 54 (20%) were unchanged. Average maximum dosage in this group of 12 patients was 4.6 mg/kg/day with a range of 1.5 to 8.2 or 370 mg/day with a range of 120 to 660 mg/day. Duration of treatment varied from 16 to 96 weeks with an average of 56. Four patients had been sensitized and treated with DNCB six years previously. Three of these 4 patients developed a marked inflammatory response in most tumors during therapy with oral 13-cis-retinoic acid. Two of the other 8 non-DNCB exposed patients developed inflammation in a few of their tumors. However, there was no difference in the final therapeutic response between these two subgroups with 21/109 (19%) tumors completely regressing in the DNCB treated group and 22/161 (14%) in the DNCB unex-

posed group. Histologically, a dense small cell infiltrate was seen in tumors undergoing inflammation, suggesting that 13-cis-RA could either be enhancing a host immunologic response or be producing a direct cytotoxic effect on the tumor. However, in 7 of 17 (41%) non-inflammatory regressing tumors biopsied during treatment islands of squamous differentiation with horn pearl formation were observed with progression in some islands to complete replacement of tumor cells by the completely differentiated horn pearl. In tumors biopsied prior to treatment, only 14 of 85 (16%) showed evidence of keratinization. This data is consistent with an enhancement of epidermal differentiation by 13-cis-RA.

6. 57 patients with cutaneous disorders of keratinization were treated with oral 13-cis-retinoic acid, an oral synthetic retinoid from Hoffman-(DD) (9 patients), lamellar ichthyosis (LI) (10), psoriasis (PSOR) (9), pityriasis rubra pilaris (PRP) (5), keratoderma palmaris et plantaris (KPP) (4), epidermolytic hyperkeratosis (EHK) (4), non-bullous congenital ichthyosiform erythroderma (NBCIE) (3), x-linked ichthyosis (XLI) (3), Hailey-Hailey (HH) (2), and 1 each with a variant form of NBCIE, erythrokeratodermia variabilis (EKV), pachonychia congenita (PC), ichthyosis vulagaris (IV), Netherton°s syndrome (NS), and porokeratosis (PORO). The patients ranged in age from 4 to 82 years. The dosage varied from 0.5 to 8.2 mg/kg/day, the duration of treatment varied from one week to over 5 years. The average maximum dosage was 160 mg/kg/day or 2 mg/kg/day. Treatment was initially given in 16 week courses of therapy with intervening 8 week treatment-free intervals. Now it is given in 6 month courses with 1-4 week intervals. Good or excellent responses were seen in DD (7), LI (8), NBCIE (3), PSOR (4), KPP (2), EKV (1), and IV (1). Partial responses were obsered in EHK (3), PRP (2), LI (1), DD (2), KPP (1), PC (1, variant-NBCIE (1). Patients showing minimal or no response included: PSOR (5), XLI (3), LP (2), HH (2), NS (1), EHK (1), PRP (1), KPP (1). One patient with PORO and one with LI stopped treatment within one week before a therapeutic evaluation could be made. The mechanism by which this synthetic retinoid alters these disease states is not known but may be related to the observed ability of vitamin A to affect epithelial differentiation. The observed variation in therapeutic response could be related to the presence or absence of specific retinoid binding proteins. Our results indicate that synthetic retinoids, such as 13-cis-RA may represent a potent new class of drugs in the treatment of cutaneous disorders of keratinization, several of which were previously treatment-resistant.

7. 75 patients with cutaneous disorders or keratinization were treated with RO-10-9359, an oral synthetic aromatic retinoid from Hoffman-La Roche, Inc., Nutley, New Jersey. This retinoid is an ethyl ester of a trimethyl-methoxyphenyl derivative of retinoid acid. Diseases included DD (18), PSOR (23), PRP (10), LI (6), EHK (4), IV (3), KPP (2), variant-NBCIE, NBCIE (1), Kyrle°s dis-(1), PC (1), HH (1), PORO (1), and LP (1). The average maximum dosage was 86 mg/day or 1.2 mg/kg/day. Duration of treatment varied from 1 week to over 3 years. Treatment was initially given in 16 week courses of therapy with 8 week treat-free intervals. Now it is given in 6 month courses with 1-4 week intervals. Good or excellent responses were seen in PSOR (8), DD (7), PRP (4), LI (3), IV (2), KPP (1), and EKV (1). Moderate responses included: PSOR (15), DD (11), PRP (6), LI (3), EHK (4), IV (1), KPP (1), variant-NBCIE (2), and

Kyrle°s (1). Minimal responses were noted in one patient with PORP, the lingual lesions of PC lesions of PC (1), and the oral lesions of LP (1). Worsening was observed in HH (1). Response to therapy was initially variable in psoriasis. 12 patients worsened for 8, 12, or even 16 weeks of therapy and then improved; 11 other patients with psoriasis improved immediately. All other patients with responsive diseases improved immediately upon beginning therapy. RO-10-9359 is clearly superior to 13-cis-retinoic acid in the treatment of psoriasis, IV, KPP, and EHK.

8. 9 patients with disorders of keratinization were treated with topical RO-11-1430 cream, 0.1%, in a double-blind manner against placebo. Diseases included PSOR (3), KPP (2), EHK (1), and variant NBCIE (1). 7 patients received retinoid cream treated sites showed improvement indicating either a beneficial effect of the vehicle or an overall seasonal improvement in the patients disease. No patient exhibited a preferential beneficial effect of the retinoid cream. No systematic or local toxicities were observed. These results indicate that either RO-11-1430 is ineffective or that the 0.1% concentration is inadequate. Similar testing with the 0.3% gel also revealed no therapeutic or toxic effects.

9. Common side effects in most patients treated with these retinoids were limited to the skin and mucous membranes and included cheilitis, facial dermatitis, conjunctivitis, xerosis, dryness of the nasal mucosa with mild nosebleeds, and easy peeling of the stratum corneum upon trauma termed "skin fragility". Aside from skin fragility, the above side effects were either more common or more severe during treatment with 13-cis-RA than with RO-10-9359. However, there were side effects that were present in higher incience in patients treated with RO-10-9359. These include: skin fragility, hair thinning (telogen), itching, palmar peeling, dry mouth with thirst, arthralgias, "stickiness" of the skin, and paronychia. Laboratory abnormalities during therapy are limited to elevations of the erythrocyte sedimentation rate, temporary low-grade elevations of the transaminases in approximately 10% of patients which return to normal values without discontinuation of therapy, and elevations of the alkaline phosphatase, LDH, and serum triglycerides in a few patients.

In the "High-Low" cystic acne study described in Methods employed, section 3d, and in Major Findings, section 3, analyses of serum lipids were performed. In those 20 patients initially receiving 1.0 mg/kg/day of 13-cis-RA, an increase of 20% in total plasma triglycerides and an increase of 6% in total plasma cholesterol was noted during therapy when compared to pretreatment values. In 9 of these 20 patients HDL-cholesterol was measured and found to be decreased by 12% during therapy. In 19 of the 20 patients initially receiving 2.0 mg/kg/day, the observed changes in serum lipids during therapy as compared to pretreatment values, were: 1) an increase of 24 mg/dl in HDL-cholesterol, 2) an increase of 5.4 mg/dl in VLDL-cholesterol, 3) a decrease of 3.6 mg/dl of HDL-cholesterol, 5) an increase of 25.5 mg/dl of total plasma cholesterol, and 5) an increase of 44.2 mg/dl of total plasma triglycerides. All values were significant at levels of p §0.01.

A third group of 9 patients were retreated with 2.0 mg/kg/day for 6 months. Data from each of these three different treatment schedules indicated no significant change in triglyceride or cholesterol values after the initial rise from base line values noted after the first week of treatment. Therefore 2 mg/kg/day of 13-cis-retinoic acid over 6 months did not lead to significantly higher triglyceride or cholesterol levels than those observed with shorter treatment periods. All values returned to base line within 4 weeks of stopping therapy.

Similar changes in serum lipids and lipoproteins have been observed in 9 patients with psoriasis treated with oral RO-10-9359 at a maximum dosage of 1.0 mg/kg/day. These changes returned towards normal with dietary management and with decreased dosage.

In general, these side effects are dose-dependent in incidence and severity, relieved by adjunctive bland therapies, well-tolerated by the patients, and totally reversible upon discontinuation of therapy.

10. To examine the association of synthetic retinoids within lipoprotein fractions, sera from 6 patients (psoriasis, Darier°s disease, pityriasis rubra pilaris) receiving etretinate and from 6 receiving isotretinoin (basal cell carcinoma, acne) were separated into lipoprotein fractions by either ultracentrifugation or by heparin-manganese precipitation. Retinoid concentrations were measured with high pressure liquid chromatography. Sera were evaluated after a 12 hour fast and four hours after the patients ingested retinoid with whole milk. 80$\pm$ 1.7% of the serum etretinate but only 27 $\pm$ 1.5% of the serum isotretinoin was found with the beta-lipoproteins. The lipoprotein bound etretinate was found in both the very low density lipoprotein and the low density lipoprotein fractions. When the fasting and four hour specimens were compared, there were no differences in distribution of these retinoids within the beta-lipoprotein fraction or the albumin containing fraction, suggesting that these associations are independent of serum concentrations of the drug. No differences were observed between individuals despite differing ages and diseases. It is not known whether the beta-lipoproteins function to transport the retinoids to cell surface receptor sites or serve only as a circulating reservoir.

11. Since etretinate is lipid soluble and may be poorly absorbed in the absence of a fat load, we sought to determine whether diet affected its absorption. After an overnight fast, 6 Darier°s disease and 2 psoriatic patients received a 1 mg/kg AM dose of etretinate with water or 1 pint of whole milk. Light protected serum samples were drawn at various times and analyzed for etretinate and its major metabolite (Ro 10-1670) by high pressure liquid chromatography. Since low levels of etretinate and Ro 10-1670 persist after chronic administration, serum levels were corrected for this baseline (zero time) value in each set of assays. At every time period, the mean corrected serum etretinate concentration

after administration with milk was higher than after administration with water. The mean corrected peak serum concentration of etretinate (2 to 6 hours) was significantly higher after administration with milk (98± 13 ug/dl) than water (29± 5 ug/dl). In each patient the peak serum etretinate after administration with milk ranged from 147% to 375% higher than after water. Over a 24 hour period there was an overall 296 ± 26% ($p \leq 0.0005$) increase in serum etretinate after administration with milk compared to water in 5 patients with Darier°s disease. In contrast, serum levels of the metabolite (Ro 10-1670) were found to be similar regardless of the mode of administration of etretinate. No difference in the pattern of absorption was observed between patients with Darier°s disease and psoriasis. These data indicate that the serum level of etretinate, but not its major metabolite can be markedly increased by administration of the drug with a fat load.

Significance to Cancer Research:

a) These are exploratory studies of a new class of drugs (synthetic retinoids) in the treatment of skin cancer. Skin cancer should prove to be a valuable lesion for the screening of these agents because of tumors are observable, measurable, and therefore can be used as an objective indicator of response to therapy. Furthermore, the patients can be expected to be in good health, which would allow for long term studies.

b) The profound beneficial effect of 13-cis retinoic acid in the treatment of acne and both retinoids in the treatment of cutaneous keratinizing diseases indicates that other keratinizing disorders of man, for instance, preneoplastic squamous metaplasia of tracheobronchial and urinary bladder epithelial, could be successfully treated with the synthetic retinoids. Treatment of these keratinizing dermatoses and acne may also provide useful information in the evaluation of newer and potentially more potent and less toxic synthetic retinoids.

Proposed Course of Project:

1) Continued treatment of patients with acne, basal cell carcinomas, and disorders of keratinization with 13-cis retinoic acid (RO-43780).
2) Continued treatment of disorders of keratinization with oral RO-10-9359.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03630-12 D

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effects of Vitamin A and Analogs on Chick, Mouse and Human Skin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.L. Peck	Senior Investigator	Derm NCI
	E.G. Gross	Expert Consultant, Dermatopathologist	Derm NCI
	P.M. Elias	Asst. Professor, Dept. of Dermatology	Univ. of California Medical Center
	J.J. DiGiovanna	IPA, Univ. of Miami	Derm NCI
	G. Chader	Acting Chief. Lab of Vision Research	NEI

COOPERATING UNITS (if any)

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2. Lab of Vision Research, NEI

LAB/BRANCH

Dermatology Branch

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) This project proposed to morphologically and biochemically define the mechanism of action of vitamin A and its derivatives (retinoids) in altering epidermal differentiation in normal skin, and in benign and malignant lesions of skin. Topical all-trans retinoic acid, but not systemic 13-cis-retinoic acid, increased gap junction density and decreased desmosome density in treated basal cell carcinomas. This indicates that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms.

A specific cytosol retinol binding protein (crbp) has been identified in mouse, normal human skin and skin and human skin from patients with Darier°s disease, psoriasis and basal cell carcinomas. A specific cytosol retinoic acid binding protein (CRABP) has also been identified in newborn mouse and normal human adult skin and newborn foreskin.

Project Description:

Objectives:

1) To investigate the mechanisms governing epidermal cell differentiation.

2) To morphologically investigate the mechanism by which vitamin A and its derivatives alter epidermal cell differentiation in normal adult skin and in benign and malignant lesions.

The Objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: Study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Material:

1) 0.2-0.4mm thick, Castroviejo keratotome slices of normal and diseased human skin. Skin from patients with Ichthyosis and Darier°s Disease (NCI-3643) has been used to date.

2) 3-4 mm punch biopsies of normal and diseased human skin both treated and untreated with synthetic retinoids (NCI-3643).

Major Findings:

1) Freeze-fracture replicas and thin sections of cell membranes of: 1) 11 basal cell cancers (BCC) treated twice daily for two weeks with topical 1.0% all-trans retinoic acid (RA); 2) 21 BCC treated for 2 to 17 weeks with oral 13-cis retinoic acid (CRA) (1.0-8.0 mg/kg/day); and 3) 17 BCC prior to retinoid treatment and/or after applications of vehicle alone. Both thin sections and replicas were examined and photographed in a single-blind fashion, and the density and size distribution of gap junctions and desmosomes were computed planimetrically. Neither RA nor CRA treatment appeared to influence hemidesmosome or microfilament populations. Structural changes in both treatment groups did not correlate with either tumor regression or inflammation. This indicates that topical and systemic retinoids may exert their antineoplastic activity by different cellular mechanisms.

2) Sucrose density centrifugation was used to identify specific cytosol retinol (CRBP) and retinoic acid (CRABP) binding proteins in newborn mouse skin. A similar amount of retinol binding in skin was found after a 15 minute, 37'C incubation compared to the standard 2 hour, 4'C incubation. Stability of binding during frozen storage was demonstrated for periods up to 4 1/2 months. The ability to augment retinol binding by 75'C with the addition of a 24-hour pretreatment of the tissue with lyophilization allowed the detection of binding in smaller qualtities of tissue.

Epidermal-dermal separation of newborn mouse skin with trypsin and the amount of binding with 2 layers was determined. Equivalent amounts of retinoic acid binding and seven times more retinol binding were found in the dermis compared to epidermis.

Specific retinol and retinoic acid binding was also identified in adult human skin. Adult human skin separated with trypsin or EDTA revealed that the epidermis bound significant levels of retinol and retinoic acid while the dermis did not bind detectible levels. Adult human human epidermis bound 10 to 20 times more retinoic acid than retinol. The ratio of Retinol/ Retinoic Acid bound in adult human epidermis was similar to unborn mouse skin.

Specific Retinol and Retinoic acid binding was identified by the skin of a patient with Darier°s disease. Specific retinol binding was found in basal cell carcinoma and psoriasis.

Significance to Cancer Research:

1) Since carcinogenesis is an instance of altered differentiation, studies of vitamin A effects on differentiation, may serve as an excellent model for investigations of cellular control mechanisms which relate to carcinogenesis. The fact that carcinogenesis is influenced very markedly by vitamin A deficiency directly links research on the epithelial effects of vitamin A to cancer research.

2) Furthermore retinoids are of value in the treatment of malignancy. The mechanisms of action of retinoids in affecting differentiation may be related to its antineoplastic activity.

Proposed Course of Project:

1) Continued ultrastructural examination of normal skin and benign and malignant lesions of skin both treated and untreated with synthetic retinoids.

2) Continued study of the specific mechanism of vitamin A and retinoid binding to normal and diseased skin with emphasis on elucidating the mechanism of action of these drugs on skin. The distribution and density of specific receptors for retinol and retinoic acid in normal and diseased skin will be studied.

Publications:

1) Elias, P.M., Grayson, S., Gross, E.G., Peck, G.L., McNutt, N.S.: Influence of Topical and Systemic Retinoids in Basal Cell Carcinoma Cell Membranes. Cancer. 48: 932-938, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03638-13 D

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Studies of DNA Repair in Human Degenerative Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.H. Robbins	Senior Investigator	Derm NCI
	A.N. Moshell	Guest Worker	Derm NCI
	R. Otsuka	Visiting Fellow	Derm NCI
	L.R. Seguin-Spillman	Staff Fellow	Derm NCI
	R.E. Tarone	Mathematical Statistician	B NCI
	L.R. Seguin-Spillman	Staff Fellow	Derm NCI
	D.A. Scudiero	DNA Repair Lab	Chem. Carcin. Prog.,FCRC
	R. Polinsky	Expert	LCS, NIMH, NIH
	C.D. Lytle	Research Biochemist. Experimental Studies Br.,	Div. Biolg. Effects., FDA.
	L.E. Nee	Clinical Res. Soc. Worker	LCS, NIMH, NIH

COOPERATING UNITS (if any)
Biometry Branch, DCCP, NCI.

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies in this laboratory are designed to elucidate the role of DNA repair processes in human diseases and in carcinogenesis and in normal and abnormal aging. Most studies have been conducted with cells from patients with xeroderma pigmentosum (XP) who have defective DNA repair plus multiple cutaneous malignancies, and premature aging of sun-exposed skin and of the nervous system. Cells from patients with ataxia telangiectasia and tuberous sclerosis, diseases with abnormal cell growth and differentiation, and from patients with primary neurological degenerations are also being studied. These studies are designed to elucidate the pathogenesis of these disorders as well as to develop presymptomatic diagnostic tests. We assess the biological effectiveness of DNA repair primarily by in vitro assays of cell survival after treatment of the cells with the DNA damaging agents.

Project Description:

Objectives:

To study DNA repair processes in normal cells, cells from patients with each of the eight genetic forms of xeroderma pigmentosum (XP), and cells from patients with other diseases in which defective DNA repair is claimed or suspected, including degenerative diseases involving the somatic and/or autonomic nervous systems, skeletal muscle, and the retina; to determine the relationship between the function of such repair processes and 1) carcinogenesis, mutagenesis, cytotoxicity and 2) the clinical findings of photosensitivity, premature aging, and UV-carcinogenesis; to develop presymptomatic diagnostic tests for the aforesaid disorders.

The objective and Approach of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Materials:

Cells are obtained from established cell repositories, from hospitalized patients and outpatients at the NIH, the University of Minnesota Hospitals, Minneapolis, Minn. (Dr. Jonathan Wirtschafter), and the Veterans Administration Hospital, Fargo, ND (Dr. Roger Brumback). Cells currently under study are dermal fibroblasts and lymphocyte cell lines.

Methods Employed:

Fibroblast and lymphocyte lines are cultured in the absence of antibiotics in laminar flow hoods.

The survival of fibroblasts after irradiation with UV or X-rays, or after treatment with chemicals, is performed by counting the number of colonies which form. The survival of lymphocytes is determined by their ability to exclude the vital dye trypan blue. Unscheduled DNA synthesis is determined by determining autoradiographically the UV-induced incorporation of tritiated thymidine. Host-cell reactivation is determined by the formation of plaques in fibroblast monolayers by irradiated herpes simplex virus (Dr. Lytle).

Major Findings:

1. We have studied cells from a patient who has both Cockayne syndrome and xeroderma pigmentosum. This combination of diseases has been seen in only one previous patient (our patient XP11BE in complementation group B). We have conducted UV-induced unscheduled DNA synthesis complementation group studies with the new patient°s fibroblasts. His cells complemented those from the previous patient as well as those from XP patients in complementation groups A,C,D, E, and G. Studies are underway

It is likely that this new patient will represent a new XP complementation group.

2. With Dr. David Lytle we have studied the host-cell reactivation capacity of fibroblasts from patients with Cockayne syndrome. Their host-cell reactivation of UV-irradiated Herpes simplex virus was less than that of normal lines. These results strongly support the conclusion that Cockayne syndrome patients have a defect in the repair of UV-damaged DNA.

3. We have determined the post-x-ray colony-forming ability of fibroblasts from patients with various neurological degenerations. We have found that fibroblasts from patients with Usher syndrome (congenital deafness and retinitis pigmentosa) are hypersensitive to x-rays.

4. With Dr. Dominic A. Scudiero we have previously detected hypersensitivity to the lethal effects of N-methy-N°-nitro-N-nitrosoguanidine (MNNG), a mutagenic and carcinogenic DNA-damaging chemical, in fibroblasts from 6 patients with Huntington disease, 4 with familial dysautonomia, 5 with muscular dystrophy and 1 with Usher syndrome. We have increased the number of normal lines studied from 13 to 21, and we have studied 3 additional Usher syndrome and 4 additional muscular dystrophy lines. Our results show that Usher syndrome and muscular dystrophy are diseases in which the patients° fibroblasts are significantly hypersensitive to the lethal effects of MNNG. Fibroblasts from patients with demyelination (e.g., multiple sclerosis, Cockayne syndrome, Charcot-Marie-Tooth disease) were not hypersensitive to MNNG. All our results show that hypersensitivity to MNNG is present in cells from patients with primary degeneration of post-mitotic excitable tissue (e.g., nerves, photoreceptors, muscle). Elucidation of the molecular basis for the hypersensitivity may shed light on why most of these disorders, while manifesting hypersensitive to the lethal effects of MNNG, are not characterized by an abnormally increased incidence of cancers. Such studies may also determine whether the hypersensitivity reflects inherited defects in DNA repair mechanisms.

5. We have obtained restored hypersensitivity in our lymphocyte-trypan blue post-x-ray survival test for certain disease lines. Ataxia homozygote and heterozygote lines and tuberous sclerosis lines again give the same survival as that reported on previously. The survival test is now being applied to lymphocyte lines from many of the diseases we expect are characterized by hypersensitivity to ionizing-radiation.

6. Cytogenetic studies are being conducted to determine if DNA-damaging agents induce abnormal numbers of chromosome abberations in human cells which have a hypersensitivity to such agents. The development of such a cytogenetic test would make it possible to detect hypersensitivity to DNA-damaging agents within a few days, as opposed to the currently employed colony-forming test which requires two to three weeks. Cells currently under study include fibroblasts and lymphocyte lines from patients with ataxia telangiectasia and Usher syndrome. Cells from patients with tuberous sclerosis and other diseases will also be studied.

evaluations of the patients are also being obtained.

8. Under our contract with the Institute for Medical Research, Camden, NJ, lymphocyte and fibroblast lines are being established from patients with degenerative diseases. Over 300 lines have already been established, making it possible for investigators to study there diseases in tissue culture.

Significance to Biomedical Research and the Program of the Institute:

UV-radiation and ionizing radiation are causes of human cancer. Patients with XP are particularly susceptible to the carcinogenic action of UV-radiation and develop multiple malignancies on sun-exposed areas of skin. Normal human cells have repair processes which rapidly and effectively repair DNA damage, while most XP patients have a marked impairment in the rate and/or efficiency of such repair. This process is involved not only in repair of UV-induced DNA damage but also in repair of damage by certain chemical carcinogens. Understanding the relationship between DNA repair deficiency and skin tumor development in XP patients would, therefore, elucidate the role of DNA repair in preventing in normal humans those cancers which may be due to certain chemical and physical carcinogens. Extension of this work to include study of diseases in which DNA repair may be defective, such as those with hypersensitivity to ionizing radiation, will further increase our understanding of the relationships between DNA repair processes and carcinogenesis.

XP patients° sunexposed skin ages much more rapidly than normal humans° skin. Some XP patients develop neurological abnormalities that are due to the premature death of neurons. Our studies of the DNA repair defects are providing an understanding of the relationship of DNA repair processes to these aging phenomena in XP. Thus, our study of aging in XP organs is providing important knowledge as to how properly functioning DNA repair processes prevent such aging in normal humans. Our studies of other diseases with premature death of nerve and muscle cells are also providing an understanding of such abnormal aging phenomena and of the relationship between ionizing radiation-type damage and carcinogenesis.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Publications:

1. Scudiero, D.A., Meyer, S.A., Clatterbuck, B.E., Tarone, R.E., and Robbins, J.H.: Hypersensitivity to N-methyl-N°-nitro-N-nitrosoguanidne in fibroblasts from patients with Huntington disease, familial dysautonomia, and other primary neuronal degenerations. Proc. Natl. Acad. Sci. USA Vol. 78: No. 10 pp. 6451-6455, 1981.

2. Scudiero, D.A., Moshell, A.N., Scarpinato, R.G., Meyer, S.A., Clatterbuck, B.E., Tarone, R.E., and Robbins, J.R.: Lymphoblastoid lines and skin fibroblasts from patients with tuberous sclerosis are abnormally sensitive to ionizing radiation and to a radiomimetic chemical. J.I.D. pp. 234-238, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03650-10 D

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Biochemical Characterization of Mammalian Melanosomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

V.J. Hearing	Research Biologist	Derm NCI
J.M. Nicholson	Professor	Howard Univ.
D. Gersten	Assoc. Professor	Georgetown Univ.
J. Marchalonis	Professor	Medical Univ. of S. Carolina

COOPERATING UNITS (if any)
Department of Chemistry, Howard University, Washington, D.C.
Department of Pathology, Georgetown University, Washington, D.C.
Department of Biochemistry, Med. Univ. of S. Carolina, Charleston, S.C.

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The abnormal proteins produced in human and murine malignant melanoma in vivo and in vitro are being studied: These studies are aimed at elucidating the mechanisms of formation of these atypical proteins, as well as their importance to the immunology of melanoma and/or possible immunotherapy potential.

PHS-6040

Project Description:

Objectives:

To characterize the structural and enzymatic composition of membrane proteins of melanocytes throughout their development *in vivo*, both in normal and in abnormal pigmentary systems.

The objective of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Our research has been aimed primarily at characterizing the chemical composition of membrane proteins in murine and human pigment systems, both in normal as well as in melanoma tissues. Our work has used both cells *in vivo* and *in vitro*. Tissues are excised and homogenized and subcellular fractions are then isolated and purified by means of an extensive series of differential centrifugations. Gel filtration and preparative polyacrylamide gel electrophoresis have enabled mg quantities of the proteins under study to be isolated in a pure state. This has allowed the characterization of these proteins with regard to their amino acid content, isoelectric point, molecular weight, prosthetic group content, end terminal sequences, and cyanogen bromide maps. In addition, antibodies to these purified proteins have been produced in both rabbits and goats, and immunochemical characterization of these proteins is underway.

Major Findings:

The melanosome in normal pigment cells is composed of multiple proteins, many of which are loosely bound and easily extracted; these constitute the proteins of the limiting membrane of the organelle. Other proteins are of lower molecular weight and are tightly bound to the granule, and probably constitute the structural, fibrillar proteins; they appear to be the proteins which complex with the melanin polymer. Melanosomal proteins from melanoma tissues vary in structure from those of normal tissue. Several of the proteins seem to be missing completely from these granules, which are also structurally distinguishable from normal granules by electron microscopy. Perhaps more importantly, many of the proteins in melanoma melanosomal membranes are unique and are not found in membranes of normal melanin granules. This has been found to be the case in human melanoma as well. In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules resolved a slight, but significant, difference of isoelectric points and a difference of 10,000 MW between the two proteins; both have amino acid contents which are identical with respect to 13 amino acids, but differ significantly with regard to four amino acids. The carboxy and amino terminals of these proteins are identical, while peptide mapping has revealed that amino acid sequences are deleted in 3 or more

regions of the abnormal protein. Other proteins in these tissues seem to differ in a similar manner. It has been found that tumor-specific proteins similar to these can be found in the serologic fluids of melanoma patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro. It has been recently shown that these abnormal proteins are shed from melanoma cells and may have a critical immunologic importance to the survival of the tumor in the host.

Significance to Cancer Research:

These observations concerning the aberrant biochemical characteristics of the melanoma melanosome indicate that the control of melanogenesis in malignant melanoma is in some manner affected by carcinogenesis. Melanomas are unusual among cancers since they do not result in complete dedifferentiation of the affected melanocyte, but allow this cell's specialization, i.e. melanogenesis, to continue. Our results indicate that although melanogenesis takes place, its metabolic patterns are abnormal.

There are a wide range of implications of this research for possible immunotherapy and/or immunoassay of human malignant melanoma. An analogous situation of altered proteins has been described in several other types of malignancies; thus this process of atypical formation of proteins may be common to neoplastic transformation. The similarity of these proteins suggests that the unique proteins present in the malignant melanocyte are an aberrant form of the analogous protein in the normal melanocyte, although the mechanism effecting this has not yet been elucidated.

Proposed Course of Project:

Since there are indications that the metabolism of melanogenesis in murine melanoma is aberrant, our research is ultimately intended to more fully characterize the production of these antigens in human melanomas, and attempt to determine the level on which these disorienting control mechanisms operate. It is hoped that further insight into such controls, be they at the level of replication, transcription, translation, or post-translation, will provide clues as to the level at which carcinogenic information is expressed.

Publications:

1. Hearing, V.J., Newburger, A.E., Ekel, T.E. and Montague, P.M.: Malignant melanoma-abnormal proteins synthesized in murine and human tissues. Pigment Cell: Phenotypic Expression in Pigment Cells 6: 112-118, 1981.

2. Gersten, D.M., Hearing, V.J., and Marchalonis, J.J.: Characterization of immunologically significant unique B16 melanoma proteins produced in vivo and in vitro. Proc. Natl. Acad. Sci. USA 78: 5109-5112, 1981.

3. Hearing, V.J., Malignant melanoma-tumor-specific markers. Amer. J. Dermatopath. 3: 411-416, 1981.

4. Hearing, V.J., Gersten, D.M., and Marchalonis, J.J.: Shed proteins in in malignant melanoma. Anticancer Res. 1: 313-316, 1981.

5. Gersten, D.M., Hearing, V.J., and Marchalonis, J.J.: Surface antigens of murine melanoma cells. Comparative Pathology, Proc. Symp. Membranes and Recognition. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03649-10 D

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Enzymatic Control Mechanisms in Melanogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

V.J. Hearing	Research Biologist	Derm, NCI
Yasushi Tomita	Visiting Scientist	Toyoko Univ Sch of Med.
John Pawelek	Asst Professor	Yale University

COOPERATING UNITS (if any)
Dermatology Branch, Yale Univ. School of Medicine, New Haven, Conn.

LABORATORY
Dermatology Branch

SECTION
NCI, NIH, Bethesda, Maryland 20205

INSTITUTE AND LOCATION

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

~~This project has partially characterized~~ the nature of tyrosinase, and determined the mechanism of normal latency of the enzyme, and its subsequent activation in vivo. The role of this enzyme in melanogenesis in normal tissue and the importance of the altered enzyme found in melanoma tissues is being studied. Further, control mechanisms involved in the regulation of melanin formation in the melanocyte are being investigated.

Project Description:

Objectives:

To investigate the control mechanisms involved in regulating melanogenesis in a variety of normal and malignant tissues.

The objective of the National Cancer Plan that this project most closely supports is 3.1: To study the nature and modification of the precancerous state and determine mechanisms accounting for high degrees of stability of cell functioning.

Methods Employed:

Tyrosinase and other enzymatic activities are investigated by histochemical, biochemical, spectrophotometric and radioactive assay methodology. Melanocytes are grown both in vivo and in vitro. Tissues are dissected, homogenized and fractionated into cellular fractions by means of density gradient centrifugation and differential centrifugation. After solubilization with detergents, enzymes are further purified by gel filtration and by preparative polyacrylamide gel electrophoresis. Samples are then incubated with the appropriate substrates and controls; the production of melanin and other reaction products can be followed spectrophotometrically, by liquid scintillation counting of the newly formed radioactive melanin or by-products, or characterized by amino acid analysis.

Major Findings:

Since only one enzyme (tyrosinase) is essential for melanin biosynthesis, it is a unique system for the study of enzymatic control mechanisms in normal and malignant tissues. We have examined the status of enzymatic control of melanogenesis and have found that tyrosinase is the enzyme responsible for melanin synthesis in many different pigmentary systems. It has always been a subject of dispute how tyrosinase, which can be easily demonstrated to be present in an active configuration in the endoplasmic reticulum and Golgi apparatus, is inactivated in vivo in mammalian systems and subsequently activated once in situ in the melanosome. L-DOPA is the natural activator of the enzyme in vivo; we have tested over twenty L-DOPA analogs substituted in various positions, and determined the enzyme is extremely specific in its requirement for L-DOPA as a cofactor. It has been found in this study that tyrosinase is under allosteric control and that phospholipids may play a part in the expression of the enzyme°s activity. It has also been found that this allosteric regulation in murine melanomas is altered; this perhaps explains the atypical melanosome formation in these tissues. Recent evidence has been found which supports the theory that the enzyme is additionally controlled by enzyme-associated factors which can either inhibit or stimulate the production of pigment, and may prove to be critical to the control of pigment formation in mammals.

Significance to Cancer Research:

In view of the difference noted between the regulation of tyrosinase in normal and melanoma tissues, we feel that at least one of the primary differences between the melanogenic capabilities of these two tissues has been revealed. The fact that these controls are operational at the post-translational level is informative; whether other levels of cellular control mechanisms are affected by carcinogenesis remains to be investigated. It is hoped that further study of the cause of these enzymatic differences in the malignant tissues will provide insights into the nature of neoplastic transformation.

Proposed Course of Project:

Differences in the control mechanisms over melanogenesis in normal and and malignant melanoma tissues will continue to be investigated. The characteristics of enzymatic activity and control in various subcellular fractions are being studied. This project has been expanded to include characterization of similar metabolisms in normal and malignant human tissues.

Publications:

1. Nicholson, J.M., Montague, P.M., and Hearing, V.J.: SDS soluble but Triton X-100 insoluble normal and malignant melanosomal proteins, in Pigment Cell: Phenotypic Expression in Pigment Cells 6: 107-112, 1981.

2. Hearing, V.J., Korner, A.M., and Pawelek, J.: New regulators of melanogenesis are associated with purified tyrosinase isozymes J. Invest. Derm. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03654-09 D

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Applications of SEM to Soft Biological Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Wetzel	Senior Investigator	Derm NCI
OTHER:	T.J.A. Johnson	Post Doctoral Fellow	Dept of Anat Col State Univ.
	R.E. Tarone	Math Statistician	B NCI
	R.M. Albrecht	Asst. Professor	Dept Pediatrics & Pharm. Univ. of Wisc.
	J. Pawley	Visiting Scientist	Dept. of Zoo.
	H. Ris	Professor	Dept of Zoo. Univ. Wisc.
	S.H. Yuspa	Research Microbiologist	LEP NCI

COOPERATING UNITS (if any)
B, NCI
LEP, NCI
LPD, NIAID

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To discover and develop new methods and interpretive criteria for the application of SEM to a wide variety of soft biological tissues, and thereby capitalize on the unique capabilities of this instrument in providing 1) improved survey and sampling as an adjunct to other studies, 2) an unparalleled three-dimensional, experiential view of cell surface phenomena, and 3) an unusual potential for cytochemical studies.

Project Description:

Objectives:

The Electron Microscope Laboratory of the Dermatology Branch studies the fine structure and cytochemistry of cells in skin and related tissues to determine their function in normal, hyperplastic and neoplastic states. This project is designed to establish the significance of cell surface structure in relation to function and cytochemistry in both normal and diseased states.

Specifically, this project includes studies on: 1) the improvement of methods to prepare biological specimens for scanning electron microscopy (SEM), with particular emphasis on retaining reliably high yields of fixed cells from suspension; 2) the visualization of native keratin filaments by means of high voltage electron microscopy (HVEM) with correlative SEM of surface features; and 3) the correspondence among topographic features, surface cytochemistry, and cell cycle in an established macrophage cell line (formerly Z01 CB 03647-08 D).

Methods Employed:

1) In order to achieve reliably high yields of fixed cells from suspensions various types of cell (trypsinized monolayer cells from an S-180 mouse sarcoma line, a P-388 mouse lymphoid line, and buffy coat cells from normal human blood) were fixed in suspension by adding an equal volume of 6% glutaraldehyde for two hours or more before rinsing in distilled water, phosphate buffered saline or 0.1M phosphate buffer. Aliquots of these cells were resuspended in the same vehicle with or without 0.05M sodium periodate for ten mintues at room temperature then rinsed in the vehicle alone. A drop containing 200,000 cells was applied to coverslips pretreated with poly-L-lysine (40,000 MW) in 0.2M phosphate buffer (pH 8.2) or in PBS for one hour or overnight at room temperature; effective poly-L-lysine coats were verified by fluorescamine binding. These preparations were photomicrographed without disturbing the settled cells on an inverted microscope with phase contrast optics at approximately 60X. The cells were left undisturbed for two hours or overnight in moist chambers at room temperature and then processed routinely for SEM by either freeze drying or critical point drying (CPD) through graded ethanols, amyl acetate and carbon dioxide. Scanning electron micrographs were taken of the same fields at the same magnification. Photographic enlargements were made of the initial light micrograph (as a negative image) and the SEM image (a positive) on 8x10 transparent film and the two images were superimposed in register. Those cells retained through SEM processing were visually cancelled by the superimposed images, but those that were lost showed up as bright spots and the yield was determined. Effects of heparin (20 units/ml) or 0.3M eth anolamine in the cell settling fluid were also tested. (With Johnson)

2) As in previous year. Special substrates have been prepared for growing

cells as whole mounts for HVEM studies. (With Pawley, Ris, and Yuspa)

3) Feline macrophage cultures plated at different densities and grown for different period have been pulsed with tritiated thymidine, fixed, stained for nonspecific esterase, mapped, and photomicrographed, and the same cells have been relocated and recorded by SEM. The criteria for subjective scoring and other studies. Accordingly, each scored cell could be a) numbered for identification, and then scored for b) continuity and overlap, c) roundness (height) and spreading, d) outline (type and degree of assymetry), e) incidence, distribution and size of microvilli, f) incidence, distribution and size of blebs, g) incidence, distribution and size of ruffles, h) incidence and distribution of retraction fibers, i) stage in the cell cycle, and j)type, extent and distribution of cytochemical staining. Micrographs of each cell were scored on plastic overlays, and the numbers compiled in a matrix for statistical analysis and graphic presentation. (With Albrecht and Tarone)

Major Findings:

1) Repeated attempts to achieve reproducibly high yields of fixed leukocytes from suspension on poly-L-lysine coated coverslips have shown erratic results and frequent low yields (below 10%), despite variations in many parameters. It now appears that our practice of including 20 units heparin per milliliter in the rinse solutions (to avoid clumping of the cells) has contributed to these poor results. Omission of heparin, however, did not ensure consistently high yields through routine critical point drying procedures with the three cell types examined, and these findings were consistent with experience in other laboratories. The addition of 0.3M ethanolamine had little effect on cell retention, and little difference was noted between samples settled for two hours and those settled overnight prior to processing.

On the other hand, brief treatment with 0.005M sodium periodate substantially improved cell retention on poly-L-lysine coated glass coverslips. Replicate samples of periodate-treated fixed buffy coat cells displayed 88% (σ = 10.5, n = 5) retention through CPD, compared with 65% (σ = 14, n = 4) of cells treated with buffer alone. Periodate treated cells processed for SEM by freeze drying showed 95% (σ = 2.5) retention in duplicate samples, and this value compared favorably with the yields of our more cumbersome filtration/ freeze dry procedure. The 0.1M phosphate buffer (ph 8.2) appeared preferable to PBS as a vehicle for poly-L-lysine trapping of fixed cells from suspension.

SEM examination of these specimens revealed no topographic changes caused by this procedure.

These findings represent the first evidence that this method employing poly-L-lysine "glue" almost universally used for retaining fixed cells from suspension for SEM, can be easily modified to ensure reproducibly high yields.

2) This work is now in progress.

3) The newly developed criteria for scoring topographic features have reduced ambiguity, added information for current or subsequent analysis, and improved the versatility of this method as applied to various cytochemical procedures and diverse cell types. For example, in place of the simple 0-4+ estimate of roundness used previously in this laboratory to score populations of dividing cells (and now in general use), a two digit code can discriminate roundness as cell height, cell outline and cell spreading on the substrate, and also defined most cell shapes and their degree of assymmetry. Similarly, previous 0-4+ estimates of the overall incidence of topographic features (microvilli, blebs and ruffles) were confounded by differences in distribution and size of these features. The present system helps to overcome these difficulties and included this information for analysis. The previous criteria were purposely simplified to enable manual tabulation without reduction of independent variables, whereas the present scoring system can be handled by data processing methods. Preliminary application of this system to the correlation of nonspecific esterase ectoenzyme localization with cell surface ruffles and tufts of microvilli indicate the efficacy of this approach.

Proposed Course of Project:

1) Demonstration of the efficacy of this procedure with other types of fixed cells and biological particles would promote its use and thus ensure more representative sampling. The losses affecting most present studies of biological particulates very likely select for or against the surface properties under investigation. Also, the consumption of periodate by fixed cells could be monitored spectrophotometrically to verify the chemistry and optimize the effect. Once verified, this method is particularly attractive for studying leukocytes, cytology specimens, ascites and a variety of cultured cells fixed in suspension, and it would greatly facilitate ancillary high resolution light microscopy, histological staining, immunofluorescence, enzyme cytochemistry, and autoradiography of the same cells that are scanned.

2) As these unfamiliar methods for whole mount monolayers and specially fixed and stained thick sections for HVEM are worked out, intracellular cytoskeletal elements can be compared under different preparative conditions, as noted previously.

3) Efforts are underway to alleviate a fundamental problem inherent in these scoring procedures, and indeed in the analysis of SEM micrographs generally, viz., the difficulty in evaluating three dimensional cell contours without tedious stereophotogrammetric procedures. Strategies for estimating cell contours in single routine SEM micrographs are being explored and

tested in order to devise a simple standardized method to approximate cell height cell spreading and normalized distributions of cell surface features. Such an approach would allow more objective appraisals of heterogenous cell populations, more realistic approximations of absolute values and promote greater correspondence among studies of different cell systems and from different laboratories.

Even with the present scoring criteria, correspondence between topographic features and nonspecific esterase ectoenzyme localization is being studied under different culture conditions which affect both parameters. In addition, the cells under study have been pulsed with tritiated thymidine, enabling identification of °S° cells to detect any influence of this phase of the cell cycle on topography or ectoenzyme distribution. Peritoneal macrophages can now be examined in the same manner for comparison with the established macrophage line (providing the periodate treatment enhances their binding to poly-L-lysine). Also, immunocytochemical localization of Fc receptors by SEM with colloidal gold labels is planned in our studies of this system.

Publications:

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Wetzel, B.: Preparing tissue culture cells for SEM. In SEM/1982 Johari, O.; ed., Scanning Electron Microscopy, Inc., AMF O°Hare, IL, In press, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03663-06 D

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Tumor virus expression in vitro and in vivo

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.R. Lowy	Senior Investigator	Derm NCI
	S.K. Chattopadhyay	Visiting Scientist	Derm NCI
	E. Chang	Expert	Derm NCI
	I. Dvoretzky	Visiting Associate	Derm NCI
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COOPERATING UNITS (if any) 1. Laboratory of Tumor Virus Genetics, NCI; 2. Laboratory of Pathology, NCI 3. Rocky Mountain Laboratory, NIAID; 4. Department of Pathology, USUHS 5.Department of Pathology, Georgetown University Medical Center; 6. Tumor Virus Laboratory, The Salk Institute

LAB/BRANCH
Dermatology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	4.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sequences which are homologous to the p21 transforming genes of Harvey and Kirsten murine scarcoma viruses have been found in normal human cellular DNA and other vertebrate DNAs. The human genome contains at least four different genes which are homologous to p21 genes. At leat one of these normal human genes can, when expressed at high levels, induce tumorgenic transformation of mammalian cells. The transforming genes of several human tumors have been shown to be p21 coding genes. The molecular genetics of the transforming function of the Bovine Papillomavirus has been further defined. The transforming gene is contained within a 2.3 kb segment of viral DNA. Long term interferon treatment of Bovine Papillomavirus transformed cells results in a decrease of viral DNA, loss of transformation, and curing of some cells of the viral genome. The importance of MCF viruses in the pathogenesis of murine thymic tumors has also been studied. Endogenous MCF-like viral DNAs have been found in all strains of Mus musculus. In ecotropic virus induced tumors, recombinant MCF viruses are found in each tumor. At least two different recombinational events between different viral genomes have occured for the formation of oncogenic MCF viruses.

Project Description

Objectives:

1. To study the structure and oncogenic potential of normal gene sequences which are homologous to the oncogenes of transforming retroviruses.

2. To analyze the normal function of genes homologous to the oncogenes of transforming retroviruses.

3. To study spontaneous human tumors for their high expression of oncogenes and for the ability of tumor DNA to induce cell transformation.

4. To improve the biologic assay of DNA-mediated gene transfer (transfection).

5. To determine the effect of a variety of physical and chemical treatments on the biological activity of specific DNAs (viral and cellular).

6. To define the portions of sarcoma virus genomes which induce cellular transformation.

7. To propagate and study papilloma viruses in tissue culture.

8. To define the functional organization of papillomavirus genomes especially with regard to their transforming and oncogenic activity.

9. To develop assays for wart viral proteins.

10. To adapt nucleic acid hybridization procedures for papillomavirus DNA.

11. To measure the relatedness of viruses in warts from different patients and species, including those from patients with epidermodysplasia verruciformis (EV), laryngeal papillomas, condyloma acuminata, flat warts, common warts, plantar warts, and warts associated with immunodeficiency.

12. To screen skin cancers of EV, kerato-acanthomas, and other tumors for papillomavirus or oncogenes.

13. To evaluate the effects of hormones and other chemicals on endogenous and exogenous virus expression.

14. To gain insight into how the state of differentiation of a cell regulates virus expression.

15. To determine structural differences between unexpressed and expressed endogenous DNA, since transfection of expressed viral DNA is more easily accomplished than that of unexpressed viral DNA.

16. To study the cellular origin of retrovirus components.

17. To study viral recombination, especially as it relates to the development of sarcoma viruses and leukemia viruses.

18. To study the evolution of retroviruses.

Methods Employed:

1. Treatment of cells or animals with hormones, other chemicals, and tumor viruses.

2. Detection of retrovirus expression biologically by XC plaque test and focus induction.

3. Specific radioimmune, fluorescence, peroxidase techniques are used for antigen detection.

4. For the isolation of genomic DNA, DNA is extracted from tissue culture cells or tumors by the Marmur technique, except that proteinase K is used instead of pronase, since higher molecular weight DNA is obtained by this modification. Unintegrated viral DNAs are enriched by the Hirt procedure.

5. Transfection of DNA utilizes the calcium phosphate technique of Graham and Van der Eb as modified by Stow and Wilkie. Cell or viral DNAs are assayed for biologic activity in the DNA transfection assay. This activity is then correlated with the expression of the transfected genes in the cells.

6. Virus is grown in sensitive tissue culture cells and purified by sucrose density centrifugation in a zonal rotor. Isotopically labeled single stranded viral DNA probes are synthesized in an endogenous reverse transcriptase reaction carried out in the presence of antinomycin D or in exogenous reactions following purification of viral RNA on sucrose density gradients.

7. Nucleic acid hybridization between specific probes and cell or viral nucleic acids is carried out in liquid or by the Southern blotting technique.

8. Cellular and viral genes are molecularly cloned and amplified in prokaryotic systems. The cloned DNAs are then used as probes, for molecular hybridization, and for structure-function studies.

9. Specific deletions, mutations, or recombinations are introduced in the cloned DNAs to map biological and biochemical functions.

Major Findings

1. Last year in collaboration with the laboratory of Dr. Peter Howley, we demonstrated that mouse tissue culture cells transformed by the Bovine Papilloma Virus (BPV) or by its viral DNA genome contain multiple unintegrated cellular viral DNA copies. These results suggested that the maintenance of papillomavirus induced transformation was probrably mediated by unintegrated viral DNA molecules. In collaboration with Dr. Howley's laboratory, we have now determined that cells transformed by the virus contain at least 5 different RNA transcripts. Each of these transcripts has the same 3' end, and each is contained within the 69% viral DNA fragment which was previously shown to contain the sequences required for the transforming function of BPV. It has also been found that when the BPV transformed cells are treated long-term with interferon the average number of copies of BPV DNA per cell decreases significantly. Following interferon treatment, it is possible to isolate cells which are no longer transformed, no longer form tumors in nude mice, and have lost the BPV DNA genome. These results suggest that interferon treatment of papillomavirus induced lesions might represent a rational therapeutic approach.

We have also studied further the molecular genetics of BPV induced cellular transformation by constructing a series of deletion mutants of the BPV DNA genome and correlating the location of each deletion with the capacity of the deleted viral DNA to induce cellular transformation. The results indicate that two discontinous segments of DNA within the 69% transforming fragment are required for the induction of cellular transformation. One segment, which is no larger than 700 nucleotides, contains a control element, while the second segment, which is no larger than 2.3 kb, encodes the transforming protein or proteins. However, sequences located between these two discontinuous segments of the 69% fragment appear to be required for maintenance of the unintegrated state of the viral DNA.

In collaboration with the laboratory of Dr. Wayne Lancaster, we have also found a second papilloma virus, the Deer Fibroma Virus (DFV), which can induce cellular transformation of mouse cells. In contrast to BPV, DFV can transform NIH 3T3 cells but not C127 cells. The mechanism of this resistance is currently under study. The cells transformed by DFV contain multiple copies of unintegrated viral DNA genomes, analogous to findings previously observed for BPV transformed cells.

2. The studies of viral and cellular oncogenes have also made considerable progress. In collaboration with the laboratory of Dr. Edward Scolnick, we have previously defined the molecular organization of the Harvey and Kirsten murine scarcoma viruses. A normal rat gene homologous to the oncogene of the Harvey virus has also been shown to have oncogenic potential when it is expressed at high levels. We have now determined that although Harvey and Kirsten viruses both encode crossreacting 21kd transforming proteins (p21), the Harvey and Kirsten virus p21 coding genes are derived from different cellular genes. Thus Harvey and Kirsten p21 genes are part of a multigene gene family. Both of these genes are conserved in evolution and are readily detected in human DNA. We have therefore molecularly cloned from normal human DNA four different p21 genes; two are closely related to the Harvey type gene and two are more closely related to the Kirsten viral gene. The

structure of one human Harvey type gene is very similar to that of a rat Harvey type gene. This normal human gene also has the capacity to induce oncogenic transformation of mouse cells when the p21 protein encoded by this gene is express at high levels by virtue of attaching a retroviral LTR in vitro. In contrast the Harvey and Kirsten viral p21 proteins, which are both phosphorylated at a threonine residue, the human and rat cellular Harvey p21 proteins are not phosphorylated, except for a very low level of serine phosphorylation. This result indicates that threonine phosphorylation is not required for p21 to be capable of inducing cellular transformation.

While there appears to be only one or two copies of Kirsten type or of Harvey type p21 genes in most normal cellular DNAs, an Asian mus species (Mus pahari) has been found which contains at least ten copies of the Harvey type p21 gene. These additional copies are rearranged with respect to the evolutionarily conserved Harvey type gene. Closely related Mus species do not contain this amplification. Analogous results have also been found with a Kirsten type p21 gene in Chinese hamsters. Such marked amplification of a unique sequence gene has not previously been described.

3. Our continuing studies of murine leukemia viruses has lead to several new observations. Last year we determined that the entire env gene of some MCF viruses was non-ecotropic. We therefore hypothesized that the MCF host range function might be encoded by endodgenous MCF-like viral DNA genomes. Using a viral probe specific for MCF and xenotropic env genes, we have now found that the germ line of many mouse strains contains multiple copies of intact MCF-like env sequences organized as proviruses. The MCF-like env sequences are similar to those of non-pathogenic MCF viruses. Under stringent molecular hybridization conditions, these sequences are found in all strains of M. musculus. In collaboration with the laboratory of Dr. M.W. Cloyd, we have also demonstrated directly that in ecotropic virus induced thymomas arising in AKR mice, specific recombinational events have taken place between the endodgenous ecotropic virus and some of the MCF sequences. The recombination is complex, since the MCF viruses associated with tumorigenisis have undergone at least two independent recombinational events. We also found that many spontaneous AKR tumors did not contain detectable unaltered ecotropic viral genomes. These results strongly suggest that the recombinant MCF viruses are a more proximate cause of the tumors than are the ecotropic viruses.

We have also, in collaboration with the laboratory of Dr. Inder Verma at the Salk Institute, studied the preintegration site in NIH cells of a previously cloned integrated AKR murine leukemia virus DNA genome. In contrast to previously reported studies of preintegration sites, we found that there was a small region of homology between the viral sequences and the preintegration site in the NIH cells.

Significance to Cancer Research:

Papillomviruses are a common cause of epidermal tumors in man. Some lesions

induced by these viruses may undergo malignant conversion. Little has been known about the functional organization of the genomes of these viruses or how lesions progress from a benign to a malignant state. The determination of the transforming sequences of BPV DNA represents a first step towards understanding how these tumors are formed. Our results indicate that the transforming protein(s) is (are) encoded in a short region of the viral genome and that transformation can occur independently of viral replication. Further studies will seek to determine the sequences which are responsible for the episomal nature of papillomavirus genomes. We will also attempt to define the viral gene product(s) associated with transformation and to relate the bovine system directly to human papillomaviruses. The finding that interferon treatment can cure some tissue culture cells of BPV sequences and revert them to the normal phenotype provides an experimental basis for attempts to treat papillomavirus induced disease with this drug.

The p21 genes are the first cellular oncogenes to be shown to be part of a multigene family. The resaons underlying their multiplicity may be important both for understanding their normal function and their possible role in specific cancers. Our data from one of the human p21 genes indicate that high levels of this normal gene product can induce tumorigenic transformation of cells. These results show that alteration in the level of expression of a normal gene may profoundly affect the proliferative capacity of the cell; they provide direct experimental evidence for the hypothesis that some spontaneous tumors may arise by such a mechanism. Using our moecular probes of Harvey and Kirsten p21 genes, several other laboratories have recently found that the transforming genes from several human tumors are Harvey or Kirsten type p21 genes. Further comparison between the normal p21 genes and those found in tumor cells should provide important molecular insights into the pathogenesis of some human tumors and may also suggest novel therapeutic approaches.

C-type retroviruses have been implicated in tumors of a wide variety of species, including man. The murine viruses of this group represent the best studied mammalian retroviruses; therefore they are an excellent system for the study of retrovirus induced tumorigenesis. Because retroviruses are composed of endogenous gene sequences, understanding how their expression leads to oncogenic transformation may be relevant to non-virus induced tumorigenesis as well. The cellular control of gene expression is also a fundamental problem of tumor cell biology. Information in this area will be critical to understand how eukaryotic genes are at different times unexpressed, induced, or constitutively expressed, and will bear directly on the approaches which will be undertaken in the future to prevent or treat malignant disease of viral or non-viral origin. The finding of specific recombination in the AKR tumors suggest that recombinational events between specific DNAs might be important in the pathogenesis of tumors, whether through the formation of a hybrid gene product or by increasing levels of specific genes.

Proposed Course:

Achieve stated goals.

Publications:

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2. Chattopadhyay, S.K., Oliff, A.I., Linemeyer, D.L., Lander, M.R., and Lowy, D.R.: Genomes of murine leukemia viruses isolated from wild mice. J. Virol. 39:777-791, 1981.

3. Chattopadhyay, S.K., Lander, M.R., Rands, E., and Lowy, D.R.: Origin of mink cytopathic focus-forming (MCF) viruses: comparison with ecotropic and xenotropic murine leukemia virus genomes. Virology 113:465-483, 1981.

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12. Chang, E.H., Gonda, M.A., Ellis, R.W., Scolnick, E.M., and Lowy, D.R.: The human genome contains four genes homolgous to the transforming genes of Harvey and Kirsten murine sarcoma viruses. Proc. Natl. Acad. Sci. USA, in press.

13. Chang, E.H., Furth, M., Scolnick, E.M., and Lowy, D.R.: Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature, in press.

14. Ellis, R.W., Lowy, D.R., and Scolnick, E.M.: The viral and cellular p21 ras gene family. In Cell-Derived Oncogenes, Advances in Viral Oncology (vol. I), Raven Press, New York, in press.

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16. Chang, E.H., Gonda, M.A., Furth, M.E., Goodwin, J.L., Yu, S.S., Ellis, R.W., Scolnick, E.M., and Lowy, D.R.: Characterization of four members of the P21 gene family isolated from normal human genomic DNA and demonstration of their oncogenic potential. In Gene Transfer and Cancer, Raven Press, New York, in press.

17. Turek, L.P., Byrne, J.C., Lowy, D.R., Dvoretzky, I., Friedman, R.M., and Howley, P.M: Interferon inhibits Bovine Papillomavirus transformation of mouse cells and induces reversion of established transformants. In Chemistry and Biology of Interferons: Relationship to Therapeutics, Academic Press, New York, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03656-09 D

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin Filaments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	L. Johnson	Senior Staff Fellow	Derm. NCI
	W.W. Idler	Chemist	Derm. NCI
	S.H. Yuspa	Branch Chief	Exper. Path. Br. DCCP, NCI
	D. R. Roop	Expert	Exper. Path. Br. DCCP, NCI
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	A.C. Steven	Visiting Scientist	Lab. of Phys. Biol., NIAMDD
	R.D. Goldman	Professor	Dept. Antmy & Cell Biol. Northwestern Univ.
	B.A. Dale	Assistant Professor	Dept. Peri. & Med. Univ. of Wash.
	G.L. Peck	Senior Investigator	Derm. NCI

COOPERATING UNITS (if any)
Experimental Pathology Branch, DCCP, NCI; Laboratory of Molecular Biology, DCBD, NCI; Laboratory of Physical Biology, NIAMDD

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.75	4.25	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The polypeptide chains which comprise the subunits of the keratin filaments of normal human, murine and bovine epidermis have been isolated and characterized. The subunits polymerize in vitro into native-type filaments. The details of filament ultrastructure are being investigated using image analysis procedures of filaments examined by transmission electron microscopic and scanning transmission electron microscopic techniques. Model structures generated from these methods will be computationally tested for compatibility with other physiophysico chemical data and amino acid sequence studies of individual filament subunits. cDNA cloned probes that encode mouse epidermal keratins have been isolated and are being used to determine the amino acid sequences of the proteins, and to study the structure and expression of keratin genes. The 10nm filaments of fibroblasts, muscle cells and neuronal tissues have been shown to be structurally similar to, but immunologically different from keratin filaments. A histidine-rich basic protein isolated from rat epidermis and the slightly different protein of mouse epidermis specifically aggregate keratin filaments and other 10nm filaments in a manner suggestive of an interfilamentous matrix component.

Project Description:

Objectives:

1) To characterize the ultrastucture of keratin filaments polymerized in vitro from the subunits of the filaments isolated form normal human, murine and bovine epidermis.

2) To characterize the polypeptide subunits of keratin filaments isolated from various benign and malignant human and mouse epidermal diseases.

3) To investigate the biosynthesis of the keratin filament polypeptide subunits in cultures of normal mouse epidermal cells and to investigate the effects of various drugs and carcinogens on the cells.

4) To study the structure and expression of mouse epidermal keratin genes by use of cloned cDNA probes complementary to keratin mRNAs.

5) To investigate the nature of the highly-specific interaction between epidermal keratin filaments and a histidine-rich basic protein isolated from the epidermis.

6) To investigate the chemical, immunological and structural similarities between epidermal keratin filaments and 10nm filaments isolated from a variety of cell types, such as BHK-21, CHO, HeLa, and PtK1 cells grown in culture, and of muscle and neuronal tissues.

Major Findings and Methods Employed:

1) The polypeptide chains which comprise the subunits of the keratin filaments of normal bovine and murine epidermis have been isolated and individually characterized by standard protein chemical techniques. The unfractionated mixture of polypeptides or combinations of two of the purified polypeptides spontaneously polymerize in vitro in dilute salt solution into filaments that are uniformally 80 A wide and up to 40 μm long. The polypeptide composition of these filaments, and their structure, based on electron microscopy and X-ray diffraction, are the same as the in situ keratin filaments. The stoichiometry of the recombination experiments reveals that the polypeptides are present in the filaments in the precise molar ratios of 1:2. This suggests that the epidermal keratin filament has a three-chained unit structure and this is supported by the X-ray diffraction analyses.

2) The X-ray diffraction and stoichiometric data implies that the filaments contain regions of three-chain coiled-coil α-helix; that is, the filament consists of a three-chain building block unit. This structural concept has been confirmed by partial proteolytic digestion of filaments and the subsequent isolation and characterization of α-helix-enriched fragments. The structural unit is about 20A wide by about 500 A long and

consists of three subunits aligned side-by-side with two coiled-coil α-helical segments each about 180 A long, interspersed by regions of non α-helix.

3) The structure of this proposed three-chain unit will be probed further to answer specific questions such as the polarity and orientation of the three protein chains within it. Isolated purified subunits will be cleaved at tryptophan and methionine residues and where possible, the fragments will be ordered along the sequence. Subsequently, intact filaments or preparations of three-chain units will be cross-linked by bi-functional reagents, cleaved and from the array of fragments obtained, orientations of the three chains will be determined.

4. In collaboration with Dr. A.C. Steven, (Laboratory of Physical Biology, NIAMDD) attempts are underway to understand the higher orders of filament structure. Good negative-stained images of filaments can be subjected to optical diffraction analysis to obtain information of prominently repeating structural elements. Many filaments have to be computationally straightened in order to eliminate "noise" introduced by the curvilinear shape of the negatively-stained filaments. Diffraction images are then computationally averaged to identify the prominent repeats, which should then provide clues as to how the proposed three-chain units are assembled into the filament. Basic structural information on filaments is also derived from scanning transmission electron microscopy (STEM) of filaments. This technique will be performed at the N.I.H. STEM facility located in the Department of Biology of the Brookhaven National Laboratory (Dr. J. Wall, Director) in Upton, New York. This technique provides information on the mass of the filaments and their composite three-chain units in relation to length. Preliminary data suggests that (1) there are 7-11 three-chain units per unit length of about 500 A of filament; and (2) at least filaments assembled in vitro are polymorphic; that is, some filaments differ in the number of units they contain per unit length. The significance of this observation is unclear but may have enormously important implications in terms of the structure and function of the filaments in cells. In addition, STEM technology will provide information on the shape of intact filaments and of the three-chain units. It is expected that the application of this new technology to the study of keratin (and other related intermediate filaments (See item 9 below) will for the first time enable the construction of working models for the filaments. Optical diffraction and STEM analysis will be performed on (1) intact filaments from a variety of keratin sources as well as on the intermediate filaments of several cell types; (2) protofilamentous forms of these filaments, obtained by dissociation in low salt or in high concentrations of sodium citrate buffer, pH 2.6; (3) particles of intermediate size obtained during various early stages of filament assembly in vitro; and (4) native filaments obtained from various types of cells where possible to provide a direct comparison of in vitro and in vivo filament structural forms.

5. High resolution transmission electron microscopy of sections through normal murine epidermis, and the outer-root-sheath component of guinea pig

hair follicles (which is contiguous to and continous with the epidermis) is being employed to study the structure of keratin filaments in intact tissue. In transverse cross-section, the filaments appear annular, that is, they have a region of reduced density in their center. Similar microscopic sections of filaments polymerized in vitro demonstrate this appearance also.

In addition, polymerized filaments negatively-stained with neutralized phosphotungstic acid display a region of enhanced electron density along centers. These observations suggest the filament is tubular in structure. Further detailed electron microscopic data as well as optical image diffraction and STEM analyses (see item 4 alone) are required to confirm this concept.

6. Comparisons of the polyacrylamide-gel electrophoretic profiles of the keratin subunits obtained from abnormal human epidermis such as Darier°s disease and lamellar ichthyosis with those of normal epidermis show prominent differences in numbers and mobilities of bands. Also, the abnormal polypeptides show limited facility for polymerization in vitro. Therefore, there may be differences in the chemical structures of the proteins. Attempts are underway to identify such differences by comparisons of two-dimensional gel electrophoretic maps of the CNBr and NBS peptides of the proteins obtained from normal and abnormal epidermis. Filaments assembled in vitro from psoriatic epidermis are also abnormal, and interestingly, form °paracrystalline° structures consisting of several filaments associated side-by-side in an apparently ordered manner. This may be due to the presence of an additional protein that perhaps functions like the basic protein (see item 9 below), or may be a feature characteristic of the filament proteins of psoriatic epidermis. Studies are underway to characterize this phenomenon further.

7. Normal mouse epidermal cells grown in monolayer culture can be made to synthesize the normal complement of keratin polypeptides. These proteins have been isolated and characterized by standard protein chemical techniques as done earlier with the bovine proteins. The reason why the mouse keratins are also being studied is that this cell culture system is currently being used for studies of in vitro carcinogenisis. Since the keratins are the principal synthetic products of the cells, they will be used as specific markers for the studies on carcinogenesis. To this end, a specific radioimmune assay has been developed to follow the changges occurring during carcinogenesis. Preliminary experiments have revealed marked alterations in the synthesis of the keratin proteins during treatment with carcinogens such as TPA and other growth effectors such as vitamin A.

8. In collaboration with Drs. D.R. Roop and S.H. Yuspa (Exper. Path. Br., DCCP, NCI) cDNA species complementary to mouse epidermal karatin have been produced and cloned in the Ecoli plasmid vector pBr322. Three cloned probes have been produced; which encode keratin of 67,59 and 55 Kdaltons in size. These cDNA species are being subjected to DNA sequence analyses by use of the Maxam-Gilbert procedures. Assignment of amino acid sequences to the DNA sequences is then planned by application of the genetic code. Preliminary results suggest that

the keratin subunits do indeed contain two long stretches of α-helical regions that are separated by a region of non- α -helical sequences, and contain regions that are rich in glycine and serine residues at the amino and carboxyl-terminal ends.

Dr. Johnson in this laboratory is using these cDNA probes to isolate from a phage Charon library the genomic versions of these keratin genes. Experience in other enkaryote gene systems indicates the coding information in genomic DNA (°exons°) is interspersed by regions of noncoding information (°introns°).

Dr. Johnson will characterize the isolated genomics with respect to the size, number, location and sequence of splice-points of the expected non-coding regions. This work will include extensive restriction endonuclease mapping and Southern (and Northern) hybridization techniques, as well as D-looping of the electron microscope level and DNA sequencing.

9. In collaboration with Dr. M.M. Gottesman (Laboratory of Molecular Biology, DCBD) and Dr. R.D. Goldman and (Dept of Anatomy & Cell Biology, Northwestern University, Chicago, IL) it has been shown that the 10nm filaments of a variety of epithelial and mesenchymal cell-types grown in culture are morphologically very similar to epidermal keratin filaments, and interestingly, also possess a three-chain unit that is structurally identical to that of keratin filaments. Partial specific cleavage of purified 10 nm filament subunits and bovine epidermal keratin subunits indicate that the subunits are all distinctly different, but they are structurally very similar. Such studies have permitted the construction of subunit domain maps that show two regions of α-helix of M_r 13,000 on each subunit, interspersed by regions of a non α-helix that vary in size between different subunits. Most of these studies have been done with the 10nm filaments of BHK-21 and CHO cells since these are readily available in large quantities, but similar comparative work is also planned or underway with the filaments of HeLa cells, and the neurofilaments isolated from cattle brain and squid giant axons. The single protein decamin and desmin, the principal intermediate filament subunits of fibroblasts and muscle cells, respectively, are capable of filament assembly by themselves in vitro; that is, they form homopolymer filaments. All keratin filaments, in contrast certain at least two demonstrably different subunits; that is they are obligate copolymer filaments. Preliminary studies suggest that certain neurofilament subunits are capable of homopolymer and/or copolymer filament assembly in vitro. Since all of these filaments are basically very similar (although subtlely different), it is perhaps not surprising that combinations from different sources also form native-type filaments in vitro. Hybrid filaments containing subunits from epidermis + fibroblasts, epidermis + smooth muscle, bovine epidermis + mouse epidermis, etc, have been formed. Such filaments are termed heterologous copolymers. A preliminary conclusion from these observations is that perhaps cells can modulate their 10nm filament composition with regard to specific functions. In support of this idea, BHK-21 cells contain two types of filament subunits, that characteristic of fibroblasts and that of muscle cells, which apparently copolymerize in situ to form a filament of properties intermediate between the two. The presence of 10 nm

filaments in cells is obviously extremely important and the structural studies of this type will provide insights into their function in normal and transformed cells.

10. Intermediate filament subunits are phosphorylated in vivo by cyclic nucleotide dependent protein kinases. Presumably cells regulate the structure and/or function of the filaments in this way. Attempts to characterize this process will initially involve estimation of the amount of phosphate bound to subunits, its location and possible function in terms of filament assembly in vitro. Filament subunits of mouse and bovine epidermis, CHO and BHK-21 cells, smooth muscle and various neuronal tissues will be examined. The serine-phosphate content will be estimated by reaction of subunits with methylamine.

11. In collaboration with Dr. B.A. Dale (University of Washington, Seattle, Wa.) it has been shown that a histidine-rich protein isolated from rat epidermis specifically aggretates epidermal keratin filaments from several species in vitro to form a highly-ordered fiber. Electron microscopy of such fibers reveals a pattern of filaments 70-80 A in diameter embedded in a darker-staining background, or matrix. This structure is typical of the "keratin pattern" seen in the fully-differentiated stratum corneum of the epidermis. This suggests strongly that the basic protein is the matrix protein of epidermis. Our work constitutes the first real evidence for and demonstration of the role of a matrix protein in the epidermis. The interaction between the basic protein and filaments is highly specific since other fibrous proteins do not form the ordered structures. Therefore, there are structural features unique to keratin filaments which recognize the basic protein. One practical limitation of such studies has been the difficulty in isolation of the basic protein. In an effort to resolve this, we have developed a very simple method for isolating large quantities of the similar protein from mouse epidermal stratum corneum which functions in the same way as the rat protein. Its chemical and functional properties will now be studied in detail. Studies on the interaction between the basic protein and defined fragments of filaments and filament subunits are underway to characterize the nature and specificity of the associations between these two components in the epidermis. Interestingly, in certain diseases of the epidermis involving abnormal keratinization, such as psoriasis, the amount of the basic protein is greatly diminished from normal. There may be a relationship between the absence of the basic protein and presence of abnormal keratin filaments which could provide important information on the disease itself. Since this basic protein also aggregates the intermediate filaments from all sources so far examined we have chosen for it a new functionally-specific name, filaggrin. Presumably the filaggrin recognises structural features common to keratin and intermediate filaments. Studies are underway to determine the physiological significance of this result; for example it is not yet known whether filaggrin-like proteins are present in fibroblasts etc.

Significance to Cancer Research:

The epidermis offers a unique opportunity for the study of tumors not only

because of the prevelance of tumors in this tissue but also because of its accessibility. One of the major problems in studying malignancies of the epidermis has been the lack of suitable biochemical markers. The keratin filaments and filaggrin are the most prominent intracellular components of all epidermal cells and therefore a study of their chemistry, structure and biosynthesis in both normal and abnormal epidermis will be of profound imp-portance in studying tumors in this tissue. The production of these protein in a well defined cell culture system will facilitate studies of carcino-genesis in vivo and in vitro.

Proposed Course of Project:

Studies on the abnormal (benign and malignant) tissues will be done in collaboration with Dr. Gary Peck. The cell culture studies will be done in the Experimental Pathology Branch in collaboration with Dr. Yuspa. The DNA cloning and related experiments will be done by Dr. Johnson and in collaboration with Dr. Roop. The biochemical, biophysical and electron microscopic studies of the structure of normal epidermal keratin filaments will be done in this laboratory. The computational image analysis studies of filaments will be done in collaboration with Dr. A.C. Steven.

Collaborative efforts with Drs. R.D. Goldman, M.M. Gottesman and B.A. Dale will continue in the areas defined above.

Publications:

1. Steven, A.C., Wall, J., Hainfeld, J., and Steinert, P.M. Structure of fibroblastic intermediate filaments. Analysis by Scanning Transmission Electron Microscopy. Proc. Natl. Acad. Sci. USA., 79 3101-3105, 1982.

2. Jones, J.C.R., Goldman, A.E., Steinert, P.M., Yuspa, S.H., and Goldman R.D. The supramolecular organization of intermediate filament netowrks in cultured epidermal cells. Cell Mobility (in press) 1982.

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6. Yuspa, S.H., Ben, T., Steinert, P.M. Retinoic acid induces transglutaminase activity but inhibits cornification of cultured epidermal cells. J. Biol. Chem. (in press) 1982.

7. Steinert, P.M. and Cantieri, J.S. Epidermal keratins in Biochemistry and Physiology of the Skin. (Goldsmith, L.A., ed.) Oxford University Press, Inc., in press, 1982.

SUMMARY REPORT
IMMUNOLOGY BRANCH
October 1981 - September 1982

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; and 4) 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. In addition, a number of collaborative studies concerning genetic control of the immune response to staphylococcal nuclease have been carried out as a collaboration between the laboratories of Drs. Hodes and Sachs. Studies in Dr. Sachs' laboratory, also involving genetic control of the immune response to nuclease, have been directed toward examination of the *in vivo* effects of treatment with anti-idiotypic antibodies.

Drs. Hodes and Singer have studied monoclonal T cell populations specific for the antigens (T,G)-A--L and KLH. Such T cells were found to be both H-2 restricted and antigen-specific, demonstrating the ability of a single T helper cell population to recognize both antigen and self H-2. Additional studies in the laboratory of Dr. Al singer have been directed towards investigations of genetic control of immune cell interactions among macrophages, T cells and B cells. It has been shown that developmentally distinct B cell subpopulations are activated by helper T cells in genetically distinct ways.

Studies in Dr. Gene Shearer's laboratory have been directed toward understanding the role of the major histocompatibility complex in regulation and restriction of T cell mediated and effected immune responses against chemical haptens and infectious viruses and toward understanding the genetic and mechanistic aspects of natural resistance to graft-vs-host (GvH) reactions. Studies of genetic control of human CTL responses against influenza-virus infected autologous cells have also been performed. These types of studies are of value in elucidating the role of HLA antigens in regulating human T cell immunity against infectious virus and in defining the cellular components involved.

B. STRUCTURE AND FUNCTION OF CELL SURFACE MOLECULES

Studies in Dr. Pierre Henkart's laboratory have continued to probe molecular features of membrane damage induced by immune mechanisms. Work in the laboratory of Dr. Howard Dickler has been directed toward mechanisms involved in the triggering and regulation of immunocompetent cells and the role that cell surface molecules play in this triggering. The recent finding that B cells can be stimulated by monoclonal anti-Fc receptor antibodies may have important implications for mechanism of B cell triggering. Additional work in Dr. Dickler's laboratory has been directed toward interactions which may take place on cell surfaces via idiotype-anti-idiotype recognition. A system has been developed which allows in vitro production of antibody responses to an antigen (T,G)-A--L (the response to which is Ir gene regulated) and the role of idiotype interactions in regulating these responses is being studied.

Studies in Dr. David Segal's laboratory have been directed toward understanding the molecular and cellular basis of the interactions of immunoglobulins with immune effector systems, and the relationship of antigenic recognition to these interactions. Morphologic studies of cellular interactions in immune systems have been carried out by Dr. Maryanna Henkart.

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. These antibodies have been used to further subdivide products of the MHC. In addition, 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.

In addition, 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 SLA antigens of all three partially inbred lines of miniature swine. Two new recombinants within the MHC have been detected within the miniature swine herd. Both recombinants involve separation of the MLC stimulatory locus (SLA-D) from the serologic loci (SLA-ABC). Transplantation studies aimed at determining the relative importance of individual MHC loci are now in progress using these new recombinant lines.

Studies in Dr. Hodes' laboratory have examined the nature of the alloreactive T cell repertoire. The findings are of fundamental importance in suggesting that the T cell repertoire for alloantigens, like that for conventional antigens, may be both MHC restricted and environmentally modified. The use of radiation

bone marrow chimeric animals in Dr. Singer's laboratory has provided a valuable approach to discerning the mechanism of generation of cell mediated immune responses to transplantation antigens. It has been shown that T cell precursors are specifically tolerized by their pre-thymic environment. Thus, these experiments suggest that T cell precursors express their receptors for allogeneic determinants prior to their entry into the thymus. These experiments have important implications for understanding how the T cell receptor repertoire is generated. Studies in the laboratory of Dr. Stephen Shaw have been directed toward the nature of human T cell recognition and activation, with a particular emphasis on the genetics of the human major histocompatibility complex (HLA). The major emphasis has been on further characterization of a new HLA gene, designated "SB" or "secondary B cell" gene. Studies have also been carried out on the relevance of these new SB markers to the diseases multiple sclerosis and dermatitis herpetiformi. Such studies may extend our understanding of the biological importance of these MHC linked antigens.

D. MOLECULAR BIOLOGY

Dr. Dinah Singer's laboratory has been involved in studies of the organization of genes encoding the major histocompatibility complex and mechanisms controlling the expression of these genes. Using recombinant DNA technology, genomic fragments containing MHC genes of both mouse and pig have been studied. Ten to 15 MHC genes have been demonstrated in the pig genome and many of these have been isolated. Transformation of mouse L cells with DNA from one porcine genomic MHC clone resulted in the expression of swine MHC antigen in association with a murine light chain on the surface of the mouse cell. It has been shown that only one-two copies of porcine DNA occur in the transformant and that these are packaged into chromatin in a manner indistinguishable from that of endogenous expressed murine sequences. In addition, a detailed analysis of MHC-linked but non-coding sequences has revealed that MHC genes are embedded in clusters of repetitive DNA. The same clusters recur non-randomly in all of the MHC gene-containing clones isolated to date. These flanking sequences may be involved in the regulation of MHC gene evolution, generation of polymorphism or expression.

E. TUMOR IMMUNOLOGY

Studies in Dr. John Wunderlich's laboratory have been directed toward identification of factors which influence host cytotoxic cell responses against syngeneic tumors. Effector cells mediating broadly reactive anti-tumor cytotoxic activity have been induced under syngeneic conditions *in vitro* in normal mouse spleen cells by polyinosinic acid. These are closely related to natural killer cells as judged by expression of cell surface differentiation antigens and target cell specificity. The response is controlled by multiple genes.

Clinical trials of immunotherapy in the treatment of human malignant melanoma have been continued. The studies involve a controlled, randomized comparison of immunotherapy to chemotherapy in Stage I and Stage II malignant melanoma.

Patients have been randomly assigned to receive treatment either with methyl CCNU, BCG alone or BCG plus allogeneic tissue-culture-grown vaccine. A fourth group consists of control patients who receive no further active treatment. A total of 181 patients have entered the trial, and patient accrual has been terminated. Studies of the peripheral blood lymphocytes of patients in this clinical trial indicated that greater than 90% of those receiving the cell vaccine generated cellular and/or humoral immune responses against one or more of the vaccine cell lines. In addition, all patients in the study had a normal capacity for generating cytotoxic cellular responses in vitro except for those patients receiving chemotherapy who showed some depression in this functional capacity. However, no significant differences between treatment groups have been detected to date.

F. FLUORESCENCE T ACTIVATED CELL SORTER

The Immunology Branch has continued to maintain an active Fluorescence Activated Cell Sorter Facility operated by Ms. Susan Sharrow. The Fluorescence Activated Cell Sorter (FACS) has been used both to analyze and to separate lymphoid populations by rapid flow microfluorometry. Cell suspensions are first treated with appropriate fluorescent reagents and then subjected to the FACS analysis. Studies performed in this facility have been an integral part of many of the investigations described in the above sections. In addition, numerous collaborative studies with other investigators at NIH and elsewhere have been performed.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05003-17 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Cell-Mediated Cytotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. R. Wunderlich	Senior Investigator	I NCI
Other:	N. A. Dorfman	Guest Worker	I NCI
	C. Muller	Visiting Fellow	I NCI
	H. Weintraub	Visiting Fellow	I NINCDS

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Effector cells mediating broadly reactive anti-tumor cytotoxic activity, induced under syngeneic conditions in vitro in normal mouse spleen cells by polyinosinic acid, are closely related to natural killer cells as judged by expression of cell surface differentiation antigens and target cell specificity. The response is controlled by multiple genes. Similar activity is generated by thymocytes stimulated in vitro by both polyinosinic acid and supernatants of Con A-stimulated cells.

The expression of MHC determinants on normal mouse spleen cells has been modulated by in vitro enrichment or depletion of membrane sterol content, as documented by fluorescent antibody probes and quantitative flow microfluorometry.

Project Description

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 is 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 monclonal antibodies and complement. Hybridoma cell lines producing monclonal antibodies have been obtained from the Salk Cell Distribution Center and recloned. 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 normal mouse spleen cells with polyinosinic acid in medium supplemented with syngeneic plasma. 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 are 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; most target cells function well if previously frozen in liquid nitrogen. Target cells also have been chemically modified in order to change the immunogenicity of cell surface antigens.

Sarcomas have been induced with methylcholanthrene (MCA) in mice which are routinely screened for potentially immunsuppressive 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.

Normal spleen cells have been treated in vitro with phosphatidylcholine or cholesteryl hemisuccinate as a model for studying the effects of changing the expression of cell surface determinants. Treated cells have been stained with fluorescent reagents to monitor the expression of cell surface MHC and theta determinants. Quantitative cellular fluorescence is determined by flow microfluorometry.

Major Findings: Previous work in this laboratory demonstrated that normal mouse spleen cells cultured for 5 days in medium supplemented with syngeneic plasma and polyinosinic acid (Poly I) generate theta-bearing cytotoxic cells, whose broad pattern of target cell reactivity in ^{51}Cr-release assays includes freshly dispersed syngeneic primary tumor cells induced by MCA but not freshly dispersed or mitogen-stimulated lymphocytes. Analysis of crosses between high and low responder mouse strains indicated that responsiveness is under the control of multiple dominant or co-dominant genes. This effort has been extended by the following findings.

The patterns of sensitivity of effector cell activity to a variety of antisera or monoclonol antibodies show a close relationship between Poly I induced cytotoxicity and NK activity. Thus, Poly I-induced C57BL/6 cytotoxic cell activity is sensitive to complement plus anti-Thy 1.2 or NK 1.2 but not to complement and anti-Lyt 1.2 or Lyt 2.2. However Poly I-induced activity, unlike NK activity, is insensitive to anti-asialo GM1 and complement.

The strain distribution pattern of Poly I-induced cytotoxicity among 12 inbred mouse strains has been categorized by cluster analysis into high and low responding strains. AKR, CBA, C3H, C57BL/6 and NZB mice are high responders whereas BALB/c, C57L, C58, DBA/2, SJL, SWR, and 129 are low responders.

Analysis of the genetic control of Poly I-induced cytotoxicity suggests that high vs low responsiveness in C57BL/6 and BALB/c mice is controlled by multiple independent genes operating in an additive fashion. Thus, the response distribution pattern of 67 (C57BL/6 x BALB/c) x BALB/c backcross mice was a unimodal distribution with a peak intermediate between the parental F1 and BALB responses. This observation is supported by analysis of inbred recombinant mice whose levels of responsiveness fall between those of high and low responding progenitors. No overlap of 40 high responding C57BL/6 and low responding BALB/c parents was observed after responses were normalized and grouped by cluster analysis. Of note, F1 responses fall between those of BALB and C57BL/6 parents.

The Poly I-induced cytotoxic responses of several inbred C57BL/6 mice carrying a limited portion of BALB/c histocompatibility genes (B6.C congeneic mice) have been reproducibly low relative to C57BL/6 controls. The reduced response of B6.C H-2^d mice suggests that the genetic control for high responsiveness is in part linked to the major histocompatibility complex (MHC). MHC-linked control of the response is weak, however, because (1) B6.CH-2^d mice generate considerably higher responses than BALB/c mice, and (2) linkage analysis of H-2 genotype and high responsiveness in (C57BL/6 x BALB) x BALB backcross mice has failed to detect a correlation.

We have not been able to find evidence of regulation of Poly I-induced cytotoxicity at the effector stage, suggesting that any regulatory influence is limited to generation of the effector cells. Thus cytotoxicity, which is induced by Poly I with spleen cells from various MHC congeneic C57BL/10 strains and tested against fibroblast cell lines from the same strains, is independent of the H-2 type of the target cells. Moreover, effector cells generated from high and low responding strains (C57BL/6 and BALB/c) and mixed at the effector stage act independently.

Genetic regulation of Poly I-induced cytotoxicity also involves gene interaction. Thus, analysis of the progeny of an F1 x parent backcross of two low responder strains, BALB/c and DBA/2, has shown that backcross mice have significantly higher responses than either parental type.

Poly I also induces broadly reactive cytotoxicity in C57BL/6 thymocytes when cultures are supplemented with ConA-induced cell supernatants. Thus, the precursor of the effector cell is probably a T-cell. Of note, the need for ConA-induced supernatants indicates that helper factors are available endogenously from spleen cells but not from thymocytes. Endogenous help in responding spleen cells is suboptimal, however, in that Poly I-induced cytotoxicity is substantially increased by supplementing cultures with ConA-induced supernatants.

Cultures of C57BL/6 spleen cells prestimulated with Poly I have been maintained for at least 6 months by supplementing growth media with supernatants of either PMA-stimulated EL4 cells (rich in IL-2 activity) or ConA-induced lymphocyte supernatants. The cultured spleen cells are Thy 1.2^{+} $Ly5^{+}$, $Lyt1^{-}2$ by fluorescence microscopy and are toxic for syngeneic chemically-induced tumor cells in growth assays but not in chromium release assays.

Enrichment of cholesterol in tumor cell membranes increases tumor cell immunogenicity, according to reports of Shinitzky et al (PNAS 76, 5313, 1979), in which treatment of host with cholesterol-enriched tumor cells increased the hosts ability to reject subsequently innoculated untreated tumor cells. We are pursuing these findings, first attempting to demonstrate that enrichment or depletion of cholesterol in normal cells changes the expression of well-known cell surface determinants. Thus, we have observed that normal spleen cells treated for 2 hours with progressively increasing doses of cholesteryl hemisuccinate (CHS) progressively lose H-2L, D, or K determinants as assessed by quantitative flow microfluorometry. Treatment with phosphatidylcholine (which depletes cholesterol) increases the expression of such determinants. By contrast, expression of Thy 1 determinants by spleen cells is changed in the reversed fashion in that CHS treatment does not decrease the expression of Thy 1 antigens whereas PC treatment decreases the expression. The findings are not in vitro artifacts insofar as similar changes have been observed using cells from mice with genetically determined lipid storage disease: in such mice expression of MHC determinants is reduced.

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. Data concerning

genetic control of the Poly I-induced response are more easily interpreted than those previously available for classic NK activity, because of the clear separation of high and low responses. Our interpretation of the genetic control, which can be applied to both classic NK and Poly I-induced cytotoxicity, is a clear departure from previous views - namely, we suggest that regulation involves multiple, independent genes cooperating in an additive fashion. The finding that this type of cytotoxicity can be generated in thymocytes is important because it will clearly establish a place for T cells in the maturation lineage(s) of NK-like cells. Finally, cultures of Poly I-induced anti-tumor effector cells have been established, which will allow more direct characterization of the cells.

Expression of cell surface determinants on normal lymphocytes has been changed by enriching or depleting cholesterol content. This finding is important because at least one set of the altered determinants, MHC products, has well described effects on immune cell responses. Thus, changes in cholesterol content could serve as a natural mechanism for altering expression of cell-surface MHC determinants and consequently immune responses. Of greater interest to us, changes in cellular cholesterol content may indeed modify the expression of cell surface determinents on tumor cells (e.g., antigens or hormone receptors), and the same quantitative techniques which have been used to demonstrate changes of surface determinents on normal cells can now be adapted to the study of tumor cells.

Proposed Course of Project: Genetic control of Poly I-induced cytotoxicity will be pursued using recently developed techniques which enhance the difference between high and low responding mouse strains. In particular we will study congeneic mouse strains and crosses between them to test the hypothesis that multiple genes controlling the response act in an independent and additive fashion and that these genes are linked to regions associated with the control of transplantation antigens.

To improve our understanding of how broadly reactive cytotoxic antitumor cells can develop, the responsiveness of thymocytes and fetal hematopoietic cells will be further explored.

Cultures of anti-tumor effector cells will be further characterized regarding cell surface differentiation antigens and anti-tumor effects. In particular we will try to derive cultures with higher levels of anti-tumor activity by isolating clones soon after initial cultures are established.

The effects of cholesterol enrichment on expression of cell surface determinants of tumor cells will be studied, particularly MHC and MULV-related determinants on chemically-induced tumor cells. In addition, we will test the effects of such treatment on tumor immunogenicity in syngeneic hosts.

Publications:

Dorfman, N., Winkler, D., Burton, R.C., Kossayda, N., Sabia, P., and Wunderlich, J.: Broadly reactive murine cytotoxic cells induced in vitro under syngeneic conditions. J. Immunol., in press, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05018-12 I

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Membrane Damage by Immune Mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.A. Henkart	Senior Investigator	I NCI
Other:	H. Streicher	Investigator	I NCI
	M. Henkart	Expert	I NCI

Collaborator: Craig Reynolds, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Immunology Branch

SECTION

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NCI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous work using the electron microscope and negative stain had demonstrated the presence of ring structures on the surface of red cell ghosts whose membranes had been damaged by cytotoxic lymphocytes in an ADCC reaction. These ring structures with a 150A inner diameter were proposed to be membrane pores inserted into the target cell membranes during the lytic process. It was found that such ring structures are generated from material in granules of the cytotoxic lymphocytes after contact with plastic surfaces coated by antigen-antibody complexes. We therefore have begun an effort to study these granules biochemically. Tumors of rat "large granular lymphosytes" (LGL) (which mediate both NK and ACDD killing) have been used as a source for biochemical isolation of the granules by Percoll gradients. In order to test whether or not granule components are inserted into membrane pores in target cells a means of measuring marker permeability into red cell ghosts is being developed so that we can perform experiments similar to those previously done with complement pores.

Project Description

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. Based on previous work by us and others, we hypothesize that killer lymphocytes implant molecules into the target cell membrane which injure its permeability properties. This is analogous to the mechanism of complement action, and other agents are known to act in this way; we are studying such agents to compare their actions to lymphocyte damage. This project seeks experimental support for the above hypothesis of lymphocyte mediated target membrane damage.

Methods Employed: (1) Classical techniques of electron microscopy were used to analyze the large granular lymphocytes adherent to plastic surfaces antigen-antibody complexes. Serial section parallel to the plastic surface were analyzed for alterations in the granular material. (2) Spleens from old Fisher strain rats were found to contain 80-90% large granular lymphocytes which were cytolytically active as NK and K cells. These spleens were used as a source of cells for biochemical fractionation in order to purify the granules. Self-generated density gradients using Percoll are used to isolate fractions which contain mixtures of mitochondria and granules as judged by electron microscopy and enzymatic assays (succinate dehydrogenase and -glucuronidase). (3) A technique for measuring marker penetration into erythrocyte ghosts was previously described and shown to be capable of showing inhibited marker permeability due to antibody against the pore material. The present efforts require a larger trapping anti-hapten antibody to be resealed inside the ghosts since the cytotoxic lymphocytes induce pores which are large enough to allow an IgG antibody to escape. IgG anti-hapten antibodies are bein cross-linked to ferritin with glutaraldihyde as one approach to this while IgM hybridoma anti-hapten antibodies are also being explored.

Major Findings: (1) Human large granula lymphocytes are both "NK cells", which kill certain tumor cells by an unknown recognition mechanism and "K cells", which destroy antibody-coated target cells (ADCC). Similar morphological changes in the granules of
these cells occur during both types of cytotoxicity and are consistent with a secretory process. The ring structures we previously described by negative stain and electron microscopy on target membranes after ADCC can be found in the lipid vesicles found in LGL granules after activation of those cells by practic surfaces coated with antigen-antibody complexes. Thus the ring structures originate from the material in the killer cell and do not require target cell components for their formation. (2) The granules in rat LGL tumor cells can be isolated in Percoll density gradients and have a density of 1.05. They are not separated from mitochondria by such gradients, however.

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: Efforts are being made to purify the rat LGL granules from mitochondria. Complete biochemical analysis of granular material will be made and efforts made to separate the morphologically different types of granule. In particular the presence of tubulin will be investigated in light of M. Henkart's immunofluorescence findings and recent reports on the interaction of tubulin with membranes. Antibodies will be raised to granular components and those tested for their ability to inhibit all types of lymphocyte-mediated cytotoxicity. Such anti-granule antibodies will also be tested to ascertain whether they block the passage of markers through the membrane pores previously demonstrated in red cell ghosts after ADCC.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 CB 05021-11 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Antigens Determined by the Murine Major Histocompatibility Locus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	J. A. Bluestone	Staff Fellow	I NCI
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	3.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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

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 ^{3}H-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-idiotype 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 50 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 amd 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.

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.

5) Anti-idiotypic antibodies reactive with three of the hybridoma anti-H-2 antibodies and with two of the hybridoma anti-Ia antibodies have been produced and have been shown to be specific by hemagglutination inhibition assays. Assessment of a variety of hybridoma antibodies and immune sera for the presence of these idiotypic specificities has indicated that at least one of the anti-Ia idiotypes is prevelant in the normal immune response.

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 idiotype 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 idiotype 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.

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 idiotype by in vivo treatment with anti-idiotype 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. Backcrosses 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.

5) The effect of in vivo treatment with anti-idiotype 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.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 05023-11 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Transplantation Antigens of Swine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	S. Rudikoff	Microbiologist	LCB NCI
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COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 characterication of intra-MHC recombinants. Two intra-MHC recombinants have been obtained and are being bred to homozygosity. These should permit assessment of the effects of matching at different MHC regions on the outcome of transplantation in this animal model.

Project Description

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 being used with these swine involves 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 six 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) are being 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-β2 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. Methods are also being developed for the preparation and isolation of peptides from these isolated antigens.

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. Typing of the fourth generation offspring yielded three breeding pairs of animals homozygous for serologically determined transplantation antigens. We therefore now have the capacity to breed three different herds of swine, each homozygous for a different MSLA antigen.

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±.89 days, while the mean survival time of skin grafts between animals of the three different herds was 7.0±.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-β2 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 anlayses 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.

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 will 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. Lines will be established and separated, so that herds of any required size in each line can be produced.

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).

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05033-11 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immunotherapy of Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. D. Terry	Director, Immunology Program	IP	NCI
Other:	R. J. Hodes	Chief, Immunotherapy Section	I	NCI
	S. A. Rosenberg	Chief, Surgery Branch	SURG	NCI
	R. I. Fisher	Senior Investigator	M	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER
X

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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

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 PPLO-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, 1981, 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	30	25	38	25
Deaths	23	22	25	19

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.

Publications

Terry, W. D., Hodes, R. J., Rosenberg, S. A., Fisher, R. I., Makuch, R., Gordon, H. G., and Fisher, S. G.: Treatment of Stage I and II malignant melanoma with adjuvant immunotherapy or chemotherapy: Preliminary analysis of a prospective randomized trial. In W. D. Terry and S. A. Rosenberg (Eds.), Immunotherapy of Human Cancer. Elsevier: North Holland, New York, p 251-257, 1982.

Fisher, R. I., Terry, W. D., Hodes, R. J., Rosenberg, S. A., Makuch, R., Gordon, H. G., and Fisher, S. G.: Adjuvant immunotherapy or chemotherapy for malignant melanoma: Preliminary Report of the National Cancer Institute Randomized Clinical Trial. Surgical Clinics of North American. Philadelphia, PA, W. B. Saunders Co., 1981, pp. 1262-1277.

Terry, W. D. and Hodes, R. J.: Immunotherapy. In DeVito, V. T., Hellman, S. and Rosenberg, S. A. (Eds.): Cancer: Principles and Practice of Oncology. Philadelphia, PA, J. B. Lippencott, Co., 1982, pp. 1788-1810.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05035-10 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Characterization of the Lymphocyte Receptor for IgG (FcγR)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	M. L. Lamers	Postdoctoral Fellow	I	NCI
	S. Heckford	Postdoctoral Fellow	I	NCI

COOPERATING UNITS (if any)
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LAB/BRANCH Immunology Branch

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	2.50	1.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are to characterize lymphocyte Fcγ receptors genetically and functionally. Recent 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. In additional, monoclonal antibodies specific for FcγR induce B lymphocytes to both proliferate and secrete antibody (function). This response does not require T lymphocytes and is specific by a variety of criteria. This is the only antibody specific for a B lymphocyte receptor which triggers function. These findings strongly suggest that B lymphocyte Fcγ receptors are central to B lymphocyte activation and immunoregulation.

Project Description

Objectives: 1) To evaluate the genotypic and phenotypic expression of the Fcγ 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 FcγR 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.

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 (Fcγ 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-Fcγ 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 Fcγ 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 Fcγ 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 Fcγ receptors whereas mixtures of the same monoclonal antibodies produce maximal inhibition; however, immune complexes bound to Fcγ

receptors do not sterically inhibit detection of Ia antigens. Thus, while the nature of the Ia antigen-Fcγ 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 Fcγ 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 Fcγ receptors. The interaction is specific and unidirectional: (a) redistribution of Fcγ 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 smilar interaction has been demonstrated for surface IgD and Fcγ 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 Fcγ receptor is also involved, and the differences between the sIgM-Fcγ receptor and sIgD-Fcγ receptor interactions provide a mechanism whereby the two antigen receptors could provide different signals to the B lymphocyte.

Normal spleen B lympohocytes in the presence of purified monoclonal antibody specific for FcγR 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-FcγR do not require T lymphocytes or their products and are limited to a major subpopulation of B cells which bear the LyB5 alloantigen. The responses are polyclonal, and mainly of the IgM class. This is the only known example of an antibody specific for a B lymphocyte receptor which by itself triggers function.

Significance to Biomedical Research and the Program of the Institute: The B lymphocyte Fcγ 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-FcγR antibodies will allow functional triggering (antibody secretion) or inactivation (tolerance) of B lymphocytes specific for any antigen. This would provide significant new therapeutic modalities for the treatment of cancer and other diseases.

Proposed Course of Project: 1) Characterization of 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 requirement for accessory cells in the response. 4) Analysis of *in vivo* effects. 5) Analysis of possible triggering of other FcγR bearing lymphocytes such as natural killer cells and suppressor T cells. 6) Analysis of the effects of molecular interactions on FcγR triggering.

Publications

Dickler, H. B. and Kubicek, M. T.: Interactions between lymphocyte membrane molecules. I. Interaction between B lymphocyte surface IgM and Fc IgG receptors requires ligand occupancy of both receptors. J. Exp. Med. 153: 1329-1343, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05036-10 I

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Genetic Control of the Immune Response to Staphylococcal Nuclease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. H. Sachs	Chief, Transplantation Biology Section	I NCI
OTHER:	R. J. Hodes	Chief, Immunotherapy Section	I NCI
	P. I. Nadler	Investigator	I NCI
	A. Finnegan	Guest Worker	I 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:
2.0	1.5	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 hemagglutination 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 idiotype 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 idiotype expression in the serum of these animals. This effect appears to involve T cells, since it is not observed in nude mice, and since idiotype-bearing T helper cells for in vitro anti-TNP nuclease response have been found in spleens from such treated animals. Isoelectric focusing studies on anti-nuclease antibodies have indicated characteristic spectrotypes for different strains which appear to correlate with idiotype expression. Several hybridomas reactive with nuclease and/or anti-idiotype have been produced.

Project Description

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. Immunoabsorptions 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 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 idiotype to allotype and/or H-2 type.

3) Backcross animals have been screened for antibody levels and for allotype and idiotype expression in order to determine linkage of idiotype 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-idiotype antibodies and the effect of this treatment on their subsequent expression of idiotype and anti-nuclease activity has been examined by means of spectrophotometric and hemagglutination assays.

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

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-idiotype 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-idiotype 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-idiotype.

Major Findings: By two separate criteria, anti-idiotype 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.AxA/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-idiotype 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-idiotype 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-idiotype 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-idiotype 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-idiotype 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 spectrotype. Development of the isoelectrofocusing gels with ^{125}I-labeled anti-idiotype 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.

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.

Publications

Nadler, P. I., Miller, G. P., Sachs, D. H., and Hodes, R. J.: Genetic control of antibody responses to TNP-nuclease in vitro. J. Immunol. 126: 1706-1712, 1981.

Sachs, D. H., El-Gamil, M., and Miller, G.: Genetic control of the immune response to staphylococcal nuclease. XI. Effects of in vivo administration of anti-idiotypic antibodies. Eur. J. Immunol. 11: 509-516, 1981.

Miller, G. G., Nadler, P. I., Asano, Y., Hodes, R. J., and Sachs, D. H.: Induction of idiotype-bearing nuclease-specific helper T cells by in vivo treatment with anti-idiotype. J. Exp. Med. 154: 23-34, 1981.

Nadler, P. I., Miller, G. P., Sachs, D. H. and Hodes, R. J.: Anti-idiotype induction of antigen-specific helper T cells for responses to TNP-nase. In Resch, K. and Kirchner, H. (Eds.): Mechanisms of Lymphocyte Activation (Proc. of the 14th Leucocyte Culture Conference). Amsterdam, Elsevier/North-Holland Biomedical Press B.V., 1981, pp. 420-423.

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Nadler, P. I., Miller, G. G., Sachs, D. H., and Hodes, R. J.: The expression and functional involvement of nuclease-specific idiotype on nuclease-primed helper T cells. Eur. J. Immunol. 12: 113-120, 1982.

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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05038-10 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cell-Mediated Immunity to Hapten Modified Syngeneic Lymphocytes in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
Other:	R. B. Levy	Senior Staff Fellow	I NCI
	H. Fujiwara	Visiting Associate	I NCI
	P. K. Arora	Visiting Fellow	I NCI
	P. A. Henkart	Senior Investigator	I NCI
	T. Tsuchida	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 isoothiocyanate 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 $-NH_2$ 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

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.; (e) to compare the in vivo priming effects on different types of T-cell mediated immunity, (e.g., cytotoxicity, delayed hypersensitivity, suppression); and (f) to compare the CTL results observed using TNP-self with those obtained using the FITC-self and AEDANS-self haptens.

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-napthyl) ethylene diamine (AEDANS-self) or various isomers of the fluorscein 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 ($H\text{-}2^{k,a}$), but not in low responder ($H\text{-}2^{b,d}$) 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 NH_2- or SH- groups. Thus, similar haptens exhibited different Ir gene pattens of response in congenic mouse strains, depending on whether they coupled with

cell surface NH_2- 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 NH_2- groups was associated with high responsiveness in H-$2^{a,k}$ and low responsiveness in H-2^b mice.

Recent data suggest that an interesting but complex pattern of Ir gene regulation controls the CTL response to SH reactive haptens. One gene which is responsible for high response potential maps to I-A, whereas a second gene which appears to down-regulate CTL responsiveness (suppression?) appears to map to I-E/C.

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., NH_2- 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.

Publications

Levy, R. B., Shearer, G. M., Richardson, J. C., and Henkart, P. A.: Cell-mediate lympholytic responses against autologous cells modified with haptenic sulfhydryl reagents. I. Effector cells can recognize two distinct classes of hapten-reactive self sites on cell surface proteins. J. Immunol., 127: 523-528, 1981.

Levy, R. B., Henkart, P. A., and Shearer, G. M.: Cell-mediated lympholytic responses against autologous cell modified with ahptenic sulfhydryl reagents. II. Analysis of the genetic control of cytotoxic responses to sulfhydryl and amino reactive reagents. J. Immunol. 127: 529-534, 1981.

Tamaki, K., Fujiwara, H., Levy, R. B., Shearer, G. M., and Katz, S. I.: Hapten specific TNP-reactive cytotoxic effector cells using epidermal cells as targets. J. Investigative Dermatol., in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05050-08 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Studies on the Structure and Function of the Constant Portions of Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) The FcR-mediated endocytosis of model immune complexes has been studied using the P388D$_1$ mouse macrophage line. Internalization increases with complex size because binding avidity increases with size and because large complexes are cleared from cell surfaces more rapidly than smaller ones. Ligand induced self aggregation greatly enhance receptor mediated uptake. About 2/3 of FcR are rapidly down modulated after binding immune complexes, the remaining 1/3 recycle between bound and unbound states. (2) The role of monomeric IgG in controlling IgG mediated effector functions has been investigated. (3) The distribution of FcR in normal human and murine cells has been examined by dual parometer flow microfluorometry. In human peripheral blood mononuclear cells, FcR distributions have been measured in cells expressing OKM1, OKT3, OKT4 and OKT8 antigens. In the mounse FcR have been measured on spleen cells which bear SIgM, SIgD, and IA. (4) We have adapted our techniques for measuring FcR to quantitating Class-I histocompatibility antigens on mouse spleen cells.

Project Description

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 artifically 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. FcR-Mediated endocytosis. We have previously used affinity cross linked oligomers of IgG to study the mechanisms by which immune complexes bind to FcR on cells from the P388D_1 mouse macrophage line. Those studies were done at 0°C in the presence of sodium azide to prevent internalization. When the cells are warmed to 37°C in media devoid of inhibitors they internalize immune complexes by an FcR-mediated process. We have recently completed a detailed investigation of the receptor-mediated endocytosis of model immune complexes by P388D_1 cells in order to probe the mechanism of this important effector function.

Cells were incubated at 37°C with radiolabeled oligomers of IgG, cross linked with one of two bivalent affinity labeling reagents, DIBADL or BDPE. Oligomers prepared with either reagent are covalently cross-linked by their combining cite regions. However, whereas the BDPE oligomers are stable with respect to size, the DIBADL oligomers aggregate non-covalently into larger complexes, unless free hapten is present in the medium. Aggregation by DIBADL oligomers occurs to an especially marked extent when the oligomers are bound to cell surfaces since their local concentrations are high in the cell-surface environment.

The internalization of BDPE oligomers occurs by two processes; FcR-mediated internalization, which is blocked by a large excess of unlabeled, cross-linked IgG, and fluid phase pinocytosis, which is not blocked. The FcR-mediated component increases gradually with oligomers size, and even monomeric IgG is endocytosed. Moreover the receptor-mediated uptake of BDPE oligomers saturates with increasing concentration in the same concentration range where binding saturates. These data suggest that FcR-mediated uptake increases with size, and concentration, in part, because the amount of oligomer bound to FcR increases with size and concentration. The data also suggest that the cross-linking of FcR is not required for the internalization of small oligomers of IgG because monomeric IgG is internalized, and because enhancement of internalization with size can be accounted for by the increased avidity of the oligomers for the cells.

The DIBADL oligomers remain cell associated for long periods of time when they are incubated at 0°C with P388D_1 cells and washed. Upon warming to 37°C, the DIBADL oligomers again remain cell associated (as determined from bound radioactivity), but surface-bound oligomers rapidly (with a $T_{1/2}$ = 5 min) disappear, as determined by the binding of fluorescent anti-IgG antibodies. Approximately 15-20 minutes after the DIBADL oligomers are cleared from the cell surface, they are degraded into smaller peptides, as determined by SDS-PAGE analysis. In parallel studies, immune complexes formed with DNP-BSA and anti-DNP antibodies behaved similarly to DIBADL dimer.

In order to determine whether oligomers of varying size are cleared at the same or different rates from cell surfaces, P388D_1 cells were incubated with either radiolabeled BDPE tetramer or DIBADL dimer, and washed. The cells were warmed to 37°C and both the total cell associated oligomer and surface-bound oligomer were followed as a function of time. The DIBADL dimer, as we have previously shown, aggregates into large polymers on the cell surface, and is therefore effectively larger than the BDPE tetramer, which does not aggregate. At 37°C, the BDPE tetramer dissociates from the cell surface more rapidly than the DIBADL dimer; in contrast the DIBADL dimer is cleared from the cell surface more rapidly than the BDPE tetramer. These experiments demonstrate that the clearance of aggregated IgG from the surface of endocytic cells increases with the size of the aggregate. Thus the enhancement of endocytic rate of immune complexes with size occurs at two stages; (1) When complexes bind to FcR from the medium, and (2) when bound complexes are cleared from cell surfaces.

We have also examined the fate of FcR during the endocytic process. This was done by following the total uptake of radiolabeled DIBADL dimer when cells were incubated at 37°C in saturating concentrations of the ligand, and comparing it with the amount of ligand bound on the cell surface (which is in turn equivalent to the number of FcR on the surface). The results of these studies show that P388D_1 cells, when warmed to 37°C in the presence of saturating concentrations of DIBADL dimer, initially bind a full receptor load of dimer, and over the next two hour period, internalize an additional amount of dimer, about 1-1.5 as much as that which was originally bound. At the same time the number of receptors on the cell surface rapidly ($T_{1/2}$ = 5 min) falls to 30% of its initial value and remains relatively constant over the next two hours. Since oligomer is taken up during this period, free receptors must have been appearing on the cell surface to replace those involved in the endocytosis. These observations were not affected by concentrations of cyclohexamide which inhibit receptor synthesis, and therefore approximately 1/3 of the FcR recycle between the bound and unbound state (as has been observed in several other systems as well). We estimate that each of these receptors recycles once every 30-40 minutes. The other 2/3 of the receptors are rapidly down modulated by immune complexes, but it is not known whether they are degraded or enter an intracellular pool of receptors. The observations that receptor cross-linking is not required for endocytosis and that approximately 1/3 of the FcR recycle suggest a mechanism by which large complexes are cleared from cell surfaces more rapidly than small oligomers. By this mechanism a subpopulation of receptors is continually entering endocytic

vesicles, reappearing on the cell surface, and diffusing toward endocytic vesicles, to complete the cycle. Large complexes would be cleared from cell surfaces more rapidly than smaller ones, because the probability that at least one IgG subunit would bind to a receptor which was near the endocytic stage of the cycle would increase with the size of the immune complex.

II. The homeostatic role of monomeric IgG in controlling IgG-dependent immune effector processes. We have known for some time that monomeric IgG can inhibit the binding of IgG oligomers or immune complexes to FcR, and we have also predicted that complement binding and activation to antibody-coated targets should also be inhibited. Since monomeric IgG is present in blood and interstitial fluids at 10 mg/ml (approximately 10^{-4} M), a value which is normally maintained with only small variations, it is clear that this IgG must exert a powerful controlling influence upon IgG-mediated effector functions. We have considered the role of IgG in three processes, the FcR-mediated endocytosis of immune complexes, the complement-mediated cytolysis of antibody-coated cells, and in ADCC (antibody-dependent cell mediated cytolysis).

First we measured the receptor-mediated endocytosis of IgG oligomers or immune complexes in the presence or absence of 10 mg/ml monomeric IgG. These experiments showed, as expected, that monomeric IgG strongly inhibits endocytosis, but that immune complexes or self-aggregating oligomers of IgG are taken up more rapidly from the medium than monomeric IgG. We observed that binding to FcR by immune complexes is undetectable by our assays, when cells were incubated with radiolabeled immune complexes in the presence of 10 mg/ml monomeric IgG. Clearly receptor-mediated internalization of immune complexes passes through an intermediate in which only very small amounts of immune complex are bound to the cell surface, compared to the total number of FcR which they express. Binding simulations, using previously determined kinetic and equilibrium constants, suggest that the preferential uptake of immune complexes over small non-aggregation oligomers or monomeric IgG derives mainly from two sources. First, more IgG subunits bind to a cell when an immune complex binds than when monomeric IgG binds; however we calculate that most subunits are not bound to FcR. For example, in the presence of 10 mg/ml monomeric IgG, an oligomer of 8 subunits would bind by an average of only 1.5 subunits. Once bound, however, the mean lifetime that an oligomer remains on the cell surface, before dissociating into the medium, increases with oligomer size. therefore the increased association of bound subunits and increased mean residence times probably are the aspects of binding which allow immune complexes to be removed preferentially from serum.

In order to test our previously published predictions that complement binding to antibody-coated cells will be inhibited by monomeric IgG, and that the extent of inhibition will decrease with the amount of cell-bound antibody, we coated TNP-P388D_1 cells with varying levels of anti-TNP antibody, and measured the ability of complement to lyse those cells in the presence of monomeric IgG. The results of these studies were in agreement with the predictions. Therefore monomeric IgG will normally determine the level of antibody on a target cell which is required for lysis.

Finally, we used equations previously developed by George Bell to estimate how monomeric IgG would inhibit cell-cell interactions between antibody-coated target cells and FcR-bearing effectors. The equilibrium constants used in these calculations were taken from our previous studies. The results of these calculations predict that monomeric IgG should inhibit cell-cell interactions, and that the ability to inhibit these interactions will depend upon both the amount of antibody on the target and the number of receptors on the effector cells. We have not yet tested these predictions experimentally, but Ziegler and Heney have already published data demonstrating that ADCC is inhibited by monomeric IgG, and that the extent of inhibition decreases as the amount of antibody on the target cell increases.

Clearly monomeric IgG is a key factor in modulating IgG-dependent effector processes. What function might this serve in vivo? We suggest that the dampening of effector functions might allow IgG antibodies to bind to self antigens to a small extent, without having effects which are detrimental to the organism. This could be a mechanism to prevent autoimmune reactions or conversely, it could provide a mechanism whereby IgG antibodies would be allowed to interact with a wide variety of foreign antigens, some of which resemble self antigens. In order to effectively control IgG-dependent functions, the concentration of monomeric IgG must be held constant, which is in fact observed. We predict that this is done via Fc receptors on some cells which respond to the binding of monomeric IgG by decreasing antibody production. Two types of cells which could be involved in modulating IgG production are B-cells and T cells.

III. The distribution of FcR within heterogeneous populations of cells. We have previously described a method for measuring FcR in heterogeneous populations of cells, using flow microfluorametry. FcR on cells are saturated with DIBADL dimer, and the cells are washed, stained with a fluorescent conjugate of goat anti-rabbit IgG antibodies, and analyzed in the cell sorter. By comparing fluorescence emission with the number of bound dimer molecules (using radiolabeled dimer) it is possible to quantitate the number of FcR on large numbers of individual cells.

More recently we have been identifying the cells which bear FcR by staining the cells with a second reagent which is conjugated with a fluorophore whose excitation and emission spectra do not overlap with the first. By using our dual laser cell sorter we are then able to determine the number of FcR on defined subpopulations of cells without isolating the cells.

In order to carry out these studies we first had to develop a fluorophore with proper chemical and spectral characteristics. This was done in collaboration with Dr. Richard Haugland, of Molecular Probes, Inc., who synthecized the sulfonyl chloride derivative of sulforhodamine 101. We termed this reagent "Texas Red", and showed that it's excitation spectrum does not overlap that of fluorescein, and that the excitation maximum is close to the 586nM krypton laser line. Likewise, the emission spectra of fluorescein and Texas Red are totally separable, and the Texas Red emission maximum is displaced far enough from the excitation wavelength to make it suitable for flow microfluoremetric studies. We showed that Texas Red is

easily conjugated to proteins, and that unlike some other fluorophores, it gives fully active, highly fluorescent reagents in high yield. Texas Red is commercially available and is being used by many laboratories. In most of our dual parameter studies we label FcR with Texas Red and other markers with FITC.

Human peripheral blood mononuclear cells express FcR in a trimodal distribution; cells in the three discernable peaks express an average of 0, $1.5x10^4$ and $4.5x10^4$ FcR/cell. By using cell isolation procedures we first showed that the high and negative peaks contained mainly T cells while the middle peak was mostly monocytes. Next we doubly stained the cells for FcR and with the following hybridoma antibodies; 3A1 a reagent specific for most human T cells, OKT3 another reagent specific for most T cells, OKT 4, a reagent which stains a subpopulation of T-cells containing helper activity, OKT8, a reagent staining cells which exhibit suppressor activity and OKM1, a reagent which has been reported as being specific for monocytes.

Our data show that OKM1, as expected, stained the peak with intermediate FcR density (monocytes) most brightly. However, we also observed that OKM1 gave a discrete peak of intermediate intensity which was almost totally comprised of the cells with the highest FcR density (T cells).

The T cells however, differ from monocytes in that they stain brightly with the 3A1 anti-T cell hybridoma, while monocytes do not bind this reagent. OKT3 labels most of the FcR negative (T non-) cells and a small subset of the T cells. It is therefore not a "pan T" reagent as was originally thought. OKT4 stains only the FcR negative cells, in agreement with others who have showed that helper T cells do not express FcR. The relationship between OKT8 and FcR is more complex. Mononuclear cells doubly labeled for both FcR and OKT8 exhibit five distinct subpopulations; cells with intermediate FcR density (monocytes) are all negative for OKT8. Cells not bearing FcR (mostly T non-cells) fall into two subpopulations of nearly equal size, those expressing no OKT8 and other bearing high levels of OKT8. The T cells also fall into two subpopulations when stained with OKT8 - cells not expressing OKT8 and cells expressing intermediate levels of OKT8. Since T cells have previously been shown to exhibit suppressor activity and to bear OKT8, the OKT8 intermediate T cells are presumably the suppressor cells. However it is not clear what the T , OKT8 negative cells are - perhaps either NK cells or ADCC effectors (K cells). Clearly a major goal of this study is to correlate the FcR-bearing subpopulations with receptor-dependent functions.

We have also examined human granulocytes in colluboration with Dr. David Bass. The FcR in granulocytes fall into two subpopulation - cells expressing a very high number of FcR ($2x10^5$/cell) and a minor, but variable subpopulation, bearing about $3x10^4$ FcR/cell. By sorting these subpopulations we have demonstrated that the FcR-dense subpopulation is totally neutrophils, while the other cells are eosinophils. Both subpopulations bind IgG1 and IgG3, but not IgG2 or IgG4. We are currently examining whether these cells also bear FcR for IgG.

In the mouse, 60-80% of spleen cells express FcR. Most FcR+ cells express low numbers of FcR ($1-2x10^4$/cell) but other cells express many more FcR. The distribution does not consist of distinct peaks, but rather a continum, trailing off into the high density range. In collaboration with Drs. Fred Finkelman and Irwin Scher we have found that virtually all cells bearing either IgD or IgM on their surfaces (i.e. all B cells) also bear FcR. The IgM+ cells express somewhat more FcR than IgD+ cells, and there is a slight positive correlation between the amount of IgM expressed and the number of FcR. No such correlation exists with IgD. Approximately 50% of the cells are B-cells, while 60% of mouse spleen cells express both FcR and I-A. Presumably this extra 10% is due to macrophages. There is a direct correlation between the IA molecules and FcR. Finally 11% of cells express FcR, but neither IgM, IgD nor IA. These are presumably T-cells, but because of difficulties in obtaining a suitable anti-Thy reagent, we have not yet been able to confirm this directly.

Finally we have begun to do functional studies with FcR bearing populations of cells. With Dr. Hillel Koren we showed that when cells from the human monocyte line, U937, are stimulated with supernatants from mixtures of allogeneic human T cells, the number of FcR increases about 4-5 fold over a 72 hr period. Concomittantly the ADCC activity of these cells increases, and the amount of antibody on the target cell required for lysis decreases. In another study with Dr. John Wunderlich, we have examined the FcR density on human lymphocytes stimulated with ConA supernatants from human T-cells. Stimulated lymphocytes increase their capacities to mediate ADCC. In this case only a small subpopulation of cells increases its FcR density, and we are currently attempting to identify those cells.

IV. Cell-cell interactions. Cell-cell interactions involve the multivalent attachment of cell bound ligands from one cell to cell surface receptors on the other. Clearly the next step in our study of multivalent interactions between ligands and receptors should be a study of cell-cell interactions. Such interactions not only occur between target and effector cells in cytotoxic processes, but are also important in such processes as antigen presentation, cellular differentiation, help, suppression and many others as well. We have therefore embarked upon a study of cell-cell interations in order to better understand the molecular bases for these phenomena.

We have begun to develop an assay for cell-cell interactions which will allow us to measure conjugates on large numbers of individual cells, that is by flow microfluorometry. We are measuring the interaction of $P388D_1$ cells with antibody coated target cells (TNP-spleen cells or TNP-$P388D_1$ coated with anti-TNP antibodies). We have chosen this system because we can alter the ligand density at will, measure (and to some extent modify) the receptor density, and because we have characterized the binding of oligomers of IgG to $P388D_1$ cells in detail. The method involves labeling the effector cells green (with FITC), the targets red (with XRITC), and allowing the cells to come into contact in suspension. The cells are then analyzed by dual parameter flow microfluorometry and the number of red-green conjugates is a measure of cell-cell interaction. Preliminary studies suggest the method will work. For

example when P388D_1 cells were mixed with TNP-spleen cells in the presence and absence of anti-TNP antibody, red-green doublets were seen only when the antibody was present. The number of doublets (approximately 20% of the cells) correlated with the number seen under the microscope, except that some doublets could be seen with the control cells in the microscope, while none were seen in the cell sorter. Presumably the flow system breaks up weak conjugates, but not those formed between antibody and receptor. One interesting observation of those studies is that each conjugate contained only one P388D_1 cell and one spleen cell, even though many different ratios of effector:target were mixed in the initial incubation.

Quantitation of histocompatibility antigens

We are currently using the technology developed in our laboratory and hybridoma antibodies developed in Dr. David Sach's laboratory to quantitate the expression of Class I histocompatibility antigens on mouse spleen cells. The binding of antibodies to H2 antigens can be a divalent interaction since antibody molecules contain 2 Fab regions. Therefore the mechanisms of binding worked out for the interaction of IgG dimers to FcR will be an appropriate description of antibody binding to Class I antigens. We are using mainly three hybridoma antibodies, 36-7-5 and 11-4-1, with anti K^k specificity, and 27-11-13 which binds to $D^{d,b}$. The mechanism of binding has not been worked out in detail yet, since we have only recently been able to obtain pure F(ab') and Fab' fragments from the hybridomas. Preliminary studies suggest that 27-11-13 and 36-7-5 bind divalently while 11-4-1 binds monovalently.

Binding studies using spleens from individual mice of varying age and sex show that the expression of at least one allele, K^k is invariant between mice. Levels of H-$2K^k$ and H-$2D^d$ expression are similar on mice of B and C backgrounds and approximately 30% higher in mice with A background. In B10.A or B10 mice the average numbers of Class I antigens expressed per spleen cell are as follows: K^k, $5\text{-}6\times10^4$; D^d, $7\text{-}8\times10^4$; D^b, $7\text{-}8\times10^4$ and L^d, $2\text{-}3\times10^4$. In F_1 animals the levels of expression drop by 50%, within experimental error. Moreover, the levels of expression of K antigens are unaffected by the haplotype at the D end and vise versa.

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 the internalization project by following the uptake of IgG oligomers by normal cells. This can be done by coating cells with oligomer at 0°C, warming to 37°C for various period, and then staining for cell bound oligomer. Cells which bind and endocytose immune complexes can be identified using a second reagent and dual parameter flow microfluorometry.

In addition we eventually plan to follow more directly the fate of FcR on $P388D_1$ cells during internalization. This could be done by labeling all FcR internally with ^{3}-S-met, and surface labeling FcR with ^{125}I. Receptors could then be isolated after down-modulation, stripping with pronase, or incubating, from either the cytoplasm, the cell surface, or from isolated coated pits using the 2.4G2 anti-FcR hybridoma, and the history of the isolated receptors determined from $^{125}I/^{35}S$ ratios.

2. We will continue to work out the mechanism of binding of hybridoma antibodies to H-2 determinants in order to quantitate H-2 expression. We are also correlating the expression of Class I antigens on cells modified with TNP or FITC with cytotoxicity in CTL reactions.

3. We are continuing to characterize cells bearing Fcr. We will finish these studies with a characterization of FcR on bone marrow, thymus and lymph node cells in the mouse. After this phase of those studies is complete we plan to study the function of the FcR in various subpopulations of cells, first in ADCC and then in the endocytosis of immune complexes.

4. Further work will be done to measure cell-cell interactions. We plan to follow the molecular events involved in cell-cell adhesions. Subsequently it should be possible to use this technique for examining which cell types interact with each other, and to follow events subsequent to conjugation (e.g. changes in membrans potentials, or transfer of antigen from one cell to another).

5. We have again begun working on the franked ADCC effector system. We are hoping to use cells franked with hybridoma antibodies against the Rauscher virus to kill implants of tumor cells which express this virus, in mice.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05055-07 I

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulatory Influences of Cell-Mediated Immune Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION Immunotherapy Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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

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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05058-07 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Immunoregulation of Antibody Synthesis and Secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. B. Dickler	Senior Investigator	I NCI
Other:	H. Weissberger	Postdoctoral Fellow	I NCI
	R. Shenk	Clinical Associate	I NCI

COOPERATING UNITS (if any)
none

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to characterize mechanisms of immunoregulation of antibody synthesis and secretion, particularly Ir genes and idiotype 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-independant anti-(T,G)-A--L antibody responses. These responses are specific at the levels of the anti-idiotype reagent, the antigen-priming, and the antibody produced. The anti-idiotype reagent stimulates function of both primed T helper lymphocytes (help) and of primed B lymphocytes (antibody secretion). The latter occurs in the absence of T lymphocytes.

Project Description

Objectives: Characterization of immunoregulation of antibody synthesis and secretion particularly by Ir genes and idiotype 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-idiotype 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 globulins. Anti-idiotype activity can be measured by inhibition of binding of anti-(T,G)-A--L antibodies to the antigen, or by direct binding to the idiotype. 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, 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-idiotype reagents in this system. It was found that antigen-primed lymph node cells responded to anti-idiotype by the production of antibody in the absence of antigen. This antigen-independant response was specific at the level of the anti-idiotype reagent in that other reagents including normal rat serum, rat anti-mouse IgG, rat anti-mouse IgM and rat anti-nuclease anti-idiotype were not active. Moreover, idiotype affinity-purified anti-idiotype 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-idiotype. 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-independant anti-idiotype 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-idiotype) responded to anti-idiotype, suggesting that the anti-idiotype was provoking function from

T helper lymphocytes. Additionally, rigorously T lymphocyte depleted antigen-primed B lymphocytes also responded, suggesting that primed B cells can be directly triggered to function by anti-idiotype antibody. Specificity by criteria similar to those for the whole lymph node was demonstrated for the above mentioned experiments.

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-idiotype reagents. This raises the possibility of the use of such reagents themselves as therapeutic modalities.

Proposed Course of Project: Further characterization of antigen-independant response to anti-idiotype as follows: 1) Phenotype subpopulations of lymphocytes which are responding. 2) Evaluate requirement for accessory cells. 3) Characterize mechanisms of activation. 4) Evaluate role of H-2 restriction and Ir genes in these responses.

Publications

None (three manuscripts in preparation).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05062-07 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Application of Rapid Flow Microfluorometry to Cell Biology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:

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B. Mathieson	Staff Fellow	LMI	NIAID	J. Smolen	Visit. Assoc.	A&R	NIADDK
D. Segal	Sen. Invest.	I	NCI	A. Steinberg	Sen. Invest.	A&R	NIADDK
J. Titus	Chemist	I	NCI	M. Waxdal	Sen. Invest.	I	NIAID
W. Paul	Chief	LI	NIAID	J. Bluestone	Staff Fellow	I	NCI
J. Kung	Visit. Assoc.	LI	NIAID	D. Sachs	Sen. Invest.	I	NCI
M. Mage	Sen. Invest.	LB	NCI	C. Reynolds	Staff Fellow	FCRF	NCI
J. Ortaldo	Sen. Invest.	FCRF	NCI	P. Morrissey	Staff Fellow	I	NCI

COOPERATING UNITS (if any)
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U. Hammerling, Sloan Kettering Institute
B. Haynes, Duke University

LAB/BRANCH
Immunology Branch

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	0.2	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using rapid flow microfluorometry (FMF) for analysis and sorting of cells, aspects of the following projects have been supported during the previous year: (1) murine T-cell differentiation and recognition of foreign MHC determinants; (2) characterization of new lymphocyte differentiation antigens; (3) identification of suspopulations of human lymphocytes; (4) analysis of antibodies induced by anti-idiotypes; and (5) characterization of new dyes for FMF.

Project Description

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).

A major segment of the maturation of lymphocytes which regulate immune responses is known to occur in the thymus. Previous work involving FMF focused on changes in the expression of cell-surface differentiation determinants on thymocytes which occur during normal fetal development and on characterization of thymocytes in bone marrow chimeras. Chimeras have been particularly useful for analyzing MHC-restricted immune responses and more recently for understanding the expression of T-cell receptors for non-self.

Recent efforts of Ms. Sharrow, Drs. Mathieson, Hammerling, Singer, and colleagues have expanded our understanding of thymocyte differentiation in the following ways. (1) Marked differences in the expresssion of cell surface determinants have been identified between the host cells which initially repopulate an adult thymus after radiation and bone marrow reconstitution versus the cells which initially settle in the fetal thymus. Thus, the first population of lymphocytes detected in the adult thymus following irradiation and bone marrow reconstitution (about 10-12 days) are radiation-resistant host thymocytes. This population is made up almost totally of $Lyt1^{+}2^{+}$ PNA bright cells; it is deficient in the $Lyt1^{+}2^{-}$ PNA dull cells which normally make up about 10% of an adult thymus. The first population of donor bone marrow cells which are detected in thymus (about 12-15 days post irradiation) have both $Lyt1^{+}2^{-}$ cells as well as $Lyt1^{+}2^{+}$ cells. Thus, the first lymphocytes of donor bone marrow origin found in an irradiated thymus are quite distinct from the first lymphocytes found in the fetal thymus, which include $Lyt1^{+}2^{-}$ but not $Lyt1^{+}2^{+}$ cells. (2) Lyt2 and PNA differentiation determinants are not expressed concomitantly by thymocytes, a finding which will affect studies of fetal thymocyte differentiation. Previous reports based on the analysis of adult thymocytes suggested that all PNA bright cells also express Lyt 2 determinants. However, all fetal and newborn thymocytes, which contain a distinct subset of Lyt2 lymphocytes, are PNA bright; and PNA dull adult cells are not detectable until 2 weeks after birth. Thus, early differentiation lineages of fetal thymocytes cannot be separated on the basis of PNA binding.

Drs. Singer, Morrissey and colleagues have used FMF cell typing to expand our understanding of cells in the T-cell lineage which express receptors for foreign MHC determinants. FMF has been used to classify cells in radiation-induced bone marrow chimeras consisting of AxB hosts bearing T-cell depleted bone marrow and a thymus from the A strain. Parallel functional studies have shown that thymocytes from these mice are specifically tolerant to B strain cells in standard cytotoxic cell responses, even though the thymocytes are derived from strain A marrow and mature in a strain A thymus. FMF analysis has verified the strain A origin of these cells and, in addition, demonstrated that no detectable MHC determinants from strain B are present within the thymus. These findings suggest that Tcell precursors from strain A marrow express receptors for MHC and are tolerized to MHC determinants in the prethymic host environment.

Ms. Titus, Dr. Segal, and colleagues. The use of two color immunofluorescence with FMF has been improved by the introduction of a new sulfonylchloride derivitive of sulfonylrhodamine 101. This reagent is readily soluble in aqueous media, binds to amino groups and emits fluorescence at wave lengths higher then standard rhodamine compounds. The new fluorochrome has been combined successfully with fluorescein in two color studies to analyze surface determinants in selected subpopulations of human peripheral blood lymphocytes.

FMF has been used to further characterize rodent lymphocytes reacting with monoclonal antibodies directed against cell surface differentiation antigens. Dual color analysis and the quantitative nature of analysis have been essential for much of this work.

Drs. Kung, Paul, Mage, Scher, and colleagues have shown that the determinant expressed by mouse B and T lymphocytes which is identified by the monoclonal antibody 14D10 is strongly expressed on 92% of the IgM^+ cells (B lymphocytes). The determinant is expressed only weakly by T lymphocytes, predominantly by the $Lyt2^+$ subclass of these cells.

Expression of a new differentiation antigen found predominately on neonatal B lymphocytes has also been analyzed by FMF. The frequency of lymphocytes expressing this determinant, identified by the 14G8 monoclonal antibody, is increased in mice with sex-linked immunodifficient B cell responses. Unlike 14G8+ cells from normal mice, those from the immunodifficient mice are unable to proliferate in response to a standard anti-IgM stimulation.

With Drs. Reynolds, Ortaldo, Herberman, and colleagues rat lymphocytes highly enriched for natural killer cell activity have been analyzed using a panel of monoclonal antibodies which react with differentiation antigens characteristic of other types of rat leukocytes. The pattern of reactions with rat natural killer cells is distinctly different from that with T cells, B cells, monocytes, and PMN's. However the sharing of some determinants between NK cells, T cells and monocytes suggests a common developmental relationship.

FMF has continued to be a valuable means for quantitatively characterizing the purity of various cell subpopulations.

Drs. Smolen and Steinberg have shown that human peripheral blood T lymphocytes proliferate in vitro when mixed with particular subpopulations of autologous non-T-cells. Using subpopulations characterized by FMF, the proliferative response is suppressed by autologous monocytes or T cells.

In a separate study with Drs. Mathieson, Haynes and Waxdal, subpopulations of human peripheral blood lymphocytes and spleen cells which bind various lectins have been characterized by FMF to determine, in part, which lectins will be useful for rapidly purifying cells.

Drs. Bluestone, Sachs and colleagues. Antibodies against unique determinants on anti H-2K^k antibodies (antiidiotypes) induce production of anti H-2K^k when injected into normal BALB/c (H-2K^d) mice. FMF has been used to quantitatively determine levels of anti H-2K^k, and the following observations have been made. (1) Production of antiidiotype-induced anti H-2K^k is genetically linked to the V_H genes for immunoglobulin, and (2) H-2K^k antigen immunization of BALB/c mice, pretreated with antiidiotype, results in antibodies up to 65% of which express idiotype whereas antisera induced in normal BALB/c mice lack detectable idiotype.

Significance to Biomedical Research and the Program of the Institute: T lymphocytes regulate immune responses in general and directly mediate normal host cytotoxic reactions against foreign cells such as tumor cells. Thus characterizing the developmental pathways of T cells is important. Monoclonal antibodies against cell surface differentiation antigens have had a major role in the characterization of subpopulations of not just T cells but also B lymphocytes and NK cells, and these reagents are particularly well suited to FMF work. The availability of monoclonal antibodies which identify new differentiation antigens and receptors on lymphocytes will expand the potential for understanding the development and function of these cells.

Proposed Course of Project: As in the past, rapid flow microfluorometry will be used for selected, appropriate projects.

Publications

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Ortaldo, J.R., Sharrow, S.O., Timonen, T, and Herberman, R.B.: Determination of surface antigens on highly purified human NK cells by flow cytometry with monoclonal antibodies. J. Immunol. 127(6): 2401-2409, 1981.

Reynolds, C.W., Sharrow, S.O., Ortaldo, J.R., and Herberman, R.B.: Natural killer activity in the rat. II. Analysis of surface antigens on LGL by flow cytometry. J. Immunol. 127(6): 2204-2208, 1981.

Kruisbeek, A.M., Sharrow, S.O., Mathieson, B.J., and Singer, A.: The H-2 phenotype of the thymus dictates the self-specificity expressed by thymic but not splenic cytotoxic T lymphocyte precursors in thymus-engrafted nude mice. J. Immunol. 127(5): 2168-2176, 1981.

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Bach, M.A., Phan-Dinh-Tuy, F., Bach, J.F., Wallach, D., Biddison, W.E., Sharrow, S.O., Goldstein, G., and Kung, P.C.: Unusual phenotypes of human inducer T cells as measured by OKT4 and related monoclonal antibodies. J. Immunol. 127(3): 980-982, 1981.

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Biddison, W.E., Sharrow, S.O., and Shearer, G.M.: T cell subpopulations required for the human cytotoxic T lymphocyte response to influenza virus: Evidence for T cell help. J. Immunol. 127(2): 487-491, 1981.

Mage, M., Mathieson, B., Sharrow, S., McHugh, L., Hammerling, U., Kanellopoulos-Langevin, C., Brideau, D., Jr., and Thomas, C.A., 3rd,: Preparative nonlytic separation of $Lyt2^+$ and $Lyt2^-$ T lymphocytes, functional analyses of the separated cells and demonstration of synergy in graft-vs.-host reaction of $Lyt2^+$ and $Lyt2^-$ cells. Eur J. Immunol. 11(3): 228-235, 1981.

Bluestone, J.A., Sharrow, S.O., Epstein, S.L., Ozato, K., and Sachs, D.H.: Induction of anti-H-2 antibodies without alloantigen exposure by in vivo administration of anti-idiotype. Nature. 291(5812): 233-235, 1981.

Broder, S., Uchiyama, T., Muul, L.M., Goldman, C., Sharrow, S., Poplack, D.G., and Waldmann, T.A.: Activation of leukemic pro-suppressor cells to become suppressor-effector cells. Influence of cooperating normal T cells. N. Engl. J. Med. 304(23): 1382-1387, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05064-06 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Genetic Control of the Immune Response In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Singer	Senior Investigator	I NCI
Other:	R. J. Hodes	Chief, Immunotherapy Section	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Requirement for self-recognition by T-helper cells of macrophage and B cell Ia determinants has been analyzed in vitro and in vivo. The self-recognition repertoires of T cells from A-->AxB, AxB-->A, and A-->B radiation bone marrow chimeras were restricted to the recognition of host, not donor, MHC determinants. While activation of T-helper cells invariably required self-recognition of macrophage Ia determinants, T-helper cell activation of B cells did not necessarily require T cell recognition of B cell Ia determinants. Indeed, it was found that those B cell responses which require T cell recognition of B cell Ia determinants were mediated by the Lyb5-B cell subpopulation, whereas B cell responses which were not T-B restricted involved the Lyb5+ B cell subpopulation.

Project Description

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 TNP 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-KLH, T-helper cells from A-->AXB, AxB-->A, and A-->B radiation bone marrow chimeras are only able to cooperate with macrophages expressing MHC determinants syngeneic to those of the chimeric host. These experiments demonstrate that activation of T-helper cells requires recognition of macrophage MHC determinants and that the specificities T cells recognize are determined by the environment in which they differentiate. In order to assess the possibility that T-helper cells recognized the same I region gene products as detected by antibodies, the ability of anti-I-A specific reagents to interfere with T-cell activation was assessed. Indeed, anti-I-A reagents specific for macrophage I-A determinants did block T-helper cell activation. Furthermore, experiments with restricted F_1-->parent chimeric T cells demonstrated that this was due to specific interference with the ability of T cells to recognize macrophage I-A determinants.

In addition, it was also found that T-helper cells could activate both Lyb5+ and Lyb5- B cell subpopulations, but that the activation of these two subpopulations was genetically distinct. Specifically, it was found that T cell activation of Lyb5- B cells was genetically restricted while T cell activation of Lyb5+ B cells was not genetically restricted.

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 not only signficantly expand our understanding of the generation of the T cell repertoire and the requirements for B cell activation, but also 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

Singer, A., Morrissey, P. J., Hathcock, K. S., Ahmed, A., Scher, I. and Hodes, R. J.: Role of the major histocompatibility complex in T cell activation of B cell subpopulations. Lyb5+ and Lyb5- B cell subpopulations differ in their requirement for MHC restricted T cell recognition. J. Exp. Med. 154: 501-516, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05065-06 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immunologic Function of Accessory Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. B. Dickler Senior Investigator I NCI

COOPERATING UNITS (if any)

none

LAB/BRANCH Immunology Branch

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0	0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long term goal is to explore the mechanism by which accessory cells process and present antigen to lymphoid cells and how they function in the regulation of the immune response. Using antigen-specific primed lymph node T cell proliferation and the in vitro primary antibody-forming cell response, the population(s) of cells possessing accessory cell function have been characterized: Thy 1.2 negative, surface Ig negative, I-A and I-E/C subregion antigens positive, radioresistant, glass adherent, phagocytic, Fc receptor positive. Accessory cell function required living cells and the efficiency of such function was directly related to the length of time the cells were exposed to antigen. Response to the antigens TNP-(T,G)-A--L and TNP-(H,G)-A--L was controlled by autosomal dominant genes located in the K or I-A regions of the responder H-2 complex and therefore mapped identically to the Ir genes controlling overall in vitro and in vivo responsiveness to these antigens.

Project Description

Objectives: 1) To phenotypically characterize the cell population(s) which possess accessory cell function. 2) To evaluate the role of Ia antigens on accessory cells in terms of the activation of subsets of B and T lymphocytes. 3) To determine the relationship between accessory cell Ia antigens and the expression of Ir genes in these cells. 4) To explore the mechanisms by which accessory cells take up, "process" and present antigen to lymphoid cells.

Methods Employed: 1) Adherent cells are purified from spleen and peritoneal exudate by adherence to glass and removal or inactivation of other cell types by various means including: a) treatment with anti-Thy 1.2 and C', b) irradiation, c) fluorescein-conjugated $F(ab')_2$ anti-Ig or fluorescein-conjugated protein coated polystyrene particles plus fluorescence activated cell sorting. 2) Accessory cells are removed from lymphoid cell populations by passage over Sephadex G10. 3) Ia antigens and other surface markers are detected by immunofluorescence. Ia bearing cells are removed by treatment with alloantisera and C'. The synthesis of Ia antigens is measured by internal labeling with ^{3}H-leucine, followed by solubilization, immunoprecipitation and gel electrophoresis. 4) Fc receptors were evaluated using antigen antibody complexes and indirect immunofluorescence. 5) The immunologic function of accessory cells is assessed by T cell activation as measured by thymidine incorporation in vitro, or by antibody formation by B cells in the in vitro induction of primary antibody-forming cell responses as measured by plaque formation.

Major Findings: T lymphocyte dependent antigen specific immune responses were evaluated using lymphocyte populations which required the addition of exogenous accessory cells in order to phenotypically characterize the cell(s) which provide accessory cell function. This function was provided by a population which was Thy 1.2 negative, radioresistant, glass-adherent, and were only functional if alive. The accessory cell function of spleen adherent cells was proportional to the length of time such cells were incubated with antigen and very small numbers of such cells provided accessory cell function. Depletion of surface Ig positive cells from the adherent population did not affect accessory cell function whereas depletion and reconstitution experiments indicated that phagocytic cells were required. Such cells also bore Fc receptors. Cytotoxic studies with subregion restricted anti-Ia antibodies and complement indicated that accessory cell function resided in a subpopulation of spleen adherent cells which bore the I-A and I-E or C subregion antigens. The function of such cells was not related to a selective ability (vs. other spleen adherent cells) to take up antigen.

The possibility was investigated that Ir genes regulate the function of cells other than T or B cells in the primary IgM PFC responses to the synthetic antigens TNP-(T,G)-A--L and TNP-(H,G)-A--L. The primary PFC responses of (Responder x Nonresponder)F_1 spleen cells to both antigens were abrogated by G-10 Sephadex passage and restored by the addition of spleen adherent

cells which had the characteristics described above. The abrogated responses of G-10 Sephadex passed (Responder x Nonresponder)F_1 spleen cells to each antigen were reconstituted only by spleen adherent cells from stains which were responders to that antigen. All the spleen adherent cells tested restored the non-Ir gene controlled response to a third antigen, TNP-KLH. The ability of spleen adherent cells to function as accessory cells in the primary PFC responses to TNP-(T,G)-A—L and TNP-(H,G)-A--L was shown to be controlled by autosomal dominant genes which were shown by the use of recombinant strains to be located in the K or I-A regions of the responder H-2 complex, the same region(s) of H-2 as the Ir genes controlling overall in vitro and in vivo responsiveness to these antigens.

Significance to Biomedical Research and the Program of the Institute: Since spleen adherent cells function as accessory cells in antigen presentation to T lymphocytes and phenotypically express both Ia antigens and Ir genes, an understanding of the mechanisms underlying accessory cell function in the immune response may lead to new modes of therapy for cancer and other human diseases.

Proposed Course of Project: Due to time commitments to other projects this project has been terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05067-07 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Genetic Control of Human In Vitro Cellular Immune Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Shaw	Senior Investigator	I NCI
Other:	B. Biddison	Expert	NINCDS
	G. Shearer	Senior Investigator	I NCI
	W. Tekolf	Guest Worker	I NCI
	D. Eckels		Georgetown University

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies are continuing on the role of HLA gene products in recognition of foreign antigen by human T cells. Studies in this project include analysis of T cell responses to the hapten TNP and to influenza virus (previously designated project Z01-CB-05078 I). The principal effector mechanism under investigation is cell-mediated cytotoxicity, but proliferation is also used as a measurement of T cell response. Population and family studies of influenza specific CML indicate considerable diversity among serologically indistinguishable molecules, and suggest that individual HLA-A3 molecules may express more than one site for recognition by MHC restricted T cells. The new pair of Bw44-related HLA antigens which have been identified (Z01-CB-05101-02 I) have been analyzed for their ability to function as restriction antigens for influenza-specific CTL. In contrast to studies of other HLA antigens, the "public" antigen Bw44 appears to function more effectively as a restriction antigen than its "private" counterparts 44.1 and 44.2. Preliminary collaborative studies indicate that the SB2 antigen (Z01-CB-05101-02 I) can restrict recognition of influenza specific proliferating T cells.

Project Description

Objectives: The primary objectives of this laboratory are to investigate the function of T lymphocytes, the role of MHC antigens in self recognition, and the mechanism by which these 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. Two model systems have been chosen for current investigations: cytotoxic and proliferative T cell responses to TNP and to influenza virus (previously Z01-CB-05078 I). Both systems have their advantages. The advantages of the TNP system are that there is more flexibility in introducing the antigen on the cell surface, and that since it is not a usual environmental antigen it is possible to analyze responses in unsensitized donors. Responses to influenza virus are stronger presumably due to prior in vivo priming, and have a restriction pattern which is more typical (than TNP) of the MHC restricted recognition characteristic of most murine responses.

Methods Employed: Human PBL are obtained from donors by phlebotomy or batch leukapheresis; mononuclear cells are prepared by density separation, and cryopreserved. For TNP responses stimulator cells are covalently modified with TNP, and cytotoxic effector function is studied after two cycles of in vitro stimulation. For influenza responses, responder cells are exposed to infectious virus, incubated for 7 days and assayed for cytotoxic activity. Cytotoxic activity is analyzed by short term ^{51}Cr release assays using as targets T lymphoblasts or lymphoblastoid B cell lines which have been TNP modified or infected with influenza virus. Analysis of the HLA markers on the donors cells is performed by microcytotoxicity testing under contract NO1-CB-04337.

Major Findings: No major investigations have been performed on TNP recognition in the last year.

Previous studies have indicated that there is greater heterogeneity in HLA class I molecules detected by influenza specific CML recognition than detected by serology. These studies have been extended to recognition of the HLA-A3 antigen. Population and family studies of influenza specific CML indicate considerable diversity among serologically indistinguishable molecules, and suggest that individual HLA-A3 molecules may express more than one site for recognition by MHC restricted T cells.

Further studies on influenza recognition involve two new HLA antigens whose definition is described under Z01 CB-05101-02 I. The two antigens 44.1 and 44.2 appear to be two different forms of the Bw44 molecule which differ in recognition by allogeneic T cells but not in their serologically defined characteristics. Studies were undertaken to determine whether these antigens function as restriction antigens for influenza specific cytotoxic responses. Analysis was difficult since overall responses to Bw44 related determinants in conjunction with influenza is weak; approaches such as cold target blocking in combinations of donors carefully selected for their HLA phenotype now

allow preliminary conclusions that most of the (relatively weak) recognition of Bw44 plus influenza can be attributed to recognition of the public rather than private specificities. This contrasts with the general finding in most MHC-restricted recognition that the most private antigens tend to dominate the self recognition profile.

Collaborative studies have been undertaken with Dr. D. Eckels at Georgetown University regarding the recognition of the SB antigens (Z01 CB-05101-02 I) by influenza specific T cells. Results of population studies of 38 stimulator cells indicate that at least one clone of influenza virus specific proliferating cells appears to recognize viral antigens in conjunction with SB2. This finding, when confirmed, will firmly establish that SB meets all the criteria as a major histocompatibility locus.

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 with 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: The studies of hapten-modified cells and virus-infected cells will be continued in parallel because of the informative similarities and differences in the two systems. The primary thrust in the TNP system will be twofold: 1) To clarify the nature of the unusual diversity of CML restriction antigens in this system. 2) To study the nature of the interaction between antigen and cell surface which is necessary to trigger an immune response; this is facilitated in the TNP system because of the flexibility inherent in chemical modification of the cell surface. With respect to the influenza system, it will continue to be exploited as a biologically relevant probe for the fine specificity of T cell recognition and the patterns of immunodominance of different MHC antigens.

Publications

Biddison, W. E., Shearer, G. M., and Shaw, S.: Influenza virus-specific cytotoxic T cells are restricted by multiple HLA-A3-related self antigens: Evidence for recognition of distinct self structures in conjunction with different foreign antigens. J. Immunol. 127: 2231-2235, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05069-06 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Expression of Ia Antigens on Functional Cell Subpopulations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Hodes	Chief, Immunotherapy Section	I NCI
Other:	P. Nadler	Investigator	I NCI
	G. B. Ahmann		
	R. E. Gress	Investigator	I NCI

COOPERATING UNITS (if any)

University of Iowa, Iowa City, IA

LAB/BRANCH Immunology Branch

SECTION Immunotherapy Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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, 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. These stimulator cells also bear I-A and I-E/C encoded determinants and their ability to stimulate allogeneic T cells could be inhibited with specific anti-Ia reagents.

Project Description

Objectives: Ia antigens are serologically demonstratable 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 the T cell-dependent in vitro primary antibody response to TNP-conjugated proteins are being studied as well as the ability of various subpopulations of spleen to stimulate in an allogeneic mixed lymphocyte response.

Methods Employed: Cell separation and reconstitution techniques are being employed and have established that accessory cells are required for both the Con A proliferative response and the primary antibody response to TNP-KLH. Kupffer cells, the tissue macrophage of the liver were prepared by sequential enzymatic digestion of liver, differential sedimentation, glass adherence and treatment with R MB + C. SAC and Kupffer cells were shown to be potent stimulators of the allogeneic one way mixed lymphocyte response in addition to serving as accessory cells for mitogen and antibody responses. Accessory populations were treated with anti-Ia reagents and complement (C) and assessed for their ability to reconstitute both responses. Accessory cells are also being fractionated into phagocytic and non-phagocytic populations by their ability to ingest fluorescent latex particles as detected by the Fluorescent Activated Cell Sorter and these fractions then evaluated for their ability to restore the Con A response and the in vitro primary antibody response.

Major Findings: (1) It has been shown that the T cell proliferative response to Con A and the T cell dependent primary in vitro antibody response to TNP-KLH require the presence of non-T, radio-resistant glass adherent spleen cells. In addition, cells within this subpopulation of cells (SAC or Kupffer cells) were shown to be potent stimulators of the murine mixed lymphocyte response. (2) Kupffer cells, the tissue macrophage of the liver were shown to be similar to splenic adherent cells (SAC) morphologically, in their expression of cell surface determinants (Ia antigens, Fc receptors and complement receptors) and functionally in the assay systems noted above. (3) Accessory cell function of these Kupffer cells and splenic adherent cells (SAC) is abrogated by treatment with anti-Ia and C. Using subregion specific anti-Ia reagents, it was shown that these cells expressed Ia determinants encoded by genes in I-A and I-E/C. In addition, these determinants are expressed on the same cells. (4) The reconstituting SAC in the Con A response were effective whether or not they were H-2 identical with the responding T cells. (5) Cells within both the SAC and Kupffer cell population were shown to be potent stimulators of the mixed lymphocyte response (MLR). The cells stimulating the MLR also bore determinants encoded in both I-A and I-E/C. Cell separation techniques showed that splenic B and T cells were poor stimulators of MLR when stimulators and responders differ at either H-2 or Mls. Depletion of splenic accessory cells by passage over G-10 Sephadex resulted in diminution of stimulatory ability in MLR whereas enrichment for accessory cells by adherence, irradiation and R MB + C treatment resulted in a population significantly more stimulatory than unfractionated spleen.

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, Ia antigens, in mitogen responses, and in antibody production should provide insight into the mechanisms of control of these immune responses. Such insight would be relevant to evaluation of the host response to allograft and tumor challenge.

Proposed Course of Project: Studies are in progress to further define the properties of the Ia^+ accessory cells and their mechanism of action in antibody responses under Ir gene control. Studies are planned to further evaluate the functional and/or structural significance of the finding that B cells which bear Ia determinants are not capable of triggering proliferation of allogeneic T cells whereas SAC bearing determinants which are serologically identical are potent stimulators of MLR.

Publications

Ahmann, G. B., Nadler, P. I., Birnkrant, A., and Hodes. R. J. T cell recognition in the mixed lymphocyte response. II. Ia-positive splenic adherent cells are required for non-I region-induced stimulation. J. Immunol. 127: 2308-2313, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05083-04 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Genome Organization of Murine Major Histocompatibility Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. Singer	Senior Staff Fellow	I NCI
Other:	D. H. Sachs	Chief, Transplantation Biology Section	I NCI
	S. Rudikoff	Senior Investigator	LCB NCI
	M. S. Satz	Visiting Fellow	I NCI
	R. Lifshitz	Visiting Fellow	I NCI
	L. Abelson	Biologist	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	5.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES x ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The aim of this work is to determine the DNA sequence organization of genes encoding the major histocompatibility complex (MHC) and the mechanisms controlling the expression of these genes. It has been demonstrated that there are 10-15 MHC genes in the swine genome and 20-30 in the mouse genome. A series of genomic clones containing MHC-homologous DNA sequences has been isolated. One of these clones (PD1) has been studied in detail, both with respect to its DNA sequence organization and regulation of expression. The organization of MHC DNA sequences in chromatin is also under investigation.

Project Description

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 and mouse genomes contain 10-15 and 20-30 MHC genes, respectively. A porcine genomic clone encoding a major histocompatibility (MHC) antigen was isolated by direct screening of a swine genomic library using a heterologous human MHC cDNA probe. Mouse L cells transformed with DNA from the clone expressed 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. It has been demonstrated that swine DNA sequences are present in the mouse transformants at a level of 1-2 copies per haploid genome. These sequences are organized into chromatin in a manner indistinguishable from that of endogenous murine DNA sequences. Further, the MHC-coding sequences are hypersensitive to DNaseI digestion.

Further characterization of the MHC-linked but non-coding sequences has revealed that the MHC gene is imbedded in a clustered array of distinct repetitive DNA sequences. The reiteration frequencies of each of these repetitive elements has been determined, as has their expression into splenic RNA.

A series of independent genomic clones containing MHC-homologous sequences has recently been isolated by screening of a porcine genome library with a DNA probe from the already isolated MHC-coding sequence subclone. These clones are currently being purified and characterized by direct DNA sequence analysis and DNA mediated transformation of L cells. It has already been found that the repetitive sequence elements isolated in the first MHC clone are non-randomly associated with all other isolated MHC clones.

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 isolated MHC genomic clones by the following lines of investigation:

(1) Genomic clones representing all of the porcine MHC genes will be isolated.

(2) These clones will be analyzed to determine which MHC products they encode.

(3) The DNA sequences of the MHC coding regions will be established.

(4) The nature of the MHC linked sequences in each of the clones will be determined with respect to their sequence families and states of expression.

(5) The relationship between the organization of MHC sequences into chromatin and their 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.

SMITHSONIAN SCIENCE INFORMATION E HANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05085-04 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Development of Syngeneic Tumor Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: G. M. Shearer Senior Investigator I NCI
Other: J. Hochman*

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:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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

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 tumorogenic (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 immunogeneic and tumorogenic lines of other murine T cell tumors can be developed.

Methods Employed: BALB/c mice were injected ip with $10\text{-}30\text{x}10^6$ TAS or 7.3 tumor cells. Mice protected by injection with 7.3 were challenged with $10\text{-}30\text{x}10^6$ 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 tumorgenic 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 T cell lymphomas are available to this laboratory for such studies.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 05086-04 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immune Response Gene Regulation of the Immune Response In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Hodes	Chief, Immunotherapy Section	I NCI
Other:	A. Singer	Senior Investigator	I NCI
	D. H. Sachs	Chief, Transplantation Biology Section	I NCI
	Y. Asano	Visiting Fellow	I NCI
	P. I. Nadler	Investigator	I NCI
	A. Finnegan	Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION Immunotherapy Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

Project Description

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: Background work has demonstrated that primary or secondary in vitro antibody responses could be generated to a number of soluble TNP conjugates of protein or polypeptide antigens. These responses are both T cell-dependent and accessory cell-dependent. The in vitro primary and secondary 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 $H\text{-}2^a$ haplotype being responders and $H\text{-}2^b$ 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 $I\text{-}A^k$ reagent have demonstrated that this reagent is capable of inhibiting the response of $(H\text{-}2^a \times H\text{-}2^b)F_1$ 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.

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.

Publications

Nadler, P. I., Miller, G. P., Sachs, D. H., and Hodes, R. J.: 1981. Ir gene control of in vitro antibody responses to TNP-nucelase. J. Immunol. 126: 1706-1712.

Singer, A., Hathcock, K. S., and Hodes, R. J.: 1981. Self-recognition in allogeneic chimeras. A radiation-resistant host element dictates the self specificity and immune response gene phenotype of T-helper cells. J. Exp. Med. 153: 1286-1301.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05087-04 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
T Cell Recognition in the Mixed Lymphocyte Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Hodes	Chief, Immunotherapy Section	I NCI
	R. Gress	Clinical Associate	I NCI
	P. Nadler	Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ability of subpopulations of murine spleen cells to stimulate a mixed-lymphocyte response (MLR) was studied. It was found that T cells and B cells were poor stimulators of an MLR across H-2 or Mls differences, while non-T, radiation resistant Ia positive splenic adherent cell (SAC) were 20-50 times more efficient as stimulators of these MLR's than unseparated spleen cells. These results suggest that Ia^+SAC may be the predominant stimulating cells in spleen cell populations, and the preferential target for T cell recognition in cell interaction events. It has further been demonstrated that T cells responding to Mls determinants recognize such determinants in the context of "self" H-2 determinants. Studies employing K region mutants have investiated the influence of chimeric maturation environment upon the alloreactive T cell repertoire to mutant determinants, and suggest that this alloreactive repertoire is in fact influenced by the environment in which T cells mature.

Project Description

Objectives: Since the MLR is a model for T cell recognition of cell surface determinants, the objective of these studies is to characterize T cell recognition of allogeneic determinants.

Methods Employed: Purified T cells (nylon non-adherent spleen cells) purified B cells (G10 Sephadex passed and RAMB + C treated) and splenic adherent cells (glass adherent, non-T radiation resistant spleen cells) (SAC) were prepared and compared for their ability to stimulate a proliferative response by allogenic whole spleen cells. Stimulator SAC populations are further evaluated by 1) treatment with anti-Ia reagents and C, 2) addition of monoclonal anti-Ia and anti-H-2 antibodies directly to the culture, and 3) fractionation of SAC on the basis of their ability to phagocytise latex particles. In vitro generation of cytotoxic T lymphocytes (CTL) was carried out by MLR cultures.

Major Findings: A non-T radiation resistant spleen adherent cell population (SAC) was up to 20-50 times more efficient in stimulating MLR on a per cell basis than an unseparated spleen cell population; and these SAC express Ia determinants encoded by genes in I-A and I-E/C. These findings were observed both for MLR to H-2-differences and for MLR to Mls stimulating determinants.

Mls encoded determinants appear to be unique in that they are the only non-MHC determinants capable of stimulating primary MLR. Studies were undertaken to determine whether responding T cells recognize Mls product alone, or recognize Mls in the context of H-2. Limiting dilution conditions were established under which the magnitude of MLR proliferative response to Mls determinants was proportional to the number of responding T cells. Experiments carried out under such conditions demonstrated that T cells do not respond to Mls determinants alone, but rather that distinct T cell subpopulations exist which recognize Mls in the context of "self" H-2 determinants.

CTL were generated by the response of B6 ($H\text{-}2^b$) spleen cells against stimulating cells differeing from B6 only by point mutations in the K^b region. These responses were compared with the responses of $H\text{-}2^b$ --> $H\text{-}2^d$ or $H\text{-}2^d$ --> $H\text{-}2^b$ chimeras to K^b mutant cells or entirely allogeneic stimulators.

The responses to selected K^b mutants were strictly determined by the environment in which responding T cells had matured, so that normal $H\text{-}2^b$ or $H\text{-}2^d$ --> $H\text{-}2^b$ chimeric cells generated strong CTL responses, while $H\text{-}2^b$ --> $H\text{-}2^d$ responding cells were selectively unresponsive.

Significance to Biomedical Research and the Program of the Institute: The mixed lymphocyte response provides a useful model for T cell recognition as well as an in vitro correlate of allograft rejection. Further understanding of the recognition process as well as the primary stimulator cell should provide insight into controlling or preventing allograft and/or tumor challenges.

Proposed Course of Project: Specific anti-H-2 and anti-Ia reagents purified from hybridoma cell lines are being evaluated for their ability to block MLR responses to either H-2 or Mls differences. In addition, further work is in progress to study the mechanism by which host environment determines the alloreactive T cell repertoire.

Publications

Gress, R. E., M. N. Wesley, and R. J. Hodes: 1981. The role of H-2 in T cell recognition of Mls. J. Immunol. 127: 1763-1766.

Ahmann, G. B., P. I. Nadler, A. Birnkraut, and R. J. Hodes: 1981. T cell recognition in the mixed lymphocyte response. II. Ia positive splenic adherent cells are required for non-I-region induced stimulation. J. Immunol. 127: 2308-2313.

Gress, R. E. and R. J. Hodes. Generation of the alloreactive T cell repertoire: interaction of T cell genotype and maturation environment. Proc. Natl. Acad. of Science in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05088-04 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effects of Graft Vs. Host Reactions on Cell-Mediated Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
Other:	U. Hurtenbach	Visiting Associate	I NCI
	J. Chalmer	Visiting Fellow	I NCI
	T. Tsuchida	Visiting Fellow	I NCI
	D. H. Sachs	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES x☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The intravenous injection of F_1 hybrid mice with parental spleen cells resulted in a loss in the ability of the F_1 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 $H\text{-}2^{k,a}$ spleen cells induced unresponsiveness, whereas $H\text{-}2^{b}$ spleen cells did not. The phenomenon is dependent on recognition of F_1 I-A alloantigens by grafted parental cells (GVH), since loss of immune activity was associated with enlarged F_1 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 F_1 resistance to parental T cells, which mapped to $H\text{-}2D^{b}$. 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.

Project Description

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 or neoplastic states following a graft vs. host reaction.

Methods Employed: F_1 hybrid mice of various strain were injected intravenously with from 1 to $40x10^6$ F_1, parental, or allogeneic spleen 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 alloantigens. 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. The abolition of cytotoxic potential: (a) appeared to depend 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 latter observation indicated that the B10 parental cells injected were rejected by the F_1 anti-parent reaction known as hybrid histoincompatibility (Hh). Genetic studies indicate that the $H\text{-}2^b$ homozygous determinant recognized by the F_1 maps to the H-2D region, which is compatible with an Hh-like phenomenon. 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.

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 medranistic relevance for these SCID's.

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) whether certain combinations of partially allogeneic (instead of F_1 and parental) cells and hosts can lead to GVH-associated immunosuppression; (g) whether the GVH reaction is actually a component of the impaired immune state; (h) whether haplotype-specific anti-T-cell receptor suppression can be induced in the F_1 parent combination; and (i) analysis of different populations of cells involved in the induction of and protection by antibody against GVH-associated immunosuppression.

Publications

Shearer, G. M. and Polisson, R. P.: Mutual recognition of parental and F_1 lymphocytes. III. Parental determinants recognized by F_1 host mice in resistance to graft-versus-host-associated immunosuppression map to H-2D^b. J. Immunol. 126: 545-547, 1981.

Hurtenbach, U., Sachs, D. H. and Shearer, G. M.: Protection against graft versus host associated immunosuppression in F_1 mice. I. Activation of F_1 regulatory cells by host specific anti-MHC antibodies. J. Exp. Med. 154: 1922-1935, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05090-04 I

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Role of Accessory Cells in B Cell Activation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Ono	Visiting Fellow	I NCI
Other:	P. J. Morrissey	Staff Fellow	I NCI
	A. Singer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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

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 clutured 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

Morrissey, P. J., Boswell, H. S., Scher, I., and Singer, A.: Role of accessory cells in B cell activation. IV. Ia+ accessory cells are required for the in vitro generation of thymic independent type 2 antibody responses to polysaccharide antigens. J. Immunol. 127: 1345-1347, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05091-04 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Target Antigen Recognition by Cytotoxic Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. A. Henkart	Senior Investigator	I NCI
	J. T. Lewis	Postdoctoral Fellow	I NCI
	J. Ortaldo		LID NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER x

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to determine the molecular nature of NK cell recognition of target cells, K562, plasma membranes were prepared from the human NK cell target plasma membrane proteins were solubilized with the detergent CHAPS, and artificial membranes bearing the membrane protein formed from exogeneous lipids after detergent removal by dialysis. These artificial membranes were found to be capable of inhibiting "congugates", i.e., killer-target pairs identifiable in the microscope, between human large-granular lymphocytes and K562 cells. The specificity of this inhibition reflects the specificity of NK killing. Solubilized membrane proteins were subjected to various treatments and fractionation procedures. It was found that the active material was heat sensitive, Typsin sensitive and could be bound to and eluted from a ConA Sephamore column.

Project Description

Objectives: To understand the molecular mechanism involved in the binding of cytotoxic lymphocytes to the target cells they specifically recognize. In the past year we have concentrated on natural killer,or NK cells whose receptor is unknown. These cells recognize and kill certain tumor cells without any immunization. We currently want to determine the nature of the target cell moiety recognized by the NK cell.

Methods Employed: Plasma membranes were prepared from the K562 mycloid leukemia cell line, which is generally regarded as the best human NK target. (The technique utilized homogenization with a polytron homogeniges followed by sedimentation on a step sucrose gradient in the ultracentrifuge.) The plasma membranes were treated with the zwitterionic cholate detergent CHAPS, to solubilize membrane proteins. These solubilized membrane proteins were then subjected to various treatments and fractionation procedures and subsequently reinserted into membranes by adding synthetic lipids to the solubilized membrane protein and dialyzing out the detergent. The resulting "reconstituted" membranes contain protein. NK-target recognition was measured by microscopically counting NK-K562 conjugates, utilizing Percoll purified human peripheral lymphocyte NK cells. The ability of the reconstituted membranes to inhibit conjugate formation was seen as a decrease in the presentage of NK cells forming conjugates.

Major Findings: Artifical membranes containing synthetic lipid plus membrane proteins extracted from the NK target K562 inhibit conjugate formation by human NK cells. The species specificity of NK killing is reflected in the ability of these artificial membranes to inhibit conjugates between human NK cells and their targets but not rat NK cells and their targets; on the other hand, artificial membranes from rat NK target cells inhibit rat NK conjugate formation but not human NK conjugate formation. Experiments with the CHAPS solubilized extract showed that the inhibitory material is trypsin digestable, and heat sensitive, this implicating a protein as the active material. The inhibitory activity is bound to ConA Sephamore and dutied with -methylmannoside, indicating the presence of sugar groups on the active protein. Subsequent gel filtration indicates that the active glycoprotein has a molecular weight on the order of 50,000.

Significance to Biomedical Research and the Program of the Institute: There is much interest among immunologists in the in vivo role of NK cells in immune surveilance against tumors. The whole phenomenon of NK killing has been plagued by the ill-defined specificity of the recognition event by which the NK cell recognizes its target cell. By putting this on a molecular basis we hope to get a more realistic idea of the possible in vivo relevance of NK cells as well as gain a better basic understanding of an interesting immunological process.

Proposed Course of the Project: Further purification of the solubilized material from K562 cells which inhibits conjugate formation will be attempted.

Publications

Levy, R.B., Shearer, G.M., Richardson, J.C., and Henkart, P.A.: Cell mediated lympholytic responses against autologous cells modified with haptenic sulfhydryl reagents. I. Effector cells can recognize two distinct classes of hapten-reactive self sites on cell surface proteins. J. Immunol. 117: 523-528, 1981.

Levy, R.B., Henkart, P.A., and Shearer, G.M.: Cell mediated lympholytic responses against autologous cells modified with haptenic sulfhydryl reagents. II. Analysis of the genetic control of cytotoxic responses to sulfhydryl and amino reactive reagents. J. Immunol. 127: 529-534, 1981.

Levy, R.B., Richardson, J.C., Cudkowicz, E., Henkart, P.A., and Shearer, G.M.: Cell mediated lympholytic responses against autologous cells modified with haptenic sulfhydryl reagents. III. Different regions of hapten-membrane protein conjugates influence CTL specificity and IR gene control of hapten-self CTL responses. J. Immunol. 27: 2218-2223, 1981.

Pehamberger, H., Levy, R.B., Henkart, P., and Katz, S.I.: Induction of cell-mediated cytotoxic self responses by epidermal cells modified with a haptenic sulfhydryl reagent. Scand. J. Immunol., 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05093-03 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Environmental Influences on Self-Tolerance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Morrissey	Staff Fellow	I NCI
Other:	A. Singer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NIC, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The influence of the prethymic compartment on the acquisition of tolerance to foreign antigens was evaluated by varying the foreign antigens to which precursor T cells are exposed in their pre-thymic environment. The experimental model consists of a host mouse which received a thymus graft followed by lethal radiation and reconstitution with bone marrow cells of the same MHC haplotype as the thymus graft. Thus, bone marrow derived stem cells are maturing in an MHC syngeneic thymus but can be exposed to novel MHC and non-MHC antigens pre-thymically by varying the genetic origin of the host mouse. The thymocytes from such experimental mice are then assayed for their reactivity against the foreign antigens which they encountered prior to their entry into the thymus. It was found that thymocytes are tolerant to foreign MHC determinants encountered pre-thymically, but appear not to be tolerant to foreign non-MHC determinants encountered pre-thymically. Thus, these results suggest that thymocytes pre-thymically express receptors specific for MHC determinants but may not pre-thymically express receptors specific for non-MHC determinants.

Project Description

Objective: These experiments will study the induction of tolerance in precursor 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. 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 receptors specific for MHC antigens and 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 and will also assess the ability of the intrathymic and postthymic T cell compartments to induce tolerance.

Publications

Morrissey, P. J., Kruisbeek, A. M., Sharrow, S. O., and Singer, A.: Tolerance of thymic cytotoxic T lymphocytes to allogeneic H-2 determinants encountered prethymically: Evidence for expression of anti-H-2 receptors prior to entry into the thymus. Proc. Natl. Acad. of Sci. (USA) 79: 2003-2007, 1982.

Bradley, S. M., Morrissey, P. J., Sharrow, S. O., and Singer, A.: Tolerance of thymocytes to allogeneic I region determinants encountered prethymically. Evidence for expression of anti-Ia receptors by T cell precursors before their entry into the thymus. J. Exp. Med. 155: 1638-1652, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05094-03 I

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Role of the Thymus in Generation of the Self-MHC Specific T cell Repertoire

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Kruisbeek	Visiting Associate	I NCI
Other:	A. Singer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to assess the role of the thymus in the acquisition of the T cell self-MHC specific repertoire, the MHC specificity of cytotoxic precursor T cells differentiating with the thymii and spleens of chimeric and thymus-engrafted nude mice was determined. The results demonstrated that cytotoxic T lymphocytes resident within the thymii of these experimental animals recognize TNP only in association with thymic MHC determinants. In contrast, cytotoxic T lymphocytes from the spleens of these same mice recognize TNP on both thymic and non-thymic host type MHC determinants. These results are consistent with the thymus being one site which determines the specificity of the T cell repertoire, but suggests that peripheral sites also play a critical role. Specifically, T cells specific for self-I are solely educated intra-thymically whereas T cells specific for self-K/D are educated both intra-thymically and extra-thymically.

Project Description

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 cells 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.

Major Findings: In both radiation bone marrow chimeras and thymus-engrafted nude mice, thymocyte T killer cells display recognition of TNP in association with thymic MHC determinants only. These results indicate that the recognition pattern observed from T cells resident within the thymus is determined by the MHC phenotype of thymic elements. Such a thymus determined T cell repertoire is also observed in the spleens of such mice. However, in the spleens a T cell repertoire specific for non-thymic K/D determinants is also found, a repertoire which was not observed intra-thymically in the same mice. Thus, whereas, I region specific T cells can only differentiate and be educated intra-thymically, these results suggest that K/D region specific T cells can differentiate and be educated both intra-thymically and extra-thymically.

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

Kruisbeek, A. M., Hodes, R. J., and Singer, A.: Cytotoxic T lymphocyte responses by chimeric thymocytes: Self-recognition is determined early in T cell development. J. Exp. Med. 153: 13-29, 1981.

Kruisbeek A. M., Sharrow, S. O., Mathieson, B. J., and Singer, A.: The H-2 phenotype of the thymus dictates the self-specificity expressed by thymic but not splenic cytotoxic T lymphocyte precursors in thymus-engrafted nude mice. J. Immunol. 127: 2168-2176, 1981.

Singer, A., Bradley, S. M., and Kruisbeek, A. M.: Role of the thymus in dictating the self-repertoire of T cells which differentiate within it: Evidence for both an intra-thymic and an extra-thymic differentiation pathway for T cells restricted to KD, but not for T cells restricted to I. Behring Inst. Mitt. 70: 140-146, 1982.

Kruisbeek, A. M., Hathcock, K. S., Hodes, R. J., and Singer, A.: T cells from fully allogeneic (A->B) radiation bone marrow chimeras are functionally competent and host restricted, but are alloreactive against hybrid Ia determinants expressed on (AxB)F_1 cells. J. Exp. Med. 155: 1864-1869, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05095-03 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Regulation of Cell-Mediated Immunity by Germ Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
Other:	U. Hurtenbach	Visiting Associate	I NCI
	W. E. Biddison	Investigator	I NINCDS

COOPERATING UNITS (if any)
Laboratory of Immunology
NINCDS

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER x

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 siminiferous 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

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: Sequential protease treatment of murine testes released two fractions of cells: a population consisting of interstitial cells; and a second population which is derived from the seminiferous tubules consisting of spermatozoa. 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. Lymphocyte reactivity was assayed by measuring the proliferative response by ^{3}H thymidine incorporation and by determining cytotoxic activity on ^{51}Cr-labelled PHA blast cells.

Major Findings: The two testicular cell populations showed different effects by lymphocyte proliferation in vitro: lymphocyte reactivity was suppressed against the syngeneic germ cell population itself as well as against autologous or allogeneic interstitial cells or against allogeneic spleen cells when germ cells were present during the sensitization phase. In contrast, the testicular cells consisting of an enriched population of interstitial cells stimulated lymphocyte proliferation. The reactive lymphocytes were T cells; suppression could be abrogated by treatment of the responder cells with anti-Ly 2.2 sera, plus complement. Lymphocyte proliferation was significantly reduced by anti Thy 1.2 plus complement treatment. Similar suppressive effects of syngeneic germ cells have been observed on the generation of cytotoxic T lymphocytes in vitro. In the presence of spermatozoa from the seminiferous tubules the reactivity against modified self or alloantigen was reduced, whereas interstitial testicular cells had no significant effect. No difference has been found in responder spleen cells between male and female mice.

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 Kaposis' 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: Experiments will be performed to investigate further the mechanism leading to the gene cell-induced suppression of the CTL response. The antigenic determinants on the germ cells responsible for the induction will be investigated using monoclonal reagents. It will be tested (a) whether antibodies directed against specific surface structures bind to germ cells and (b) whether induction of suppression can be prevented by preinjection of the specifc monoclonal reagents. In addition, variation of antigen expression will be studied, since induction of suppression seemed to be related to the age of the germ cell donors. The target cell of the germ cell within the lymphoid cell population will be determined using physical or serological separation methods and consecutively their suppressive ability on normal spleen cells in co-cultivation experiments will be tested. Recombinant mouse strains will be used to determine whether there is genetic restriction at the level of the germ cells for induction of suppression and/or at the level of the interaction of the suppressor cells with the target lymphoid cell. Studies will continue in the murine model to elucidate the events leading to suppression, as well as the mechanism of suppression, including possible synergistic effects between sperm and cytomegalovirus, and sperm and nitrite drugs frequently used by homosexuals. Peripheral blood leukocytes (PBL) from male homosexuals not known to have immunological disorders will be tested for their CTL potential against influenza virus, Epstein-Barr virus and alloantigens. If some of these donors exhibit reduced response potential, functional analysis will be made of CTL precursors, helper, suppressor and accessory cell populations to determine the defect(s). Also, the immune potential of healthy homosexuals will be followed with time to determine whether they develop reduced immune potential.

Publications

Hurtenbach, U. and Shearer, G. M.: Germ cell-induced immune suppression in mice. Effect of inoculation of syngeneic spermatozoa on cell-mediated immune responses. J. Exptl. Med. in press (June, 1982).

Shearer, G. M. and Hurtenbach, U.: Is sperm immunosuppressive in male homosexuals and vastectomized men? Immunology Today in press (June, 1982).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05096-03 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Identification and Function of Intracellular Calcium-Containing Organelles

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. P. Henkart	Expert	I NCI
Others:	C. E. Fiore	Physical Scientist	BEIB, DRS
	P. J. Millard	Biologist	I NCI
	R. W. Tucker	Asst. Professor	Dept Oncology Johns Hopkins Med Sch, Balt.

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 21205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 form 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. This study thus far has been devoted to development and testing of the methods.

Project Description

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) In previous studies (in collaboration with Dr. T. S. Reese of NINCDS) cells were rapidly frozen and substituted with acetone 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 using the analytic electron microscope facility being developed by BEIB in DRS.

A rapid freezing device (Polaron "Slammer") has now been obtained. Various methods of tissue handling prior to freezing, variations of the freezing technique itself (different specimen stages, different shock-absorbing substances, liquid N_2 vs liquid He as coolants), and variations of freeze substitution protocols are being tested.

Major Findings: In previous studies related to this problem I have shown that the endoplasmic reticulum of neurons is a calcium-sequestering compartment. In macrophages areas of both smooth and rough endoplasmic reticulum also contain calcium. The ER of macrophages 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". Other organelles also contain calcium. One general class includes organelles whose membranes circulate through the surface membrane via endocytosis and exocytosis. This class includes pinosomes and phagosomes, lysosomal structures and secretory granules. Some cisterns and vesicles in the vicinity of the Golgi apparatus also contain dense deposits, but these are vey small structures and it has not yet been possible to identify them in the images thus far obtained under conditions for x-ray analysis. Calcium has also been identified in the periphery of lipid droplets and in the space between the inner and outer mitochondrial membranes, but rarely is found in mitochondrial matrices.

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: Plans for this project still involve refinements of the techniques and determination of their limitations. We have just begun a study of the distribution of calcium in BALB/c 3T3 cells as a function of their growth status. Initial plans are to compare 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.

For identification of Ca containing compartments in cells we plan to use (in addition to energy dispersive x-ray microanalysis) the technique of electron energy loss spectroscopy now being developed in the BEIB facility. This approach should be more sensitive and may have other advantages over x-ray analysis.

Publications

Henkart, M.: Identification and function of intracellular calcium stores in neurons. Introduction to Symposium. Fed. Proc. 39:2776-2777, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05098-02 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Non-H-2-Linked Genetic Control of Cell-Mediated Cytotoxic Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
	P. K. Arora	Visiting Fellow	I NCI
	H. Fujiwara	Visiting Associate	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Spleen cells from mice of different inbred strains sharing the same H-2 haplotype 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 histiocompatibility complex (MHC) genes in regulating CTL responses to foreign antigens recognized in association with self MHC gene products.

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 ^{51}Cr-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 vise 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 $H\text{-}2^d$ strains--Balb/C, high responder; DBA/2 and B10.D2, low responders; among $H\text{-}2^K$ strains--C3H, high responder; AKR/J, intermediate responder; B10.BR, low responder; among $H\text{-}2^b$ 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, F_1 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.

Publications

Fujiwara, H., and Shearer, G. M.: Non-H-2-associated genetic regulation of cytotoxic responses to hapten-modified syngeneic cells: Effect on the magnitude of secondary response and helper T cell generation after in vivo priming. Eur. J. Immunol. 11: 700-704, 1981.

Arora, P. K., and Shearer, G. M.: Non-MCH-associated genetic control of immune cell-mediated lympholysis to trinitrophenyl-modified syngeneic cells. J. Immunol. 127: 1822-1828, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-05099-02 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Immunogenetic Effects of Murine Cytomegalovirus on Induced and Natural Immunity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	G. M. Shearer	Senior Investigator	I NCI
OTHER:	J. Chalmer	Visiting Fellow	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES x ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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, and then to a hightened state of immune potential detected by the hapten-self CTL only. The 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).

Project Description

Objectives: The purpose of this project is to investigate the immunological and genetic effects of MCMV infection and of MCMV infection plus a GVHR on (a) acquired T cell immunity to hapten-self antigens and alloantigens; 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 GVHR.

Methods Employed: Sublethal doses of MCMV (prepared from salivary glands of infected mice) were injected intraperitoneally into various inbred and F_1 hybrid mouse strains. Also F_1 mice were injected intraveneously with known concentrations of parental spleen cells, and F_1 mice were also injected with MCMV plus parental cells. 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 ^{51}Cr-release assay.

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 allo-geneic CTL systems (days 9-13). The injection of F_1 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. However, it was also found that in those F_1 and parental combinations in which natural resistance to GVH was observed, there was a synergistic effect of resistance to GVH when CMV was given at the same time.

Significance to Biomedical Research and the Program of the Institute: Cyto-megalovirus 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 paramaters 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.

Proposed Course of Project: A number of inbred, recombinant and F_1 hybrid mouse strains will be studied for their ability to be resistant or suscep-tible to immunosuppression rsulting from MCMV infection. We shall also investigate the genetic and mechanistic aspects associated with the syner-gistic effects of MCMV and the GVHR on immunosuppression.

Publications:

Shearer, G. M.: Resistance to murine cytomegalovirus infection: H-2 linked genetic regulation of an immunopathological condition? Immunol. Today 2: 60, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05100-02

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
The Role of HLA Genes in Human Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. Shaw	Senior Investigator	I NCI
Other:	R. Hall	Clinical Associate	Derm NCI
	S. Katz	Chief, Dermatology Br	Derm NCI
	W. Tekolf	Guest Worker	I NCI
	P. Rubinstein	New York Blood Center	
	L. Ercolani	Veterans Administration Hosp, Iowa	

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have recently defined a new HLA locus (SB) which maps centromeric to the other new genes of the HLA complex. During this year studies have included the importance of the genetic region marked by this gene in three different human diseases: dermatitis herpetiformis, juvenile onset diabetes, and kidney transplantation rejection. Previous studies of patients with dermatitis herpetiformis have been extended particularly with respect to family analysis. The results indicate that the positive association of DH with DR3 and the negative association with SB2 are most consistent with the hypothesis that risk is conferred by at least one as yet unknown HLA gene which occurs generally on HLA haplotypes which encode DR3 but encodes SB alleles other than SB2. Collaborative studies have been undertaken to use SB as an informative marker to map more precisely the susceptibility gene for juvenile onset diabetes; initial studies suggest that there is no SB antigen characteristic of "diabetic" haplotypes. The prediction has been made that the SB locus will function as a barrier to allogeneic tissue transplantation. "HLA-identical" donor/recipient pairs in which kidney transplantation has failed are being collected to test this prediction.

Project Description

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 Z01-CB-05101 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 Z01-CB-05101 I; they are also serotyped for other HLA antigens under contract N01-CB-04337.

Major Findings: Among the 43 patients studied with classic dermatitis herpetiformis, the SB as well as DR phenotypes were found to be associated with alteration of risk. The presence of DR 3 conferred greatest risk; however, among individuals with DR3, the risk of DH was increased about 2-3 fold by having the SB1 antigen but decreased about 5 fold by having the SB2 antigen. Several hypotheses were advanced to explain the modifying effect of SB phenotype; family studies are beginning to distinguish among these hypotheses. Families have been studied of the three atypical patients with both DR3 and SB2, the SB2 and DR3 antigens segregate on opposite haplotypes in all three families. These data tend to support the hypothesis that the SB2 marker helps to distinguish statistically among DR3 haplotypes as to which carry are likely to carry as an yet unknown gene which confers risk for dermatitis herpetiformis.

Collaborative studies have been undertaken to use SB as an informative marker to map more precisely the susceptibility gene for juvenile onset diabetes, since that gene has been postulated to map centromeric to DR (the genetic region for which SB would be an important new marker). Initial studies of six families suggest that there is no SB antigen characteristic of "diabetic" haplotypes.

We have predicted that the SB locus will function as a barrier 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 occured may have an increased frequency of SB/DR recombination. "HLA identical" donor/recipient pairs in which kidney transplantation has failed are being collected to test this prediction.

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 transplantation.

Proposed Course of Project: If resources are available, further studies will be performed in DH patients and families to distinguish statistically among the hypotheses to explain the current data.

Twenty to thirty JOD families will be studied, particularly those with known DR/GLO recombination involving one of two affected HLA-identical siblings.

At least five pairs of "HLA-identical" recipient/donor kidney transplantation pairs s/p rejection will be tested to determine if any have detectable SB/DR recombination.

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.

Publications

Shaw, S., and Shearer, G. M.: Cytotoxic T cell interactions with antigen: potential relevance for drug-related systemic lupus erythematosus. Arthritis and Rheumatism. 24: 1037-1042, 1981.

Kaslow, R. A., and Shaw, S.: The Role of HLA in infection: A review and perspective. Epidemiologic Reviews. 3: 90-114, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05101-02 I

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Definition of Gene Products of the Human Major Hostocompatibility Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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LAB/BRANCH

Immunology Branch

SECTION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3 (NIH)	1.6 (NIH)	0.7 (NIH)

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using secondary lymphocyte proliferative responses and secondary cell-mediated cytotoxicity we have continued to probe the complexities of the alloantigens encoded in the HLA region. The discovery of a new gene "SB" in our laboratory has fostered a large number of collaborations and rapid progress has been made in defining the genetics, structure and function of the SB antigens. A monoclonal antibody and alloantisera have been identified which react with the gene product. Initial structural studies indicate that the SB molecule has the structural characteristics typical of Ia-like antigens, and has amino acid sequence homology to HLA-DR (and H-21E). The genetics of this locus has been explored in a number of populations in USA, Europe and the Orient. Furthermore, T cell cloning has facilitated definition of these antigens. Analysis of other complexities of HLA markers has allowed the definition of two antigens intimately associated with Bw44. These antigens are of particular interest since they shed new light on linkage disequilibrium of the Bw44-related antigens, and they may have important biological relevance since they appear to regulate the level of expression of the Bw44 antigen on platelets.

Project Description

Objectives: Genes of the HLA region are important in tissue transplantation, immune regulation and disease association. 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 intricacies 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. Once appropriate tools have been 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.

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 No1-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 ^{3}H-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.

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. Considerable effort has been expended in screening monoclonal antibodies from other laboratories for their ability to interact with the SB gene product. Among about 25 antibodies tested one, ILR1, appears to react with some SB molecules. Three lines of evidence indicate that the ILR1 molecule identifies an epitope on some alleles of the SB gene: 1) The polymorphism of ILR1-reactivity in the population correlates with SB2, SB3; 2) T-cell proliferative response to SB2 and SB3 are specifically inhibited by ILR1; and 3) ILR1-reactivity is exactly concordant with the expression of SB2 in a panel of HLA-deletion mutant lymphoblastoid cell lines. Together with previous studies, these results indicate that the SB antigens are on Ia-like molecules. Furthermore, the serologic studies of HLA-deletion mutant cell lines demonstrate that there are two HLA regions contromeric to HLA-B which control expression of Ia-like molecules: a region toward HLA-B which controls expression of HLA-DR, and a region toward GLO which controls expression of SB. It is of particular interest that the ILR1 antibody also appears to bind to one allele of another gene product, DR5. The sharing of such an epitope between DR and SB would appear to reflect extensive homology between these two distinct

gene products. Another study of monoclonal antibody inhibition of proliferation also suggests that SB and DR share serologically defined determinants. In that study, some monoclonal antibodies specific for monomorphic epitopes on human Ia molecules were able to inhibit proliferation of T cells specific for all HLA-DR and SB determinants tested. Data from these two studies are consistent with the hypothesis proposed previously that SB and DR genes were derived from duplication of an ancestral gene(s). Although ILR1 is not exclusively SB-specific, the data indicate that ILR1 can be used to identify SB molecules in informative donors -- i.e., who are positive for SB2 or SB3 but negative for DR5. Previous immunochemical studies on cells now known to be informative for ILR1 binding to SB, indicate that ILR1 precipitates a glycoprotein composed of two polypeptide chains with molecular weights of 29,000 and 34,000 daltons. Preliminary results of further structural studies of the ILR1-reactive molecule indicate that it has an alpha chain amino acid sequence which is similar or identical to the HLA-DR alpha chain, but has a novel beta chain. These results support the hypothesis that SB molecules are typical Ia-like molecules.

The reagents generated at NIH have been shared in collaborative studies with approximately 15 laboratories worldwide for studies including characterization of the SB antigens in different ethnic/racial groups. There is good agreement in results between the three studies of American Caucasians; as might be expected from the polymorphism in the system, there are substantial differences between these groups and a German, Southern French, and Nigerian population.

Serologic identification of the SB antigens using alloantisera has been difficult. However, by appropriate screening and absorption proceedures, three sera have been identified which appear to include reactivity with SB4. These sera are interesting since they detect antigens which correlate highly with SB4 in the population but are much better expressed on monocytes than on B cells. It remains to be determined if this reflects differences in threshold, or genuine differences in tissue distribution from the DR antigens.

Studies of cloned T cells derived from SB-specific primed cells indicate heterogeneity of the broadest SB antigen (SB4) but amazing lack of heterogeneity of other antigens (particularly SB3). Several lines of independent evidence indicate that SB4 will prove to be a public specificity including many distinct molecules.

Analysis of other complexities of HLA markers has allowed the definition of two antigens intimately associated with Bw44. Possible immunogenic heterogeneity of the HLA-Bw44 antigen was investigated by using cytotoxic T-lymphocytes (CTL) generated between donors identical for HLA-A2,3,-B7,w44. Highly discriminatory CTL combinations were identified which define two subgroups of Bw44, designated 44.1 and 44.2. Out of 47 Bw44-positive donors tested in a population study, 30 were lysed by the CTL defining 44.1 and 19 were lysed by the CTL defining 44.2. All Bw44 cells could be typed as either 44.1 or 44.2, except two Bw44-positive cells that were phenotypically homozygous for the serologically defined Bw44 antigen and were lysed by both CTL. CTLs were also raised between responder/stimulator combinations mismatched for Bw44. These CTLs lysed all Bw44-positive target cells, indicating a CML antigen shared by all Bw44 cells. But clear discrimination of the 44.1 and 44.2 subgroups was

obtained when appropriate cold target blocking cells were added. There was an exact correlation between these subgroups and the quantity of serologically detectable Bw44 on platelets ($p < 0.005$). Furthermore, population studies indicate that 44.1 is in strong positive linkage disequilibrium with HLA-A2 whereas 44.2 is in strong positive linkage disequilibrium with HLA-DR7. These data suggest the existence of two genetically and functionally different subgroups of Bw44 antigens. These antigens are of particular interest since they shed new light on linkage disequilibrium of the Bw44-related antigens, and they may have important biological relevance since they appear to regulate the level of expression of the Bw44 antigen on platelets.

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 pehnomenon 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. Futhermore, 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). Furthermore, initial studies (project Z01-CB-05100 I) suggest that it may be a useful new genetic marker for the disease dermatitis herpetiformis

Proposed Course of Project: 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.

We will also be pursuing this approach in definition of other segregant series of antigens such as the Bw44 related antigens alluded to above. In particular, we will begin to explore molecular mechanisms which could account for both the antigenic differences between 44.1 and 44.2 and the associated differences in their expression on platelets.

Publications

Shaw, S., Kavathas, P., Pollack, M. S., Charmot, D., and Mawas, C.: Family studies define a new histocompatibility locus, SB, between HLA-DR and GLO. Nature. 293: 745-747, 1981.

Kavathas, P., DeMars, R., Bach, F. H., and Shaw, S.: SB: A new HLA-linked human histocompatibility gene defined using HLA-mutant cell lines. Nature. 293: 747-749, 1981.

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Pawalec, G., Shaw, S., and Wernet, P.: Analysis of the HLA-linked SB gene system with cloned and uncloned alloreactive T-cell lines. Immunogenetics. 15: 187-198, 1982.

Pawalec, G., Shaw, S., Ziegler, A., Muller, C., and Wernet, P.: Differential inhibition of HLA-D or SB-directed secondary lymphoproliferative responses with monoclonal antibodies detecting human Ia-like determinants. J. Immunol. In press, 1982.

Pawalec, G., Schneider, M., Blaurock, M., Frauer, M., Shaw, S., and Wernet, P.: Population studies of the HLA-linked SB antigens and their relative importance in primary MLC-typing. Human Immunology. Submitted for publication, 1982.

Shaw, S., DeMars, R., Schlossman, S. F., Smith, P. L., Lampson, L. A., and Nadler, L. M.: Serologic identification of the human secondary B cell (SB) antigens: Correlations between function, genetics and structure. J. Exp. Med. Submitted for publication, 1982.

VanLeeuwen, A., Termijtelen, A., Shaw, S., and VanRood, J. J.: The recognition of a polymorphic monocyte antigen in HLA. Nature. Submitted for publication, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05102-02 I

PERIOD COVERED
October 1, 1981-September 30, 1982

TITLE OF PROJECT (80 characters or less)
Morphologic Studies of Cellular Interactions in the Immune System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Others:	P. A. Henkart	Chemist	I NCI
	P. J. Millard	Biologist	I NCI
	A. Shekhtman	Visiting Fellow	
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	C. Reynolds		FCRC

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Interactions of positively identified human natural killer (NK) cells with appropriate target cells have been studied in serial thin sections with the electron microscope. These studies have been extended to human effectors of ADCC rat NK cells and rat tumor cells with NK and ADCC activities. These cells have been examined (1) under non-activated conditions, (2) at various times during killing of targets and/or (3) under conditions mimicking signals that activate them to kill (adherence to antigen-antibody complexes or activation by lectins). Several murine cytotoxic T cell clones are also being studied and compared with non-cytotoxic clones. All cytotoxic lymphocytes studied thus far contain membrane bound granules, although the granules differ in details of morphology from one species to another. 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 membraneous structures which often bear ring-shaped profiles on their surfaces. Studies are in progress to identify the granule contents in situ by light and EM immunocytochemistry, and by isolation and characterization by biochemical techniques.

Project Description

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 human natural killer cells with appropriate target cells.

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 fluroescent 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. To ensure that all lymphocytes forming conjugates are active killers, the lymphocytes are treated for 2 hours before conjugate formation with human interferon. Under these conditions virtually all lymphocytes that bind target cells kill them within 4 hours.

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 ^{51}Cr 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. Rat natural killer cell-like tumors 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).

Cytotoxic T-cells: A number of cytotoxic and non-cytotoxic T-cell clones have been obtained from laboratories of S. A. Rosenberg, A. M. Schmidt-Verhulst and R. Schwartz. Their morphology is being studied as they grow and after attachment to plastic surfaces coated with lectins or poly-L-lysine (PLL). These coated surfaces are intended to be models of target or non-target surfaces. For example, a phytohaemaglutanin (PHA) coated target would be killed by these effectors while a PLL-coated cell would not.

Granule isolation: In an attempt to isolate and purify the granule from NK-like cells, various cell disruption techniques are being tested in combination with various disruption buffers. The cell homogenates are then separated on sucrose or Percoll density gradients. The fractions obtained are harvested and assayed for β-glucuronidase (a lysosomal marker) and succinic dehydrogenase (a mitochondrial marker). Portions of each fraction are also studied in negative stain and fixed and thin sections prepared and studied by electron microscopy.

Models of tubulin-membrane interactions: Small unilamellar dipalimitoyl-phosphatidyl choline liposomes were prepared and phosphocellulose-purified tubulin was inserted into the membranes by passage through the phase transition temperature as has been described (Klausner et al. J. Biol. Chem. 256: 5879, 1982 and Kumar et al ibid p. 5886.) The morphology of these structures (LT) was studied by negative stain and in thin sections for the electron microscope. For studies of the possible cytotoxicity of LT, various concentrations of LT were added to microtiter wells containing ^{51}Cr labelled red cells. The LT were added with varying Ca concentrations or under Ca-free conditions. LT were centrifuged down onto a monolayer of ^{51}Cr-labelled red cells and then Ca was added to promote fusion of LT (see below).

Major findings: NK cells contain a variety of granules distinguished on the basis of their morphologies. In NK cells that have formed conjugates with appropriate targets the contents of granules becomes more heterogeneous, apparently because of fusion of several granule types. In some cases tubule bundles are seen in the same granules as numerous small vesicular structures similar to those contained in classical multivesicular bodies. Material similar in appearance to the heterogeneous granule contents including fragments of tubule bundles and membrane vesicles are also found in the extracellular space between killer and target cell. The membranous extracellular material sometimes bears superimposed ring-like profiles similar in diameter to microtubule cross-sections. The simplest interpretation of the morphologic observations is that the mixed contents of the granules are secreted by exocytosis. The images seen in the EM suggest further that the membranous material released from the killer cell may fuse with the target cell.

Morphologic studies of cytotoxic effector lymphocytes have been extended from human natural killer cells to effectors of antibody dependent cellular cytotoxicity (ADCC). In this preparation as in the human NK cells we have observed the fusion of lymphocyte granules and secretion of granule contents. Secretory product can be observed in association with red cell target membranes or deposited on plastic surfaces coated with antigen-antibody complexes. The material secreted by ADCC effectors also includes membranous structures bearing ring-shaped profiles.

The possibility that the ring-forming material released from the killer cell may be tubulin is being pursued by immunocytochemistry with several polyclonal and monoclonal tubulin antibodies. Results thus far at

the light microscope level indicate that material in granule-like structures in human ADCC effectors, human NK, rat NK and mouse CTL lines build anti-tubulin antibodies. Thus, tubulin is probably a part of the granule contents in all cytotoxic lymphocytes. We are currently extending these studies to the EM level to confirm the identity of the granule-like structures.

Examination of six lines of cytotoxic T-cells has shown that they contain characteristic granules. The morphology of the granules in mouse CTL lines differs from that of the granules in human NK and ADCC effectors, but they appear to be secreted under conditions in which the CTL are stimulated to kill. Two non-cytotoxic lines thus far examined have not had comparable granules.

Since morphology and preliminary results with immunocytochemistry suggested that tubulin might be a component of the granule contents and secretory product, we studied the morphology of a model system in which tubulin was inserted into liposomes (LT). Lt were found to aggregate and, in the presence of mM Ca, to fuse extensively. In thin sections of this material, globular structures 50^{o}_{A} in diameter and occassional ring shaped profiles made up of 50^{o}_{A} diameter subunits were seen in the plane of the membranes. These structures resemble the membranous material secreted from NK and ADCC effectors and indicate that tubulin is capable of producing ring-shaped structures in membranes. No cytotoxic effect of LT on ^{51}Cr-labelled red cells was observed, however.

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 cytoxic lymphocyte function for prevention or therapy of neoplasms.

Proposed Course of Project: Further studies with monoclonal anti-tubulin antibodies are being undertaken at the light and EM levels. We intend to study CTL in the process of killing by using highly cytotoxic cloned T-cells and cell lines as targets. Conjugates will be formed and their cytotoxic function tested in a single conjugate assay. Then the conjugates will be studied in serial thin sections with the EM. In parallel we intend to study the morphology of a number of cytotoxic cloned lines and compare them to cloned lymphocyte lines which lack cytotoxic function. We intend, in addition, to pursue the isolation, purification and characterization of the granules from NK cells.

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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05103-01 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Structure and Function of Cloned Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	T. Soares	Microbiologist	I NCI

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A series of cloned T lymphocytes from C57Bl/10 mice has been examined by two-dimensional gel electrophoresis to try to define the proteins which are responsible for the differentiated functions of these lymphocytes, especially cytotoxicity. By using ^{35}S methionine-labeling, over two hundred proteins can be reproducively resolved from the total cell extracts. All of these except two are found in all the clones examined. The two spots which are variable are found in some clones which are cytotoxic and some clones which are not. Non-cytotoxic variants of clones which originally were cytotoxic have identical patterns of proteins to the cytotoxic parent clones. We thus conclude that proteins uniquely responsible for the differentiated functions of lympohocytes are not among the major proteins species synthesized by the cells.

Project Description

Objectives: The objective of this project is to define the biochemical differences between various T lymphocyte subpopulations, especially with respect to their unique functions, i.e., cytotoxicity, help, suppression, and lymphokine secretion. To date the principal means of classifying lymphocytes has relied on the presence of membrane antigens, which in some cases correlate with functional properties. We propose to examine a large number of cloned T lymphocytes whose functions have been studied by us and others, and analyze the protein content of the whole cells and various subcellular fractions by 2D gel electrophoresis. We will look for the presence of certain proteins which correlate with lymphocyte function.

Methods Employed: The in vitro culture and cloning of T lymphocytes requires the use of "T cell growth factor", or IL2, which is produced by in vitro stimulation of the lymphoma EL4 with phorbol myristal acetate or by stimulation of rat spleen cells in vitro with Con A. Various sources of immune and non-immune T lymphocytes are used. The cells are cloned by limiting dilation. These cloned cells are labeled in vitro with ^{35}S-methionine and then the labeled cells fractionated by various means. The labeled proteins are subject to 2D electrophoresis and analyzed by autoradiography.

Major Findings: At this stage we have analyzed 17 lines of cloned T lymphocytes of which 9 are cytolytic and 7 non-cytolytic. We have also examined non-cloned splenic ConA blasts, 2 T cell lymphomas and the macrophage-like cell line P388D_1. When whole cell extracts are examined, examination of the 2D gel autoradiograms allows visual comparison of over 200 proteins. For the cloned T lymphocytes all but two are qualitatively identical in all 17 lines examined. Two proteins are not detectably expressed in several of the lines; however this pattern of expression does not correlate with any known function or with Lyt phenotype. The protein pattern of the splenic T cell blasts is distinctly differenct and more complex than the cloned T lymphocyte patterns examined. This presumably reflects proteins made by T cell clones of a type we have not yet examined.

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: We plan to extend this work by first fractionating cells into nuclear membrane, membrane, mitochondrial, granular and cytoplasmic fractions, and we will compare the protein patterns of each of these fractions from each of the functional types of clones. We also plan to analyze many more different types of cloned lymphocyte lines as they become available through collaboration and our own cloning efforts.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05104-01 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Detection and Analysis of H-2 Variant Cell Lines from Murine T Cell Lymphomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
Other:	Keiko Ozato	Senior Staff Fellow	LND CH

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)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

Project Description

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 "^{14}C-leucene 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 phenomenology is more general, and could be demonstrated for other murine T cell lympomas. Some 40 lines are available for these studies.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05105-01 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Specificity of Human Cytotoxic Effector Cells Generated by Stimulation with Conconavalin A

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. M. Shearer	Senior Investigator	I NCI
Other:	S. Payne	Technician	I NCI
	S. Rosenberg	Branch Chief	S NCI

COOPERATING UNITS (if any)

Surgery Branch, NCI

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

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☐ (a) HUMAN SUBJECTS* ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Peripheral blood leukocytes (PBL) from normal donors stimulated with Conconavalin 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.

Project Description

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 ^{51}Cr-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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05106-01 I

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Analysis of the T Cell Alloreactive Repertoire

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard Hodes	Chief, Immunotherapy Section	I	NCI
Other:	Ronald Gress	Investigator	I	NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
	1.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 F_1 responding T cell populations contain distinct subsets capable of recognizing Mls^c encoded determinants in the context of parental MHC determinants. These findings demonstrate that non-MHC Mls^c determinants are recognized by responding T cells in association with MHC encoded determinants. 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.

Project Description

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 determants were assayed by proliferation in mixed leukocyte response (MLR) cultures. Responding spleen cells were mixed in microculture with irradiated stimulating cells which were identical in the MHC but which differed in the non-MHC Mls locus, such that stimulating cells presented alloantigenic products of the Mls^c allele. 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.

Studies of responses to the mutant K^b products employed a series of mutants including the K^{bm1} and the K^{bm6} mutants. 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 cytolysis assayed at the end of this period by ^{51}Cr release. Responding populations included normal spleen cell populations, radiation bone marrow chimeric spleen cell populations, and spleen cells from neonatally tolerized animals. Long term radiation bone marrow chimeras were prepared as previously described (see Project No. _____). Neonatal tolerization was performed by the intravenous injection of parental type spleen cells into newborn F_1 mice. Cold target inhibition studies were carried out by the addition of unlabelled target cells to cultures of cytolytic effector T cells and ^{51}Cr labelled targets.

Major Findings:

Responses to Mls^c determinants were studied in F_1 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. Responding F_1 cells recognized Mls^c determinants in association with either parental MHC type, and with approximately equal efficiency for the two parental haplotypes. 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 F_1 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.

Wild type B6 T cells generate strong CML responses to the B6 mutant strains bm1 and bm6. In addition, the $H\text{-}2^d$ strain D10.D2 also can respond to these mutant determinants, as demonstrated by cold target inhibition studies to distinguish mutant-specific response from anti-$H\text{-}2^b$ response; and by the ability of B10.D2 mice neonatally tolerized to $H\text{-}2^b$ to generate a mutant-specific response. B10.D2 --> B10 chimeric cells were similarly able to respond to bm1 and bm6 determinants. In contrast, however, B10 --> B10.D2 chimeric T cells had a markedly reduced response to bm1 and were entirely unresponsive to bm6, in spite of their normal alloreactivity to third party B10.BR. 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 $H\text{-}2^b$ or $H\text{-}2^d$ T cells can respond) or of the T cell maturation environment alone (since T cells which have matured in either an $H\text{-}2^b$ or $H\text{-}2^d$ 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.

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 addition, subregion mapping to determine those MHC regions critical to restriction of the alloreactive repertoire will be carried out.

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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB-05107-01 I

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

T Cell Responses to Minor Histocompatibility Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. C. Groves	Medical Staff Fellow	I NCI
Other:	A. Singer	Senior Investigator	I NCI

COOPERATING UNITS (if any)

LAB/BRANCH Immunology Branch

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 thus far have demonstrated 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. It is anticipated that studies in chimeric mice will help elucidate the mechanisms of tolerance induction with the specific intent of determining whether tolerance induction is H-2 restricted.

PHS-6040
(Rev. 2-81)

Project Description

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.

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: Chimeric mice will be utilized to determine whether the induction of tolerance to minor H antigens is H-2 restricted.

SUMMARY REPORT

MACROMOLECULAR BIOLOGY SECTION, IMMUNOLOGY PROGRAM
DCBD, NCI

October 1981 - September 1982

The Macromolecular Biology Section in the Immunology Program achieved major results in the elucidation of specific macromolecular changes on the surface of mammalian cells, how such changes relate to certain normal (differentiated) cell surface functions, and to the appearance of cellular tumorigenicity.

Sid Shifrin continues productive cooperation with the investigators centered around Dr. L. D. Kohn in the LBP at NIADDK, in receptor structure and function, using for an exploratory approach the thyrotropin receptor as a vehicle. They study the binding of thyroglobulin to the thyroid receptor in the bovine thyroid plasma membrane. By sequential glycohydrolase treatments of the thryoglobulin and subsequent binding studies Sid Shifrin found that the receptor prefers to recognize the mannose residues that are exposed after treatment with β-N-acetylglucosaminidase. He also initiated physicochemical studies on thyroglobulins extracted from thyroid goiters and from thyroid adenomas, and found significant structural differences from that of normal thyroglobulin.

Dr. Chandrasekaran, together with Mrs. V. W. McFarland and Dr. D. Simmons at the University of Delaware, found that an ∿55,000 MW cellular protein which is "induced" after SV40 infection of cultured mammalian cells, is also present in embryo primary cells which were not exposed to the SV40 virus. The mouse embryo protein and the "induced" protein in SV40 transformed mouse cells when labelled with ^{35}S-methionine have virtually identical 2D tryptic peptide fingerprints. Both proteins are phosphorylated *in vivo* in the serine residues. They also possess associated phosphokinase activity in the immunoprecipitate (with C.Parrott). Mouse, rat and hamster embryo ^{35}S-labelled 55K proteins and the 55K SV40 induced proteins from the same species are structurally identical, but have species divergence approximating the expected evolutionary order and divergence (with D. Simmons). These make it likely that the 55K protein has (or had) an important, i.e. evolutionary conserved, cellular function. We do not know what this function is, but we find that it is important in embryonic differentiation: Dr. Chandrasekaran and Mrs. McFarland showed a significant decrease during the development of the embryo: the amount of the 55K protein in the primary cells or in the organ culture from 12 day old mouse embryos is high, but decreases to a very low level from 16 day old mouse embryos. Replating midgestation embryo primary cells from mouse, rat and hamster leads to a great decrease in the 55K protein. Thus established cell lines and clones, which are mainly fibroblasts, have only very low amount of the protein. This is so regardless whether they are normal cells or highly tumorigenic spontaneously transformed (sarcomagenic) cells. In both the spontaneously transformed cells and in normal cells, the half life of the protein is very low ($\leq$ 60 min.). In SV40 transformed cells the SV40 coded T antigen stabilizes the 55K protein by a direct interaction. However, there is no interacting protein in those cells which are not transformed by SV40, but contain

constitutively high level of stable 55K protein: i.e. embryo primaries, mouse embryonal carcinoma cells, L cells, neuroblastoma cells, placenta cell cultures.

Our pioneering finding on the 55K protein is being rapidly confirmed in other laboratories, usually studying transformed or tumorigenic cell lines. However, in our carefully matched cell families we do not find correlation with tumorigenicity when the spontaneously occurring tumorigenic transformed mouse fibroblast cells (clones) are compared with the normal clonal parent cells. We feel that the primary importance of our discovery is in the finding that the 55K protein is being expressed constitutively in normal embryogenesis, and only secondarily in the finding that the same cellular gene expression being modulated in many transformation processes such as in SV40 transformation. The latter, however, can lead now to the convergence of two large fields of investigations: There is a potential of applying molecular biology techniques and reagents which are so well defined in tumor virology, to molecular changes which occur in embryogenesis.

The 55K protein together with the SV40 T antigen, appears on the surface of the SV40 transformed cells, as well as in their nuclei (Drs. Luborsky and Chandrasekaran). Dr. Luborsky postulated that the 55K cellular protein may represent as it does a "contact point" reaching back to the DNA level in the regulatory pathways in DNA replication and cell division in two systems: The differentiating embryo cells and the SV40 transformed cells. He stuided the interaction of the 55K protein and of the SV40 T antigen with cellular DNA. A fraction of the SV40 T antigen and also of the 55K protein, isolated from nuclei or from surface membranes of SV40 transformed cells, appears to interact with calf thymus DNA, as eluted only at elevated pH and salt concentrations, similarly to a fraction of the 55K proteins from mouse embryonal carcinoma (F9) cells which contain the 55K protein but not the SV40 T antigen. However, the data on the 55K protein is not yet sufficiently strong to definitely establish interaction with DNA.

In other studies, we obtained a collection of large number of spontaneously transformed clonal mouse cells in "closed families" with careful control on the pedigree and of the selection factors both in vivo and in vitro (clones obtained with V. McFarland and L. Waters). This now allows a systematic analysis of the until now seemingly intractable problem of "spontaneous" (chance) transformation, including cloning of transforming DNA fragments.

In characterization of the well pedigreed families of normal mouse cells, or cells transformed spontaneously or with SV40, we also found that spontaneous transformation (cf. by somatic mutation) is sufficient to explain the acquisition of cellular tumorigenicity of the SV40 transformed mouse fibroblasts. There is no binding correlation between cell growth in viscous medium, of tumorigenicity in vivo in both syngeneic and in nude mouse and of the SV40 expression in mouse fibroblasts. This extends our previous finding that on the balance the phenotypic changes pertaining to in vivo properties of cells attributed to SV40 early gene coded T antigen are predominatly expressed as cell surface antigens which cause immune recognition and rejection in the mouse. This is the first model when a tumor associated "transplantation" antigen was fully defined molecularly: i.e. the product of (part) of the early half of the SV40 genome.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05097-03 IP

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Structure of Thyroglobulin and Interaction with Membrane Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sidney Shifrin	Chemist	IP NCI
Other:	L. D. Kohn	Medical Officer	LEP NIADDK
	J. E. Rall	Director	NIADDK
	Salvatore Aloj	Visiting Scientist	LEP NIADDK
	Carlo Rotello	Visiting Scientist	LEP NIADDK
	William Habig		Bureau of Biologics

COOPERATING UNITS (if any) Frank Maley, N.Y. State Dept. of Health, Albany, N. Y.; William Valente, Univ. of MD; Eduardo Consiglio, Salvatore Aloj, Paolo Lacetti, Univ. of Naples; Roy Sundick, Wayne State Univ.; Ephraim Yavin, Weizman Inst. of Science, Rehovot, Israel; Roberto Toccafundi, Univ. of Florence, Italy

LAB/BRANCH
Immunology Program

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to examine the amino acid and carbohydrate structures of thyroglobulin, thyrotropin (TSH), interferon, cholera toxin and tetanus toxin in order to elucidate the functional groups which are necessary for interaction with receptors on thyroid membranes and to examine the properties of thyroglobulin isolated from thyroid adenomas.

Project Description

Objectives: The purpose of this project is to examine the amino acid and carbohydrate structures of thyroglobulin, thyrotropin (TSH), interferon, cholera toxin and tetanus toxin in order to elucidate the functional groups which are necessary for interaction with receptors on thyroid membranes and to examine the properties of thyroglobulin isolated from thyroid adenomas.

Methods Employed: Affinity chromatography, two dimensional gel electrophoresis, chemical modification of proteins and receptors, chemical modifications and isolation of glycopeptides from glycoprotein hormones, preparation and characterization of monoclonal antibodies, purification of enzymes involved in ADP-ribosylation, ultraviolet absorption spectroscopy, fluorescence, circular dichroism, light scattering, radioautography.

Major Findings: The removal of the polymannose (A unit) chain by β-endoglycosidase H requires pretreatment of thyroglobulin with 0.5M sodium thiocyanate, a chaotropic agent which will alter the conformation of thyroglobulin without denaturing the endo H. The structures of the released polymannose units have been determined using H-nmr spectroscopy. The thyroglobulin which remains after removal of the polymannose (A unit) aggregates to 27S material which binds to thyroid membranes to a greater extent than the original 19S material.

Radioiodination using either the lactoperoxidase procedure or the chloramine T method results in the breakdown of the 27S protein to 12S material which binds poorly to thyroid membranes. Successful radiolabelling of thyroglobulin was affected using the iodogen procedure.

The necessity for adding thiocyanate to thyroglobulin in order to affect removal of the A unit suggested that the polymannose unit may be hidden and not readily accessible as is generally believed to be true for the polysaccharide units of glycoproteins.

We are currently measuring the rate at which the A unit is removed from 12S succinyl thyroglobulin, trinitro-phenyl-thyroglobulin and 27S agalactothyroglobulin.

These results demonstrate that the polymannose (A unit) is not essential for binding to thyroid membranes but is necessary for the maintenance of the stable 19S conformation.

Bovine thyroglobulin has been treated sequentially with sugar-specific glycohydrolases as follows:

1) neuraminidase
2) β-galactosidase
3) β-N-acetylglucosaminidase
4) α-mannosidase
5) α-fucosidase

The intermediate glycoproteins were isolated and their physicochemical properties examined as well as their ability to bind to thyroid membranes.

We previously reported that the removal of sialic acid and galactose from thyroglobulin molecules containing a high level of iodine results in aggregation to 27S material. 19S Thyroglobulin molecules with low iodotyrosine content remains as 19S after removal of the two terminal sugars. The 19S agalactothyroglobulin can be made to aggregate by chemical iodination of 19S agalactothyroglobulin. The 27S agalactothyroglobulin is processed differently than 19S thyroglobulin.

19S Agalactothyroglobulin is completely converted to 27S material by the removal of the next sugar; namely, β-N-acetylglucosaminidase. The spectrum of this compound compared with the spectrum of 19S agalactothyroglobulin indicates that a new chromophore is formed as a result of glycohydrolase treatment which is characterized by a new band, at 304nm and at 248nm and numma at 290nm and at 282.5nm. There is also a small decrease in fluorescence intensity at 330nm that accompanies the removal of the GlcNAc residue.

The binding data indicates that the receptor prefers to recognize the mannose residues that are exposed after treatment with β-N-acetylglucosaminidase and upon remval of the mannose residues with α-mannosidase there is a decreased ability to bind to thyroid membranes. The results indicate that the oligosaccharide chains of bovine thyroglobulin are not essential for binding to the thyroid membrane but may be necessary for proper biosynthesis or biodegradation. We are currently exploring these two pathways.

Physicochemical Properties of Thyroglobulin from Goiter and from Adenoma.

Thyroglobulin extracted from thyroid goiters has a large proportion of 27S material in addition to 19S material. Both 19S and 27S thyroglobulin break down upon removal of sialic acid with neuraminidase which is unlike the behavior of normal thyroglobulin. We are currently examining the chemical structure of thyroglobulin from goiters.

Very little thyroglobulin can be extracted from thyroid adenomas. The thyroglobulin which is extractable from the thyroid adenomas contains an inordinate amount of 27S material whose absorption spectrum is not like any thyroglobulin examined previously. On the other hand, the spectrum of 19S thyroglobulin looks by the absorption spectrum like bovine 27S thyroglobulin suggesting that there are abnormal structures in the adenomatous thyroglobulin.

Significance to Biomedical Research and the Program of the Institute: By characterizing the abnormal structure of the carbohydrate and the polypeptide chains of thyroglobulin isolated from human adenomas we hope to be able to determine which enzymes necessary for normal biosynthesis are defective in the adenomas. We also are isolating the receptor on the thyroid which binds thyroglobulin and are examining its physicochemical properties.

Proposed Course of the Project: Derivatives of thyroglobulin interfere with the binding of TSH and the activation of adenylate cyclase. We are using monoclonal antibodies to the TSH receptor to examine the relationship between TSH and thyroglobulin binding sites.

Publications:

Shifrin, S., and Kohn, L. D.: Binding of thyroglobulin to bovine thyroid membranes: Role of specific amino acids in receptor recognition. J. Biol. Chem. 256: 10600-10605, 1981.

Consiglio, E., Shifrin, S., Yavin, Z., Ambesi-Impiombato, F. S., Rall, J. E., Salvatore, G., and Kohn, L. D.: Thyroglobulin interactions with thyroid membranes. Relationship between receptor recognition of N-acetylglucosamine residues and the iodine content of thyroglobulin preparations. J. Biol. Chem. 256: 10592-10599, 1981.

Kohn, L. D., and Shifrin, S.: Receptor structure and function: An exploratory approach using the thyrotropin receptor as a vehicle. Horiz. Biochem. Biophys. 1-41, 1981.

Shifrin, S., Kohn, L. D., Salvatore, G., and Consiglio, E.: 27S thyroid iodoprotein: In vitro formation from 19S thyroglobulin by glycohydrolases and its binding to thyroid membranes. J. Biol. Chem. 257: (August) 1982.

Shifrin, S., Consiglio, E., Kohn, L. D., Rall, J. E., and Salvatore, G.: The role of tyrosine and N-acetylglucosamine residues of thyroglobulin in binding to thyroid membranes. In Andreoli, M., Monaco, F., and Robbins, J. (Ed.): Advances in Thyroid Neoplasia 1981. Field Educational Italia, 1981, pp. 53-60.

Consiglio, E., Kohn, L. D., Salvatore, G., Shifrin, S., Cavallo, R., and Formisano, S.: Mannose phosphate as a specific signal in the lysosomal biodegradation of thyroglobulin. In Andreoli, M., Monaco, F., and Robbins, J. (Ed.): Advances in Thyroid Neoplasia 1981. Field Educational Italia, 1981, pp. 61-69.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05545-02 IP

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Expression of Cellular Antigens in Transformed Cells and in Embryonic Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Chandrasekaran	Visiting Associate	IP NCI
Other:	P. T. Mora	Chief, Macromolecular Biology Section	IP NCI
	V. W. McFarland	Chemist	IP NCI
	J. C. Hoffman	Microbiologist	IP NCI

COOPERATING UNITS (if any)
Dr. Daniel T. Simmons, University of Delaware
Dr. L. Nagarajan and Dr. W. Anderson, Laboratory of Pathophysiology, NCI

LAB/BRANCH
Immunology Program

SECTION
Macromolecular Biology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The purpose of this project is to identify, quantitate and characterize an SV40 induced 53,000 MW (p53) cellular protein in embryonal carcinoma cells (EC), embryonic cells and in various other transformed cells. Undifferentiated EC cells and primary cells prepared from mid-gestation mouse, rat and hamster embryos all expressed the protein p53 without SV40 infection. The 2D tryptic peptide map obtained from EC and embryo cells showed that this protein is similar to SV40 induced p53 and is conserved evolutionarily in both embryonic and in SV40 transformed cells. The amount of the protein determined quantitatively in both cells were found to be half that of SV40 transformed cells. The amount of p53 decreased with differentiation of EC cells, and with the increasing age of the embryo. The protein was also detected in various tumorigenic mouse cells such as L cells, neuroblastoma cells, placental cells and 3T12 cells. In the established mouse cells prepared from AL/N strain mouse embryo, the expression of p53 did not correlate with tumorigenicity. The turnover of this protein was found to be rapid in tumorigenic and in non-tumorigenic cells whereas in SV40 transformed cells the protein was found to be stable due to its interaction and complex formation with large T antigen.

Project Description

Objectives: To determine whether the SV40 induced 53K cellular protein is expressed in embryonal carcinoma and embryonic cells; is it related to differentiation of embryonal carcinoma and embryonic cells; whether the presence of p53 is related to tumorigenic transformation or not; and also what are the control mechanisms in the regulation of expression of this protein in SV40 transformed cells and in other cells.

Methods Employed: Maintenance and growth of cells in culture. Radioactive labelling, immunoprecipitation, gel electrophoresis, thin layer chromatography and fluorography.

Major Findings: A specific 53,000 dalton protein was shown to be present in SV40 transformed cells in addition to SV40 coded large T and small t antigens, first reported by us but rapidly confirmed by others. Studies (with D. Simmons) on tryptic peptides revealed that this is an evolutionarily conserved protein. The same or similar protein was also detected by others in a variety of transformed cells induced by various agents such as meth A, x-ray and RNA tumor virus. Furthermore, the same or similar protein was also detected by others in embryonal carcinoma cells. We have now shown the p53 to be present in embryo primary cells without SV40 infection. The objective of our most current study was to find out (a) the correlation between the "differentiation" of embryonal carcinoma cells and the amount of p53 (b) the correlation between the embryonic development and amount of p53 (c) correlation between the amount of p53 and tumorigenic transformation (d) control mechanisms involved in the regulation of expression of p53 in these cells.

A) 1. p53 is an Embryo Protein. Mouse embryos at various post implantation stages(10-16 days) were investigated and compared. Primary cultures from 10 and 12 day old mouse embryo showed the presence of specifically immunoprecipitable p53. Undifferentiated mouse embryonal carcinoma cells also showed the presence of specifically immunoprecipitable p53. The presence of this protein in these two cells was further confirmed by

a. immunoprecipitation of p53 from embryonal carcinoma cells and from primary embryo cultures with a monoclonal antiserum prepared against SV40 induced p53,

b. the comparison of ^{35}S-methionine labelled tryptic peptides of p53 from embryonal carcinoma cells and from embryo primary cells with SV40 induced p53 showed similar tryptic peptide pattern (in collaboration with Dr. D. Simmons),

c. p53 in embryonal carcinoma cells and in embryo cells were labelled with ^{32}P indicating the protein to be a phosphoprotein similar to SV40 induced p53.

2. Correlation of p53 with Mouse Embryo Age and Tissue Culture Transfer. The presence and the amount of the p53 was then investigated and quantitated from different day old mouse embryos. The amounts were both expressed as %, compared to p53 in an SV40 transformed mouse cell line taken as 100% and were also compared with the amount of the SV40 induced p53 in an SV40 transformed mouse cell. The amount of the protein present in 12 day old embryo primary cells was

found to be about 50% compared to SV40 induced p53. The amount of the protein in older embryos decreased with the age of the embryo, as shown - 13 day - 40%, 14 day - 25%, 15 day - 7.1% and 16 day - 2.6%. Thus the expression of the protein correlated with the age of the embryo.

Attempts were made to identify the organs which are responsible for the synthesis of this protein in embryos. In collaboration with Dr. M. Dziadek we dissected and labelled various organs from 12 day and 16 day mouse embryos. The results showed that in 12 day old mouse embryo the following organs were found to possess the p53 - liver, brain, heart, carcass and possibly visceral yolk sac whereas the same organs isolated from 16 day old mouse embryo did not possess the protein. The expression of this protein was studied upon tissue culture transfer (t.c.t.) of the embryo primary cells. The 12 day mouse embryo primary cells which possessed the protein when trypsinized and replated were found not to contain the protein (the first t.c.t.). Similar results were obtained during the second t.c.t. also. Thus there could be a tissue culture selection against these cells which synthesize the p53.

3. Presence of 53K Protein in Other Murine Embryos. The presence of the protein in midgestation embryos was further confirmed by studying the embryos of other species, i.e. rat and hamster. Immunoprecipitation with the SV40 T serum and with monoclonal serum indicated the presence of p53 in 14 day old rat and 10 day old hamster embryo primary cells. The amount of the protein precipitated was found to be 60% in hamster embryos and 45% in rat embryos compared to SV40 induced p53. The first tissue culture transfer cells derived from hamster embryo primary cells synthesized about 10% of p53 whereas the second t.c.t. cells did not show the presence of p53. On the other hand the first t.c.t. cells from rat embryo primary cells did not show the protein. Thus the hamster embryo primary cells which synthesize maximum amounts seem to retain some cells after first t.c.t. and therefore continue to show the presence of p53 whereas other embryo primaries did not. The structure of the p53 in various embryos was compared by 2D tryptic peptide map after ^{35}S meth label. Such 2D maps of hamster embryo and rat embryo are found to be similar to that of p53 from SV40 transformed hamster and rat cells respectively. Thus the embryo p53 protein also seems to be evolutionarily conserved as is the SV40 induced p53 in mouse, rat, hamster, monkey and human cells (work with D. Simmons).

4. Correlation of p53 with Differentiation of EC Cells. Embryonal carcinoma cells (F9 and OTT6050) were tested and found to express the p53 protein. The methionine containing tryptic peptides were found to be similar to SV40 induced p53. The EC cells were then subjected to differentiation by treatment with retinoic acid and the quantitation expression of p53 in such cells were determined. The results indicated that the amount of the protein decreased with differentiation of embryonal carcinoma cells in culture (work with Drs. Nagarajan and W. Anderson).

B) 1. Expression of p53 is not a General Correlate of Cellular Tumorigenicity. We present here a summary of experiments on two families (two AL/N strain) of mouse clonal cell lines, and derivative tumor lines with respect to the expression of p53.

The first family of cells originated from a spontaneously transformed highly malignant (median tumorigenic dose, $TD_{50}=10^2$ cell/mouse) AL/N mouse embryo cell clone 104C. We have shown previously that after SV40 transformation the tumorigenicity of 104C cells in immunologically competent syngeneic mice becomes lower ($TD_{50}=10^4$), apparently because of the expression of the SV40 T and transplantation antigens. One such "daughter" cell, the SV40 T antigen positive clone *106CSC was shown to contain 1 copy number equivalent of SV40 DNA per cell DNA. When we injected *106CSC cells into the syngeneic mouse, a negative immunologic selection resulted in T antigen negative revertant derivative tumor lines and clones that originated from a rare cell(s) through some DNA rearrangement. These T antigen negative revertants (i.e., the 124, 127, 128 CSCT and 134CSCTC cells) retained "late" SV40 DNA sequences, and probably also a region (Taq 1 - Hha 1, 0.73 - 0.55 map units) which includes the sequence coding for small t antigen (0.65 - 0.55 map units). In the tumor lines and clones (128, 127, 124CSCT and 134CSCTC) negative for large T antigen there was no specific p53 band detectable, just as in the distant parent 104C cell before the SV40 transformation, while the proximal parent T antigen positive *106CSC cell possessed the p53. Thus the presence of one copy number equivalent of early SV40 DNA encompassing sequences coding for large T (but not necessarily for small t) antigen, and the resulting synthesis of SV40 large T antigen, is required and is sufficient in this family of mouse embryo fibroblast cells for the production of (stable) p53. Our results with the T antigen revertant cells extend the findings of others by demonstrating clearly that the continued presence of the A gene and synthesis of large T antigen is required for the maintenance and production of stable p53. In SV40 transformed cells large T antigen is shown to form a complex with p53 and therefore the precipitation and stabilization of p53 could be due to this complex formation. To confirm the absence of p53 in the tumor lines and clones the monoclonal antibody prepared against p53 was also used. The results showed that there was no immunoprecipitable p53 in these tumor lines and clones prepared from AL/N strain mouse embryo. These results are interpreted to mean that the highly malignant ($TD_{50} \leq 10^2$) 124 CSCT and all the other T antigen negative cell lines do not synthesize (detectable amounts of stable) p53.

The second family of cells studied originated from a clone (210C) of AL/N mouse fibroblast cells which possessed very low tumorigenicity ($TD_{50} = 10^{6.5}$). However, when injecting 10^7 210C cloned cells, tumor lines of high tumorigenicity ($TD_{50} < 10^2$) can be obtained, such as the 219CT. The simplest explanation is that in 210C cells variant (mutant) "spontaneously" transformed highly tumorigenic cells(s) arise at low frequency, and these cells are selected for tumorigenicity by the in vivo passage. Both the non tumorigenic 210C cells and the highly tumorigenic 219CT cells possess no p53. When the 210C cells were infected with SV40 and immediately recloned, the derivative T antigen negative daughter clone 213CSC had no p53, while the independently transformed SV40 T antigen positive daughter clones *214CSC and *215CSC both had the p53. Note that the transformation of 210C with SV40 resulted in the T antigen positive *214CSC and *215CSC clones which have similar low tumorigenicity as the 210C, thus SV40 transformation does not ensure increased tumorigenicity. From 10^7 *215CSC cells the tumor lines obtained from separate mice all had high tumorigenicity (TD_{50} 10^2-10^3), irrespective of whether they were T antigen positive (*221CSCT, *222CSCT, and *CSCT) or T antigen negative (223CSCT); and 223CSCT being probably a similar revertant to those discussed in the first cell family. Only the SV40 positive clones were shown to contain p53.

2. Detection of p53 in Other Tumorigenic Cells. In the third family, derived from a Balb/c strain tumor, the highest relative tumorigenicity was the property of the parent line 301T. This cell line negative for SV40 T antigen was found to contain the p53. The clone derived from this tumor line 312TC was also found to express p53. This clone when transformed with SV40 and recloned namely 318TCSC was also found to express p53. Thus p53 is detected in some tumor lines and clones such as 301T and 312TC and not detected in others such as 219CT, 124CSCT, 130CSCTC and 219CT. We then attempted to find out whether the lines derived from particular germ layer might be responsible for the difference in the detection of p53. Established lines derived from embryonic ectoderm, endoderm and mesoderm were tested for the presence of p53. The cell lines used were neuroblastoma neuro 2A; parietal yolk sac; PYS-2; and L cells derived respectively from the germ layers of ectoderm, endoderm and mesoderm. All the three lines were found to express stable p53. Extraembryonic placental cells were also tested for the presence of p53 and they were also found to contain p53. Thus some tumor lines were found to express p53 whereas other tumor lines and clones where the tumorigenicity is well characterized there was no detectable amount of p53. We are currently attempting to find out the reason for this difference.

3. Regulation of Expression of p53. The absence of detection of this protein in tumorigenic cell lines could be due to differential turnover of this protein in these cells and the SV40 virus transformed cell lines. To study this the cells were labelled for a short period of time (30 min.) and then chased for various periods of time. The cell extracts were then immunoprecipitated and the p53 band was quantitated either by direct counting or by integrating the peak area. The results showed that during a 30 min. pulse label all the non-tumorigenic and tumorigenic non-SV40 transformed, as well as SV40 transformed cell lines showed the presence of p53. The half life of the protein was found to be different. The p53 in non-SV40 transformed tumorigenic and non-tumorigenic cell lines had a half life of about 15-60 minutes, whereas the p53 in SV40 transformed cells had about >600 minutes. Thus there is a post translational regulation involved in the amount of detectable p53 in SV40 transformed cells, apparently through complexing and stabilization with the SV40 T antigen. We are further investigating this phenomenon.

Significance to Biomedical Research and the Program of the Institute: Re-expression of embryonic antigens can occur in transformed cells. Analysis of the identification of these proteins could provide more insight into the mechanism of transformation, and the phase specific expression of this protein may be used at the molecular level to study embryonic differentiation.

Proposed Course of the Project: Attempts will be made to determine if this protein would serve as a marker of differentiation of embryonic cells and embryonal carcinoma cells. We plan to detect this protein in pre-implantation embryos. We also plan to use the monoclonal antibodies against p53 to detect the presence of this protein in spontaneous transformed tumorigenic cells and study further the regulation of expression of p53 in these cells. The nature of phosphorylated amino acid in the p53 from both SV40 transformed cells and embryo cells will be studied in an attempt to find its biochemical role in transformation and embryonic development. The Principal Investigator for this project is going to continue this work until the end of September, 1982, in this laboratory, and after that at the Laboratory of Drs. Pierre and Evelyn May at the Institut de Recherches

Scientifiques Sur Le Cancer in Villejuif. Further work related to this subject is going to be continued under P. T. Mora, as given in Z01 CB 05546-02 IP, and Project Report Z01 CB 05545 IP will be terminated.

Publications:

Chandrasekaran, K., McFarland, V. W., Simmons, D. T., Dziadek, M., Gurney, E., and Mora, P. T.: Quantitation and characterization of a species-specific and embryo stage-dependent 55-kilo dalton phosphoprotein also present in cells transformed by simian virus 40. Proc. Natl. Acad. Sci. USA 78: 6953-6957, 1981.

Mora, P. T., Chandrasekaran, K., Hoffman, J., and McFarland, V. W.: Quantitation of a 55K cellular protein: Similar amount and instability in normal and malignant mouse cells. Mol. Cell. Biol., in press, 1982.

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., in press, 1982.

Mora, P. T., and Chandrasekaran, K.: Role of SV40 induced antigens in transformation and rejection of malignant mouse cells and the detection of an embryo protein. In Manson, L. and Nowotny, A. (Eds.): Biomembranes. New York, Plenum Publishing Corporation, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05526-14 IP

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cell Surface Changes in Spontaneously or SV40 Transformed Mouse Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. T. Mora	Chief, Macromolecular Biology Section	IP	NCI
Other:	S. W. Luborsky	Chemist	IP	NCI
	V. W. McFarland	Chemist	IP	NCI
	K. Chandrasekaran	Visiting Associate	IP	NCI

COOPERATING UNITS (if any)
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LAB/BRANCH
Immunology Program

SECTION
Macromolecular Biology Section

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the cell surface membrane, how it may change in transformation to malignancy, either by spontaneous induction, or as induced by SV40 virus; to study the nature of biochemical and biologic changes associated with the cells being transformed by either SV40 or by spontaneous event(s).

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 glycoproteins, glycosaminoglycans and glycolipids, 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. Analysis of Glycosaminoglycan Metabolism. Our role in this project is now only to supply Drs. Winterbourne (London) and Kent (Oxford) with selected clonal derivative mouse cells from "families" of clonal cells developed for comprehensive biochemical and biological studies, to provide the biologic data, and to evaluate 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. The same lesion was now also observed in derivative spontaneously transformed mutant tumor cell clones, as compared to the parent non-tumorigenic clone. 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. Dr. Winterbourne found that the changes in heparan sulfate metabolism relate to this, and this somehow over-rides the normal control of metabolism in both of the two different forms of transformations, SV40 induced and 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. An Approach to Analyze the "Spontaneous" Transformation of Cells. We concentrated in obtaining biologic data on genetically well defined families of mouse fibroblast cells with very different cellular tumorigenicity. For example from a clone (210C) we have isolated 10 independent tumor lines by a single in vivo passage through the syngeneic AL/N mice (work with L. Waters). Each tumor line had $TD_{50} \tilde{\sim} 10^2$, both in the syngeneic and in nude mice, and this phenotypic property is 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 ($TD_{50} \tilde{\sim} 10^2$). Thus the tumorigenecity, it once appears, does not revert, and does not appear to "segregate". These families of cells represent an unique collection to study (on a statistically significant level) the biochemical correlates of spontaneous ("chance") transformation on various molecular levels (DNA, RNA, proteins etc.). For example we are evaluating whether our cells can serve as paradigm of control study of transfection of DNA fragments from the non-tumorigenic parent clone (i.e. 210C) into the direct derivative tumor clones. Collaborative work is being set up with many laboratories.

On the biologic level we have found that the highly tumorigenic mouse clonal cells, as a rule, do not grow without anchorage in viscous medium. Thus while cells which grow well without anchorage are generally tumorigenic, the opposite is not true. Obviously, for in vivo tumor growth further "factors" are required in the interaction of these cells with the host, which do not show up in the anchorage independent in vitro growth experiments in the viscous medium. Our cells therefore also are useful to study such in vivo factors, both in the syngeneic and in the nude mice.

Third type of cell families, originating from Balb/c strain mouse embryo fibroblast clones, are also under study. Several derivative subclones were obtained (as in the above described AL/N cell families) both after spontaneous and after SV40 induced transformations. Numerous clones which were obtained by independent SV40 transformations have been valuable in the SV40 T antigen studies, and in the SV40 induced 55K protein studies (see CB 05545-02 IP and CB 05546-02 IP, and also below).

III. SV40 Transformation, Cell Biology and Surface Antigen (Protein) Studies. The analysis which led to an absolute correlation of the expression (detectability) of the cellular 55K protein with the presence of functional T antigen in the above referred numerous clonal derivative cell lines has been reported in original papers and in reviews; the recent results are summarized by Dr. Chandrasekaran (Z01 CB 05545-02 IP). The studies on the binding of the 55K protein and of T antigen to DNA, and the presence of these proteins on the cell surface is reported by Dr. Luborsky (Z01 CB 05544-14 IP). The identification of the embryo 55K protein with the SV40 induced 55K protein is in collaboration with D. Simmons, University of Delaware, and the major results are reported in Z01 CB 05545-02 IP and in Z01 CB 05546-02 IP.

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 which is responsible for transplantation rejection of SV40 transformed cells. This is the first time when a "tumor specific" transplantation antigen has been fully defined molecularly: the T antigen is encoded in the early half of SV40 genome.

Dr. C. Chang, in Taiwan, is continuing collaborative studies on the SV40 T and surface antigen recognition by T cells. He also employs our cells and antisera in further collaborative work on (other) tumor specific antigen studies in human biopsies.

Further collaborative studies: Dr. Timothy Rose used our SV40 transformed clones to extend studies he began in this laboratory on the SV40 cell surface and antigens in the Department of Molecular Biology in Geneva (with Professor R. Weil) and now at the Cancer Research Center in Seattle (with Hellströms). Dr. David Lane, Imperial College, London, studies the interaction of the SV40 T antigen and the 55K cellular protein; Dr. W. Deppert and R. Henning at the University of Ulm, West Germany, studies subcellular distribution of the antigens; Drs. Pierre and evelyn May, Villejuif, France, isolation of the gene for the 55K protein; Dr. A. DiMarco, Milan, studies on cAMP independent ATPases, and the effects of certain DNA binding anthracycline drugs.

In this laboratory (Drs. Chandrasekaran and Luborsky) used the SV40 transformed clones to detect the SV40 T antigen also the specific induced 55K protein in the cell surface (plasma membrane) enriched subcellular fraction. The accessibility of these proteins on the cell surface was confirmed by radioactive iodination as catalyzed by lactoperoxidase from the outside of the cell, and also by susceptibility of the protein to removal by gentle trypsinization (Dr. Chandrasekaran). Mrs. McFarland is developing fluorescent staining techniques for the detection of SV40 T antigen and for the 55K protein on the cell surface (in collaboration with Drs. Edidin, Johns Hopkins, and D. Solter, Wistar Institute).

Using the families of clonal cells transformed spontaneously and/or by SV40, we have concluded that spontaneous transformation (cf. by somatic mutation) is sufficient to explain the acquisition of cellular tumorigenicity of the SV40 transformed mouse fibroblast. When considering on the balance the in vivo phenotypic changes in mice, the SV40 early gene coded proteins are more "dominant" as cell surface antigens, facilitating immunologic recognition and rejection of mouse cells, rather than causing tumorigenic transformation. There is no binding general correlation between cell growth in viscous medium, of tumorigenicity in vivo, and of the SV40 antigen expression in the mouse fibroblast cells we have investigated. This of course does not mean that the SV40 early gene, its expression, and the role of the T antigen is not a suitable system to study the correlation between certain biochemical controls in cells and their phenotypic tissue culture growth, when such correlations can be made (such as between DNA synthesis and focus formation in culture). Our research, however, emphasizes a growing recognition that for the acquisition of tumorigenic potential the transformation by SV40 is not a sufficient cause, and not even a crucial contributory event in most species, such as the mouse. A comprehensive study is being published summarizing the utility of the SV40 transformation to elucidate immunologic changes and changes in cellular tumorigenicity in the various species.

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 syngeneic and nude mouse systems. Studies on the molecular level will also include regulatory events which may be due to association of the SV40 A gene product with selective (e.g. nuclear membrane associated) portions of the cellular DNA. We will further study 1) the relationship between expression SV40 TSTA (part of the T antigen and the cell surface, 2) control of gene expression for the newly discovered 55K protein and its relation to transformation and its role in embryogenesis, 3) the basis for the interaction of the 56K cellular protein with the various antisera, including monoclonal hybridoma sera, 4) in collaborative work we will also attempt isolations (cloning) and characterizations of 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:

Chang, C., Chang, R., Mora, P. T., and Hu, C-P.: Generation of cytotoxic lymphocytes by SV40-induced antigens. J. Immunol. 128: 2160-2163, 1982.

Mora, P. T.: The Immunopathology of SV40-Induced Transformation. In Miescher, P. A. (Ed.) Klein, G. (Guest Editor): Springer Seminars in Immunopathology. Heidelberg, Springer-Verlag, 1982, in press.

Winterbourne, D. J., and Mora, P. T.: Cells selected for high tumorigenicity or transformed by Simian Virus 40 synthesize heparan sulfate with reduced degree of sulfation. J. Biol. Chem. 256: 4310-4320, 1981. (Correction in journal citation.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05546-02 IP

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

A Common Protein in Embryonic Differentiation and in Cellular Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. T. Mora	Chief, Macromolecular Biology Section	IP	NCI
Other:	K. Chandrasekaran	Visiting Associate	IP	NCI
	V. W. McFarland	Chemist	IP	NCI
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LAB/BRANCH
Immunology Program

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A specific 55,000 MW phosphoprotein was found in murine embryo cells. This protein is very similar, if not identical, to an SV40 induced protein, isolated earlier from SV40 transformed cultured fibroblasts. The amount of the embryo phosphoprotein is high in cells taken from midgestation stage mouse embryos. We are studying the biochemical nature and the possible function(s) of this embryonal protein. This (or similar) protein(s) appear in stable form in cultured cells which were transformed by various other means including DNA and RNA viruses, but in much lower amounts in normal or spontaneously transformed cells. The amount of the stable protein is not a correlate of cellular tumorigenicity or cell division rate. The function of this embryo protein is unknown.

Project Description

Objectives: To isolate and characterize a specific cellular phosphoprotein of ~55,000 MW which is present up to midgestation stage in embryo primary cells, and also in many cells transformed by various agents.

Methods Employed: The biochemical and tissue culture methods are outlined in Project Reports Z01 CB 05526-14 IP and Z01 CB 05545-02 IP. 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: I. Related results are presented in Z01 CB 05545-02 IP by K. Chandrasekaran. Additional major findings and correlations include the following:

1) The embryo protein is a phosphoprotein. From 14 day old rat embryo primary cultures, labelled with inorganic ^{32}P, the 55K protein was specifically precipitated with a monoclonal antibody prepared against the 55K protein from SV40 transformed mouse fibroblast (monoclonal Ab from E. Tucker Gurney, University of Utah). A related 55K phosphoprotein from SV40 transformed mouse cells was found to possess phosphoserine residues and small amount of phosphothreanine residues, but no detectable amounts of phosphotyrosine residues (with C. Parrott and T. Martensen). We (C. Parrott) also showed that an cAMP independent $\gamma^{32}P$ ATP phosphokinase activity is associated with the 55K protein. 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 55K proteins which are not in complex with a stabilizing protein, with that of the complexed and the SV40 T antigen stabilized 55K protein.

2) Distribution of the 55K embryo protein during embryogenesis. For detection in various organs and in various phases of the developing embryos, immunofluorescent methods are being developed using fluoroscein labelled monoclonal antibodies to stain in situ sections of (mouse) embryos in different stages of development (V. W. McFarland in collaboration with Davor Solter, Wistar Institute). Cell lines cultivated from normal placentas also contain the 55K protein (work with Dr. K. S. S. Chang, LCBGY).

3) Cell division rate and the 55K protein. Careful cell growth rate measurements and quantitations of the ^{35}S-Met labelled 55K protein 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 55K protein in the cells. The absence of significant correlation in embryonal carcinoma cells with cell growth rate, but strong correlation with the state of differentiation is reported in CB 05545-02 IP (work with Dr. Chandrasekaran and Dr. W. Anderson, LPP).

4) The detection and possible biologic correlates of the 55K embryo protein in human tumors, and in various (transformed) mouse cell lines.

A) As many (but not all) established human tumor lines were shown by others to contain the 55K protein 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 the 55K protein, with information on optimizing quantitation of the 55K protein. This collaborative project is still in an early stage for deciding whether there is any utility to follow the quantitation of this new (class of) embryo "antigen(s)" for diagnosis of any human malignancies.

B) More careful analysis is being 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 (see Z01 CB 05545-02 IP and Z01 CB 05526-14 IP). Several highly tumorigenic clones ($TD_{50} \leq 10^2$) form rapidly ($\leq$ 8 weeks) lethal fibrosarcomas in contrast to the normal parent clone ($TD_{50} > 10^6$), and both possessed very little 55K protein (1/50th to 1/100th) when compared to an SV40 transformed clonal derivative cell.

However, we found other mouse cells (clones), which are considered malignant, which possess the 55K protein in ample amounts. These cells (in addition to RNA virus or methylchorantrane transformed cells investigated by others) include a mouse neuroblastoma clone (neuro 2-A) and an L cell clone (#929). All of these cells, however, do not have closely matched normal counterparts, and have been in culture for a long time. Clearly our newly isolated and characterized families of cloned cells with careful pedigree as described in Z01 CB 05526-14 IP are the choice for controlled biochemical and biologic studies. In these mouse fibroblasts clones there was no correlation found with tumorigenicity in either the syngeneic or in the nude mice.

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: Specific plans and work underway are given in Z01 CB 05545-02 IP. On a general level those avenues will be explored which use the molecular biology techniques and reagents so well defined in SV40 virology, to explore related changes which occur in embryogenesis, and may also occur during cell division. It is hoped to characterize this cellular gene which is constitutively expressed in normal embryogenesis, and which also is modulated in many (but not all) transformations. Various potential molecular mechanisms for the modulation of the gene expression will be explored. The nature of the phosphokinase activity in embryo cells and in embryonal carcinomas will be further studied.

Publications:

Mora, P. T., and Chandrasekaran, K.: Role of SV40 Induced Antigens in Transformation and Rejection of Malignant Mouse Cells, and the Detection of an Embryo Protein. In Manson, L. and Nowotny, A. (Ed.): Biomembranes. New York, Plenum Publishing Corporation, 1982, in press.

Chandrasekaran, K., McFarland, V. W., Simmons, D. T., Dziadek, M., Gurney, E. G., and Mora, P. T.: Quantitation and characterization of a species-specific and embryo stage-dependent 55-kilodalton phosphoprotein also present in cells transformed by simian virus 40. Proc. Natl. Acad. Sci. USA 78: 6953-6957, 1981.

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., 1982, in press.

Mora, P. T., Chandrasekaran, K., Hoffman, J. C., and McFarland, V. W.: Quantitation of a 55K cellular protein: Similar amount and instability in normal and malignant mouse cells. Mol. Cell. Biol., 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05544-14 IP

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Cell Surface Changes in Transformed Mouse Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Calf thymus DNA (ct-DNA) stimulated immunoprecipitation (IP) by antibody (a55), and inhibited IP by normal mouse serum (N), from extracts of SV40 transformed T antigen (TA) positive mouse embryo cell cultures (line 215CSC); maximal effects for both reagents, at 0.1 mg/ml DNA. These IP all contained roughly equal amounts of both 94K TA and 55K protein (SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses). The two proteins were eluted from gels following SDS-PAGE separation of IP from 215CSC cell extracts. The TA was bound; 55K protein was not bound to a ct-DNA-cellulose column. Similar binding tests using extracts of 215CSC cells showed both 94K and 55K proteins present in both non-bound and bound column fractions. Embryonal carcinoma F9 cell extracts (contain 55K but not 94K protein) were passed through a ct-DNA-cellulose column. Both non-bound and one bound fraction eluted under same conditions as from 215CSC extracts; a new additional more tightly bound fraction was obtained, eluted by high pH buffer containing 0.7M NaCl. Inconsistencies in analyses (variously indicating 55K, or 94K, or both protein bands) were traced to variability both in F9 cells (lost 55K protein during tissue culture passage), and in antibody preparations (changed specificities). These problems have now been corrected and work continues.

Project Description

Objectives: To gain insight into the biological activity and function of proteins involved in the basic control mechanisms of cell growth and division and the maintenance of the normal phenotype, and to study the interaction and regulation of cellular and viral control proteins; to improve methods for their isolation and study.

Methods Employed: Tissue culture cell cultivation; cell cloning procedures; polyacrylamide gel electrophoresis; column chromatography, including DNA-cellulose chromatography; ultracentrifugation; 2-dimensional thin-layer electrophoresis and chromatography; various immunological assays to detect tumor antigens.

Major Findings: The tumor antigen (TA) of SV40 is thought to be responsible for malignant conversion and the maintenance of the transformed state of the cell by its effect on cell control mechanisms for growth, division and differentiation. It is known to bind specifically to SV40 DNA near the origin of replication, and non-specifically to cell DNA, and to play a role in the control of viral DNA replication and gene expression. The host 55K protein, somehow derepressed/stimulated by the viral infection is specifically associated with the viral TA. Although its activity in the cell is unknown, its appearance and its association with the TA following viral infection provide a basis for the expectation that this 55K host protein too might somehow be involved in similar control processes in the normal cell. The detection of this 55K protein in normal uninfected, midgestation murine embryos supports this presumption. It was felt that the 55K protein-host DNA interaction represented a contact point in the regulatory pathways of both the normal cell and the virus-cell systems. The nature and properties of this 55K protein became a focus of major interest. We wished to isolate this protein in relatively pure native form for further study.

Preliminary tests were performed first to help select a suitable isolation procedure. We determined the effect of added calf thymus DNA on immunoprecipitation of extracts of the 215CSC cells. The antibody preparation used was a monoclonal antibody against the 55K protein (a55) in SV40 induced hamster tumors. Normal hamster sera (N) was used as a control. The 215CSC cell line is an SV40 transformed, T antigen positive mouse embryo cell line. It was labelled with ^{35}S-methionine when the cells were (60-75)% confluent (visual estimate, under microscope), and was extracted in pH 9.0 buffer containing 0.5% each of deoxycholate and of Triton X-100, then dialyzed into phosphate buffer, 0.1M NaCl, pH 6.2, for studies of DNA effects. Aliquots of cell extract were incubated for 15 min., 5°C, with levels of DNA up to 2.5mg/ml, prior to immunoprecipitation with a55 media or with N sera. While the effect of added DNA was small and not outside the limit of experimental error, it was consistent and near this limit. All levels of DNA added produced a stimulation of precipitation by a55 media, and an inhibition of precipitation by N sera, with the maximal effect for both a55 and N occurring near 0.1 mg/ml DNA. Thus the ratio of radioactivity precipitated by (a55/N), after pre-incubation of the extract with this DNA concentration, increased to over 3.5X from the usual 2.0X in the absence of added DNA, while at higher levels of DNA the inhibition of precipitation by N serum was large enough that (a55/N) decreased; eventually this ratio reached a stimulation of only 1.1X at 2.5 mg/ml DNA. Polyacrylamide gel electrophoresis (PAGE) analysis of these immunoprecipitates showed that all contained both 94K and 55K species

in roughly equivalent amounts.

To examine further the DNA interaction with these proteins, tests were carried out using the individual purified proteins. Low levels of these ^{35}S-proteins were obtained by elution of the separated bands from a polyacrylamide gel after electrophoretic separation of the immunoprecipitate of the extract of 215CSC cells previously labelled with ^{35}S-methionine. These tests suggested that while the 94K SV40 TA did bind to a calf thymus DNA-cellulose column, the 55K cellular protein did not bind to this material. It was felt that the treatments to which these proteins had been subjected would have altered/denatured them differentially.

Experiments were carried out to determine binding of undenatured proteins to calf thymus DNA and the degree of separation that might be obtained between the 94K and 55K proteins by passage of labelled cell extracts through a calf thymus DNA-cellulose column. In most experiments the possible overloading of the column with excess cell extract was monitored by re-running an aliquot of the eluted non-bound fraction on a fresh DNA-cellulose column to insure that it contained a suitably low proportion of ^{35}S-material still capable of binding to the DNA. Subsequent polyacrylamide gel electrophoresis (PAGE) analysis of both non-bound and bound fractions from the DNA-cellulose column showed all the usual anti-T serum reactive protein species (94K, 55K and 19K) to be present in both fractions from cell extracts. Thus, the 55K cellular protein was present in both bound and non-bound fractions from cell extracts. These results are only apparently in disagreement with the preliminary indication that the 55K protein did not bind to DNA, obtained using the SDS-PAGE pruified 55K protein. The cell extracts, however, are known to contain 94K-55K protein complexes. Thus the 94K protein in these complexes must mediate binding of the 55K protein to DNA. In this case, attempts to elute these proteins from a DNA-cellulose column might contribute to effecting their separation.

We had previously examined the behavior of ^{35}S-215CSC cell extracts on DNA-cellulose columns (cf. this project report, last year), and obtained some preliminary data using embryonal carcinoma cells, EC F9, in much the same fashion as the 215CSC cells. It was hoped that as the EC cells contain the 55K cellular protein but not 94K TA, they might prove a useful source from which to purify protein. A large non-bound fraction eluted first, followed by two well separated bound peaks, the first eluted by the higher pH buffer (pH 8), the second, by this buffer containing 0.7M NaCl. These three peaks constituted (65-89)%, (20-6)% and (4-3)%, respectively, of the effluent among seven experiments performed. Clearly, F9 cell extracts contain protein which binds to the DNA-cellulose with two different affinities. The properties of the two fraction - one which does not bind, one which requires only the higher pH buffer for its elution - are similar to the two fractions obtained from the SV40 transformed 215CSC cells; the third fraction from the F9 cell extract, which requires both high pH and salt for it's elution, was not present in the 215CSC cells.

From the labelled F9 cells, immunoprecipitation and PAGE analysis of the fractions eluted from the DNA-cellulose columns produced variable results. In some F9 cell extracts no 55K protein band was found; in others, bands were found at the 94K region; and in others, both at the 94K and 55K regions. In some F9 cell extracts, however, only the 55K protein band was detected, as expected.

These variable results were finally traced to the reagents; to insufficient attention to monitoring of cells and antibody preparations. The 55K protein is known to be present in F9 cells only as long as the cells remain in an undifferentiated state; when the cells are induced to differentiate (e.g., by retinoic acid treatment), or simply "drift" to a more differentiated state during tissue culture passage, the 55K protein is lost (Oren et al., Mol. Cell. Biol. (1982) Chandrasekaran et al., ibid. (1982)). Apparently, F9 cells differentiated during growth on a few occasions, and the negative experimental results (the loss of the 55K protein) were at first puzzling until this factor was recognized. When fresh F9 cell stocks of lower passage number were obtained from frozen cell supply, they were found to possess the 55K protein. Other problems were encountered with variability and/or loss of activity of the antisera and of the monoclonal anti-55K media. These problems have now been corrected.

Significance to Biomedical Research and the Program of the Institute: It is important to try to understand better the nature and properties of the cell components which exert important influences upon cell growth characteristics and the ability of certain cells either to replicate normally, to form tumors or be rejected by the host, or to differentiate in a normal orderly fashion. The 55K protein apparently provides a unique activity for study, representing as it does a contact point in the regulatory pathways of two systems, the normal cell and the virus-cell systems. It is hoped that evaluation of the ability of the 55K protein (complex) to associate with DNA will provide a model for understanding better such interactions within the cell. These interactions may be the basis of the effect of some proteins in regulating differential cell growth and differentiation, since a similar 55K protein has been reported in EC cells (Linzer and Levine, Cell 17: 43 (1979)) and in embryos (Mora et al., Nature 288: 722 (1980)). As already pointed out, we have found this protein together with the SV40 TA, on the surface of SV40 transformed murine cells, as well as in their nuclei. The presence of such a regulatory protein on the surface may provide a link to mechanisms of surface control of cell growth and division. Many cell systems have recently been shown to possess this 55K protein, including cells of both virus infected or transformed or tumor origin, and uninfected normal cells, including midgestation murine embryos which we have studied (Z01 CB 05545; Z01 CB 05526). Such widespread occurrence underscores the apparent biological importance of this 55K protein (complex). It is hoped that evaluation of its ability to associate with DNA will provide a model for understanding better such interactions within the cell. These interactions may be the basis of the effect of some proteins in regulating differential cell growth and differentiation. Study of its behavior in these systems should contribute to a better understanding of basic cell processes at the molecular level.

Proposed Course of the Project: Work will continue on the following topics when I return from a work/study year abroad. The nature of the binding of the 55K protein to DNA will be investigated, first to determine whether it can bind directly to DNA or requires a mediator, in the form of another protein such as the SV40 TA. Attempts will be made to fractionate the SV40 anti-T reactive proteins from various sources to obtain each free of the others and not denatured by high concentration of detergent, urea or other denaturing solvent, to compare the 55K proteins from each source. Particular attention will be focussed on possible differences in properties and/or function of the 55K protein obtained from virus transformed cells or from normal embryos of F9 EC cells. We will attempt to

better understand the relevance of the various changes in cell biochemistry and immunochemistry, and the presence and function of the 55K protein, to spontaneous and to viral induced transformation, as well as to normal differentiation of cells.

Laboratory of Immunobiology

SUMMARY REPORT

October 1, 1981 to September 30, 1982

INTRODUCTION

The year 1982 was characterized by changes and uncertainties. 1981 was a difficult year because the serious illness of the Chief put undue stress on the senior members of the Laboratory of Immunobiology; the tragic death of the Chief in September 1981 induced even more stress on the staff. Nevertheless, productivity suffered little from these severe stresses and many important advances were achieved in the program of the Laboratory. Several changes in research emphasis also occurred after the death of the former Chief. Studies on bovine cancers were phased out (a continuation of the process already initiated last year) and studies on chemically induced breast cancer of rats received new directions. All research activities were eliminated from the Office of the Chief and a closer collaboration among the three sections has been encouraged. From all of these changes a stronger and more effective program has emerged: a program whose main aim is studying and elucidating on a broad basis the mechanisms of the effector arm of the immune system. Specifically three major areas are under study each of which relates to and interacts with the other two. In the Humoral Immunity Section the direct cytotoxic effect of complement on target cells is studied. The main questions are: by what mechanisms do immunoglobulins acquire complement fixing and activating properties, the relation of chain structure to binding and activity of complement components, especially C4, and the mechanism of the damage producing steps. Another major effort is directed at elucidating the behavior of cells under attack by complement, particularly the biochemical events that govern the maintenance of cell integrity and cell susceptibility in face of humoral immune attack. Monoclonal antibodies are produced to cell antigens and to drugs to elucidate the mode of action of antibodies at cell surfaces and to understand better the utilization and metabolism of drugs.

Several of the complement components serve as sources of kinins and other signaling molecules for many of the cellular components of the immune system. The program of the Immunopathology Section concerns two major cellular effectors of the immune system. One area concerns the chemotaxis of leukocytes, especially the questions of what causes the accummulation of leukocytes in inflammatory sites and why chemotaxis is depressed in patients with cancer. To facilitate these studies on a strict quantitative basis methods were developed using image analysis for counting migrating cells; active collaboration with the Surgical Service of the Bethesda Naval Hospital will allow a study of chemotactic responses of patients with breast and colon cancer. The second area in the Section concerns the mechanism of activation of macrophages to kill tumor cells. The key element is the in vitro assay of tumor cell killing by macrophages which has a high specificity and sensitivity. The high activity required for this assay is induced by a limited number of agents and persists for a short time. The availability of the assay makes purification of various signaling mechanisms possible and permits investigation of cell membrane and metabolic events that occur during activation.

The in vitro interaction of the effector arms of the immune system with cell surface factors has in vivo counterparts. In the Cellular Immunity Section studies have continued on development of methods for modifying host response to weakly or nonimmunogenic tumor cells. The transplantability of two nonimmunogenic guinea pig fibrosarcoma cell lines was profoundly changed after in vitro infection of the tumor cells with an amphotropic murine leukemia virus. Rejection of virus-infected cells appeared to be a host-mediated event since murine leukemia virus infection had no detectable cytopathic effect on the tumor cells. Tumor eradication occurred without development of immunity to cryptic, intrinsic tumor antigens. In progress are studies to evaluate the effects of in vivo infection of tumors with murine leukemia viruses. Growth of uninfected hepatoma cells or fibrosarcoma cells was suppressed at the site of rejection of virus-infected tumor cells. The model of breast cancer described in the previous annual report has been adapted to study the antitumor effects associated with administration of protein A. Studies of the basis of adoptive transfer of tumor immunity have continued to elucidate types of cells that are important in the donor population and to identify the cells that the host contributes.

I. HUMORAL IMMUNITY SECTION

In the Humoral Immunity Section the role of immunoglobulins at cell surfaces in binding and activating complement by the classical pathway and the cytotoxic effects of the activated C has been further analyzed. One of the major problems that have been intriguing immunologists is the relation between structure and function of the Ig molecule. It has been known for several years that Igs of the appropriate class (IgG and IgM) as a result of interaction with antigens may acquire the ability to bind and activate (fix or consume) complement. A major effort in the past year has been directed at elucidating the conditions that confer upon Igs C binding and activating properties. Advantage was taken of the fact that sera of many mammals contain C binding anti-methotrexate (MTX) IgM antibodies and that highly avid rabbit anti-MTX IgG antibody was available. These antibodies were capable of inducing C mediated lysis of sheep red cells to which MTX was coupled covalently. It was reported last year that IgM anti-MTX antibodies were bound to cells in three different forms: one that bound the first component of C and could activate the lytic sequence, another that also bound the first components but could not lyse the cells and a third that could do neither. The ratio of the different forms depended on the density of MTX coupled to the cell surface. From this it was concluded that hapten density induced "deformation" in the IgM molecule was a prerequisite for IgM to acquire C fixing property. These studies have been extended to IgG anti-MTX antibodies. In contrast to IgM, which is a "pre-aggregated" molecule, individual IgG molecules are incapable of activating C sequence even when in combination with appropriate antigens. Moreover, while there is evidence that soluble IgG can interact with C1q, the antibody binding subcomponent of C1, such interactions cannot be shown to occur at cell surfaces. Studies from this laboratory now showed that for IgG to acquire C1 binding and C activating properties, two signals were necessary: C1 binding that depended on the proximity of at least 2 IgG molecules not more than 30 nm apart and C activation that most probably depended on the angle of the Fab arms of the IgG molecules. Both of these functions depended on the distribution and density of hapten. The optimal distance between haptens for inducing C activating property in IgG was calculated to be about 5 to 7 nm which indicated an angle of about 50 to 60°. It was found that this is the same distance that induces optimal C activating property in the IgM molecule.

In concurrent studies on the mechanism of cell lysis by IgM antibodies and C, the hemolytic efficiency of cell bound IgM anti-Forssman antibody was studied. As mentioned above, not all C1 fixing cell-bound IgM molecules were capable of initiating or completing the lytic sequence. These studies established the fact that the hemolytic efficiency of rabbit anti-Forssman IgM antibody depended on the source of C components. With whole guinea pig C as few as one in 10 IgM molecules were hemolytic; with purified guinea pig components C1, C4 and C2 and with guinea pig C3-C9 one in three was lytic. When rat C3-C9 was substituted all IgM molecules were capable of lysing the cell. Evidence was obtained that the block occurred at the C4 level: at least two different kinds of C4 molecules were generated by the IgM-C1 complex one of which was incompetent with homologous reagents. From these studies it was concluded that C4 was a pivotal molecule in the classical pathway of C activation and that the form in which C4 appears in the sequence depended upon the Ig-C1 complex, which, in turn depended upon the distribution and density of the hapten interacting with the Ig.

These results were further accentuated by studies on the binding and chain structure of C4. Since it was shown in this laboratory that IgM, in contrast to IgG, was incapable of binding C4 to itself, it was concluded that during the activation of the classical C sequence, C4 had to bind to cell receptors at some distance from the IgM molecule. Furthermore, while at that distance, C4 had the capability of interacting with and binding C2 to generate the C42 enzyme. In studying the effect of removing the IgM from the cell surface by treatment with reducing agents (such as 2 mercaptoethanol) it was found that C4 was left intact on the cell surface. This was unexpected for C4 is comprised of three chains held together by disulfide bonds; these bonds can be easily broken by MSH when C4 was in the fluid phase. At present the increased resistance of bound vs fluid phase C4 to MSH is studied and is correlated to the activity of the various forms of C4.

To gain better understanding of the mechanisms whereby Igs interact with and activate C, monoclonal antibodies to methotrexate were prepared and characterized. Female BALB/c mice were immunized with conjugates prepared by covalently binding either a relatively high or low number of methotrexate molecules to keyhole limpet hemocyanin as the carrier immunogenic protein. An important discovery was that the efficiency of producing hybridomas with FO myeloma cells was shown to depend strongly on the molar ratio of drug to carrier: when the immunizing conjugate contained 400-500 molecules of methotrexate per hemocyanin molecule, the efficiency of producing hybridomas that secreted only anti-methotrexate was 39-58%. In contrast, only 3-13% of the hybridomas produced only anti-hemocyanin. When the immunizing conjugate contained only 50 molecules of drug per carrier molecule, the efficiency of producing hybridomas with only anti-methotrexate activity was only 4%, whereas hybridomas producing only anti-hemocyanin were produced in 27% efficiency. These results demonstrate the ability to influence the number of fusion products secreting monoclonal antibodies with desired specificity by manipulating the epitope density. Consistent with these results, mice immunized with the high drug/carrier immunogen had significantly higher levels of antimethotrexate in the plasma although the binding affinity of the antibodies was the same regardless of whether the mice had been immunized with an immunogen containing a high or low level of methotrexate.

Three IgG_1, two IgG_{2a}, and two IgG_{2b} monoclonal antibodies have been produced in the ascites fluid of mice at concentrations in the range of 2-3 mg/ml. The

IgG_1 antibodies bind neither complement C1 nor protein A of S. aureus, whereas all the IgG_{2a} and IgG_{2b} antibodies bind both proteins. These antibodies offer the advantages of chemical and functional purity, and are being used to extend our studies on the role of antibody aggregation and structural changes on the ability of IgG to interact with the components of complement and protein A.

The monoclonal antibodies are more specific for methotrexate compared to conventional antisera prepared in mice, rabbits, or a goat, and offer advantages for use in immunoassay. The antibodies produced in the ascites may prove to be useful standard reagents in immunological determination of methotrexate levels in cancer patients undergoing chemotherapy with methotrexate.

As in the past years efforts continued aimed at better understanding of the behavior of nucleated cells under immune attack. Various target cells have now been studied. As was shown originally the sensitivity of guinea pig hepatoma cells, L1 and L10, to C-mediated killing was dependent upon their lipid and/or fatty acid composition. Studies were initiated with human lymphoblastoid (Raji and PY) and mouse mastocytoma (P815) cell lines to determine if the sensitivity of these lines was also dependent upon the lipid or fatty acid composition. These cells show cell growth dependent variation in sensitivity to C-mediated killing. Cell cycle specific populations of cells did not demonstrate any variation in sensitivity. Lipid analysis and membrane fluidity were compared at the times the cells were sensitive and resistant to C-mediated killing. No correlation was found between lipid synthesis, membrane fluidity and sensitivity to C attack. There is suggestive evidence that the fatty acid composition of the lipid or lipid containing macromolecules varies in the cells. Like the hepatoma cells increase in unsaturated fatty acid content appear to be correlated with an increase in sensitivity to C killing. When the sensitive Raji cells were cultured at low density in media obtained from any phase of cell growth or in fresh media they became sensitive to C killing within 4-18 hr after culture. In contast cells at high density became susceptible only when cultured in fresh or log phase media.

It was also demonstrated that the susceptibility of these human and murine cells could be increased by treatment with selected chemotherapeutic drugs while certain hormones had no effect or rendered PY and Raji cells more susceptible to C killing. The drug effects were dose dependent and reversible upon culture of the cells for 4-18 hr in drug-free medium. In addition, most drugs were effective with log and late stationary phase cells but least effective with early stationary phase cells.

II. IMMUNOPATHOLOGY SECTION

Definition and characteristics of migrating and non-migrating leukocyte subpopulations. It was previously shown that the number of human blood monocytes responding to chemotactic stimuli under optimal conditions in vitro was less in cancer patients than in normal controls or hospitalized patients without cancer. Questions about the nature of this defect have led to studies of monocyte subpopulations, chemotaxin receptors, maturation dynamics and factors affecting chemotactic response. Studies were facilitated by development of a multiwell chamber for measuring chemotaxis, use of an image analyzer for counting migrated cells, and construction of a separation chamber that allows for collection of migrated and non-migrating cells.

Among normal human blood monocytes, 20-40% respond to chemoattractants. In the case of the peptide attractant, f-met-leu-phe (FMLP), the non-migrating monocytes lack the receptor for the attractant. This laboratory is now determining whether absence of FMLP binding by non-migrating monocytes is an isolated difference or whether these cells differ in many respects from the morphologically similar migrators. Populations of migrating and non-migrating monocytes were obtained by use of the chemotaxis separation chamber. Phagocytic capacity for IgG coated sheep erythrocytes was the same for the two populations. Respiratory burst activity was also studied by continuous measurement of superoxide anion production in response to phorbol myristate acetate (PMA) or chemotactic peptides. Migrating monocytes exhibited a respiratory burst to FMLP, whereas the non-migrators had little or no response. Relative to the migrators, the non-migrators had a much lower response to PMA. Thus, in addition to lack of the FMLP receptor, these subpopulations differ biochemically in other respects.

In an approach to the question of how maturation might affect the proportion of migrating and non-migrating monocytes, chemotaxis assays were made on young monocytes that repopulated the circulation during leukopheresis of normal human donors. Over a period of 2 hours, donors were depleted of approximately 10^9 monocytes, equivalent to the total in the circulating blood. New monocytes entered the circulation, since monocyte counts were normal at the end of the procedure. Blood samples were drawn at the start and end of leukopheresis and 3 hours later. The number of monocytes migrating to optimal chemotaxin concentrations was decreased to about half at the end of leukopheresis, with recovery 3 hours later. Since these changes were not observed in 3 sham donors who went through the same procedure without withdrawal of cells, they reflect functional characteristics of newly circulating monocytes. Chemotaxin bindings studies of these cells are in progress.

Among normal human peripheral blood neutrophils there is also a subpopulation, comprising 60-70% of the total, that is non-responsive to chemoattractants. In contrast to the non-responsive monocytes, the non-responsive neutrophil subpopulation has receptors for FMLP. Despite the absence of a migratory response, the non-migrating neutrophils produce superoxide in response to FMLP. Thus, subsequent to ligand-receptor binding there are 2 pathways, one leading to migration and the other leading to superoxide release; only the latter pathway is intact in the non-migrating neutrophil.

Chemoattractants and the differential accumulation of inflammatory cells. What mediators cause accumulation of inflammatory cells and how are differential patterns of inflammatory cell foci achieved? The chemotactic peptide FMLP was oxidized at the methionine residue to the sulfoxide or sulfone form. Both oxidized forms of the molecule failed to attract human neutrophils, but remained chemotactic for human monocytes despite lower binding affinity for both monocytes and neutrophils. This is an interesting model for differential accumulation of leukocytes at inflammatory sites.

C5a, derived by activation of the fifth component of complement, is the major chemoattractant that can be produced from serum. It was found that modification of C5 in a process that does not involve complement activation results in potent chemoattractant activity. This was done by ultracentrifugation of mouse serum and collection of the concentrated high MW residuum remaining in the bottom of the tube. Chemoattractant activity of this material was detected at dilutions relative to serum as high as 1/900 - comparable to the potency of serum-derived

C5a. The attractant was heat-stable. The precursor, or one of the reactants involved in attractant formation, was heat-labile. Neither classical nor alternative complement pathways appear involved, since attractant was formed during centrifugation in the presence of 0.01M EDTA. These findings suggest that at local inflammatory sites enzymatic modification of C5 may generate chemotactic activity without complement activation.

Macrophage activation for cytotoxicity. Macrophages activated in vivo or in vitro develop capacity to kill any of a diverse group of tumor cells. The same macrophage population exerts potent microbicidal activity against intracytoplasmic bacteria, Rickettsia tsutsugamushi, intraphagolysosomal protozoa, Leishmania tropica and extracellular helminths, Schistosoma mansoni. With each target, cytotoxic activity is short-lived with progressive and irreversible decay to baseline by 24 hours. Macrophages remain viable after 24 hours and are often able to kill the same target by other mechanisms such as specific antibody or phorbol myristate acetate (PMA)-mediated cytotoxicity. Characterization of activation signals and mononuclear phagocyte subpopulations that respond to these signals to develop this subset of transient, cytotoxic responses are major areas of investigation:

Macrophage activation by lymphokines (LK) for nonspecific tumor cytotoxicity requires multiple reactions. Stable, noncytotoxic cellular intermediates between immature mononuclear phagocytes and fully activated tumoricidal macrophages can be identified. For example, LPS at ng/ml concentrations synergistically increases cytotoxicity by macrophages treated with low LK concentrations (1/500). That cells can be activated by LK alone at high concentrations (1/20), however, suggests an LPS-like signal may be present in LK. Macrophages cultured 4 hours in low concentrations of LK (1/500) developed cytotoxic activity only after subsequent exposure to LPS or 1/20 LK for 1 hour; macrophages treated with LPS or LK alone were not cytotoxic. Thus, cells exposed to one signal (low concentrations of LK) enter into a receptive, primed state in which they are not yet active but can then be triggered by another signal (LPS or LK) to develop full cytotoxic activity. Activation by LK signals required a defined treatment sequence: optimal cytotoxicity occurred with cells treated (primed) 4 hours with low concentrations of LK and then triggered 1 hour with high concentrations.

Supernatant culture fluids from a phorbol myristate acetate (PMA)-stimulated subline of the murine EL4 thymoma activated inflammatory macrophages for nonspecific tumoricidal activity in vitro; active supernatants from PMA-stimulated EL4 were not directly toxic to tumor target cells in the absence of macrophages. Additionally, macrophages treated with supernatant fluids of PMA-stimulated EL-4 also developed potent microbicidal activity against the protozoan, Leishmania tropica. Macrophages treated with supernatant fluids of unstimulated EL-4, concanavalin A-stimulated EL-4 or PMA-stimulated BFS cultures (an IL-2 independent T cell line unrelated to EL-4) were not cytotoxic. Supernatant fluids from 10 other continuous T cell lines stimulated with PMA were also inactive. The titer of activity in cytokines from PMA-stimulated EL-4 was similar to that found in LK. The time courses for development of macrophage cytotoxic activity with EL-4 or LK activities were identical. Both EL-4 and LK activities were nondialyzable, destroyed at 100°C for 10 minutes and could be separated from IL-2 macrophage-granulocyte colony stimulating or B cell growth factors. Activities in the EL-4 fluids but not in LK were stable at pH 2. Further physicochemical characterization of the EL-4 cytokines showed 2 distinct activities that activated macrophages for cytotoxicity: one was indistinguishable from gamma interferon (50,000 mw, pI 5.2 to 6.0, pH 2 labile and neutralized by anti-gamma interferon

sera); the other was clearly distinct (23,000 mw, pl 4.4 to 5.2, pH 2 stable and unaffected by anti-gamma interferon sera). These results suggest that the EL-4 cell line may produce one or more of the macrophage activation factors present in LK.

Macrophages continuously exposed to LK and target cells throughout the 48 hour cytotoxicity assay exhibit 3-fold more tumoricidal activity than do cells optimally treated with LK before addition of tumor cells. Increased cytotoxic activity induced by continuous LK treatment was not due to direct toxic effects of LK on tumor target cells or to alterations in target cell susceptibility to cytopathic effects of LK-activated macrophages. Moreover, sensitivities of responsive macrophages to LK activation signals and time courses for onset and loss of tumorcidial activity during continuous exposure or LK pulse were identical. Analysis of macrophage or or LK dose-responses and time courses for development of cytotoxicity each suggest that differences in tumorcidal activity between macrophages continuously exposed or pulsed with LK were quantitative: the number of cytotoxic events was increased 3-fold during continuous LK treatment. Optimal levels of macrophage tumoricidal activity then occur only if effector cells, target cells and activation stimuli are simultaneously present for a defined time interval: tumor cells need not be present during the initial 2 to 3 hours of culture; LK can be removed after 8 hours with little or no loss of cytotoxic activity. However, removal of LK or target cells during the critical 4 to 8 hour interval decreased levels of cytotoxicity 3-fold. Thus, nonspecific effector function by LK-activated macrophages is controlled by both the physicochemical nature of the LK mediator and the time interval effector and target cells are exposed to LK.

III. CELLULAR IMMUNITY SECTION

Superinfection of nonimmunogenic guinea pig fibrosarcomas with murine leukemia viruses. To develop an approach to control of non-immunogenic tumors, fibrosarcomas were infected in vitro with murine leukemia viruses and the consequences of this infection on tumor growth, immunogenicity and cell viability was measured. Infection of two guinea pig fibrosarcomas with an amphotropic murine leukemia virus had no detectable cytopathic effects. Injection of virus-infected fibrosarcoma cells into syngeneic guinea pigs led to the formation of tumors which grew temporarily and then regressed; in contrast, uninfected tumor cells grew progressively leading to the death of the recipient. Animals injected with virus-infected tumor cells developed an immunologic response to virus-infected tumor cells; sera obtained from animals 21 days after injection of virus-infected tumor cells contained antibodies that reacted with virus-infected but not uninfected tumor cells. Antibodies in the sera of animals injected with virus-infected tumor cells could be detected by binding of Staphylococcal protein A, by antibody dependent cell cytotoxicity but not by complement mediated cytotoxicity. No evidence was obtained for virus-mediated augmentation of immunity to intrinsic tumor antigens. Animals immunized repeatedly with virus-infected tumor cells were as susceptible as unimmunized, control animals to a challenge with noninfected tumor cells.

To gain some insight into the processes involved in this form of tumor rejection a variety of in vivo and in vitro experiments were performed. Histologic studies performed in collaboration with Dr. Stephen Galli showed the accumulation of basophils between day 5 and 9 at sites of injection of virus-infected fibrosarcoma cells. Growth of uninfected line 10 hepatoma cells or uninfected fibrosarcoma

cells was suppressed at sites of rejection of virus-infected tumor cells.

Tumor rejection antigens of chemically-induced murine fibrosarcomas. To gain some insight into the nature of a viral-determined rejection antigen on a chemically-induced murine fibrosarcoma, C3H/HeN MTV- mice were immunized with an allogenic embryo cell line infected with 1 of 4 murine leukemia viruses and then challenged with living tumor cells. Murine leukemia viruses were selected so that they shared some but not all of the epitopes defined by a panel of monoclonal antibodies to antigens of endogenous murine leukemia viruses. Cells infected with the murine leukemia virus isolated from the transplantable tumor contained all epitopes defined by the panel of monoclonal antibodies. Cells infected with certain recombinant murine leukemia viruses expressed p15(E) epitopes but not some gp 70 epitopes. Transplantation protection experiments showed a correlation between the expression of gp 70 epitope "a" on the immunizing cells and the development of a degree of resistance against tumor challenge.

Adoptive transfer of tumor immunity. As part of a continuing effort to understand the mechanisms of tumor eradication by adoptively transferred immune lymphoid cells, we have examined the possible contribution of the host primary immune response to tumor antigens to the efficacy of adoptive transfer. The antimetabolite methotrexate was used to inhibit the host primary immune response to tumor antigens. Methotrexate prevented guinea pigs, immunized with a mixture of adjuvant and tumor cells, from developing the ability to reject a challenge with living tumor cells. Methotrexate-treated guinea pigs were fully able to serve as recipients of adoptively transferred immune lymphoid cells. Recipient T cells may cooperate with adoptively transferred spleen cells in effecting tumor rejection. To evaluate recipient T cells as a source of cytolytic precursor cells, guinea pigs were depleted of T cells by adult thymectomy, lethal whole body irradiation and bone marrow reconstitution. Guinea pigs immunized with mycobacteria and then treated with this T cell depletion procedure failed to express delayed cutaneous hypersensitivity to mycobacterial antigens and did not contain lymphoid cells that proliferated in response to mycobacterial antigens. T cell depleted guinea pigs were fully able to reject tumors after transfer of immune lymphoid cells.

We studied the influence of several inhibitors of host immunity on the adoptive transfer of tumor immunity. Lethal whole body irradiation of recipient animals did not interfere with expression of adoptively transferred tumor immunity. Carrageenan treatment of recipients did interfere with transfer of adoptive tumor immunity; inhibition of transfer did not appear to be the result of a direct toxicity of the carrageenan on effector lymphocytes.

Immunologic studies of primary mammary adenocarcinomas in Buffalo/N rats. Studies are in progress to develop a small animal model for Staphylococcal protein A mediated tumor regression. Preliminary studies suggest that primary breast carcinomas induced by N-methyl N-nitrosourea in Buffalo/N rats may be suitable for this purpose. Regression of primary mammary tumors has been observed after intravenous injection of plasma absorbed with protein A-sepharose or sepharose alone.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08525-06 LIB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immunotherapy of Primary Autochthonous Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Zbar	Chief, Cellular Immunity Section	LIB NCI
	J. Langone	Expert	LIB NCI
	S. Sukumar	Visiting Associate	LIB NCI
OTHER:	J. T. Hunter	Expert	LIB NCI
	E. Yarkoni	Guest Worker	LIB NCI
	N. Terata	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
Cellular Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 or Sepharose alone.

PHS-6040

Project No. Z01 CB 08525-06 LIB

Project Description

Objectives: The primary objective of this project is to test a variety of therapies in animal with primary cancer in the hope of providing guides to the immunotherapy of human cancer.

Methods Employed: Breast cancer was induced in rats by a single intravenous injection of N-nitroso N-methyl urea into approximately 50 day old animals. A dose of carcinogen was used that produced malignant disease in about 30 percent of the animals. A majority of animals with breast cancer developed only a single lesion. Animals with single primary autochthonous breast cancer (5-12mm in diameter) were treated by repeated intravenous injections of plasma absorbed with protein A-Sepharose, or Sepharose alone. Plasma donors were untreated control rats or rats with mammary tumors. Rats were killed 50 days after initiation of treatment; each mammary tumor was weighed; comparisons between groups were made with the Mann-Whitney U test.

Major Findings: Tumor bearing rat plasma absorbed with Sepharose or protein A Sepharose inhibited growth of primary mammary adenocarcinomas in Buffalo/N rats. Tumor weight in animals treated with absorbed plasma obtained from tumor bearing animals was significantly less than tumor weight in animals treated with absorbed plasma from untreated control rats or tumor weight in untreated control rats.

Significance to Biomedical Research and Program of the Institute: Recent clinical studies indicate 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 greatly facilitate investigation of these questions.

Proposed Course of Project: Experiments will be performed to determine whether the observed antitumor effects are reproducible. Antitumor effects observed with plasma from tumor-bearing animals absorbed with Sepharose alone were unexpected; all plasmas will be tested for the presence of endotoxin since this product can cause regression of experimental tumors. A second trial of efficacy of plasma therapy will be performed; rats will be treated with unabsorbed plasma from control or tumor bearing rats, or absorbed plasma from control or tumor bearing rats. We will test the transplantable rat mammary adenocarcinoma 13762 to see if this tumor responds to treatment. The antitumor activity of protein A alone will be evaluated.

Publication

Rapp, H.J.: Immunotherapy of animals with primary induced cancer. Letter to the Editor, Cancer Immunol. Immunother. 11: 181-182, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08527-06 LIB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immunotherapy of Animals with Clinical Stage II Malignant Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Zbar	Chief, Cellular Immunity Section	LIB NCI
OTHER:	S. Sukumar	Visiting Fellow	LIB NCI
	E. Yarkoni	Guest Worker	LIB NCI

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to establish the conditions and methods best suited to the prevention and treatment of cancer by Immunostimulation. The area of current interest is active specific immunotherapy of animals with microscopic lymph node metastases or occult visceral metastases remaining after surgery.

PHS-6040

Project Description

Objectives: The primary objective of this project is to define the methods and conditions best suited for immunotherapy of metastases remaining after surgical removal of primary tumors.

Methods Employed: Tumor. A transplantable syngeneic cavian hepatocellular carcinoma, designated line-10, was used.

Cavian model for clinical stage I malignant disease. Line-10 cells were injected intradermally into strain-2 guinea pigs. When dermal tumors were palpable, (day 7) animals were treated by active specific immunotherapy.

Treatment of visceral tumor implants. Line 10 cells (10^6) were injected into the dorsal penal vein of each strain 2 guinea pig.

Major Findings: Previous reports have described in detail the efficacy of active specific immunotherapy in the treatment of the line 10 hepatoma of strain 2 guinea pigs. The findings made during the past year can be summarized as follows.

Established dermal tumors and microscopic lymph node metastases were eradicated by active specific immunization. Effective vaccines contained irradiated line 10 tumor cells and an oil-in-water emulsion of heat-killed cells of Mycobacterium bovis strain bacillus Calmette-Guerin (BCG). Squalane or squalene-in-water emulsions, prepared by ultrasonication, containing mg doses of mycobacterial cells were effective adjuvants.

Established visceral micrometastases of the line 10 hepatoma could also be eradicated by active specific immunotherapy. Mycobacterium phlei was an effective substitute for BCG.

Emulsions containing killed BCG were good adjuvants after prolonged storage at 4° C, but lost most of their adjuvant activity after autoclaving or freezing.

Significance to Biomedical Research and the Program of the Institute: The results of these investigations have defined conditions essential for the success of active specific immunotherapy in one transplantable tumor model system. Considerable progress has been made within this model system in determining dose requirements for tumor antigen and adjuvant. Information has been obtained on optimal adjuvant formulation. These results may ultimately be valuable in the design of active specific immunotherapy trials in humans.

Proposed Course of Project: Work on this project has been completed. Attention will be directed in the coming year to development of alternative methods for influencing the growth of nonimmunogenic tumors. Superinfection of tumor cells with murine leukemia viruses and induction of mutations in tumor cells will be evaluated as potential methods for modifying host response.

Publications

Ashley, M.P., Zbar, B., Hunter, J.T., Sugimoto, T., and Rapp, H.J.: Post-surgical treatment of microscopic lymph node metastases with vaccines containing tumor

cells and BCG cell walls. In Israel, L., Lagrange, P., and Solomon, J.C. (Eds.): Les Colloques de l'INSERM, Cancer Immunology and Parasite Immunology. Paris, INSERM, 1980, Vol. 97, pp. 243-253.

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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., in press.

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Yarkoni, E., Hunter, J.T., Sukumar, S., and H.J. Rapp.: Active specific immunotherapy of guinea pigs with visceral tumor implants. Cancer Immunol. Immunother. 12: 273-275, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08528-06 LIB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Mechanism of Delayed Hypersensitivity and Tumor Graft Rejection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	N. Terata	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to develop methods for modification of host response to nonimmunogenic tumors. The current area of interest is the biologic and immunologic consequences of superinfection of cavine tumors with murine leukemia viruses.

Project Description

Objectives: The goals of this project are to develop methods to modify host response to nonimmunogenic tumors, to study the basis of rejection of murine leukemia virus-infected tumor cells and to define the nature of cross-reacting tumor rejection antigens on chemically-induced murine fibrosarcomas.

Methods Employed: Animals. C3H/HeN MTV- mice were obtained from the Charles River Laboratories, Wilmington, Mass. Strain 2 guinea pigs were obtained from the Experimental Animal Breeding Facility of the National Cancer Institute, Frederick Cancer Research Facility, Frederick, Maryland.

Murine tumors. A transplantable syngeneic fibrosarcoma, designated 1063, was induced in the subcutaneous tissues of a C3H/HeN Icr mouse with a pellet of 1% 3-methylcholanthrene. Tumor cell suspensions were prepared by enzymatic digestion of minced tumors.

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.

Immunization of mice with allogeneic, virus-infected embryo cells. SC-1 cells, a clonal line of wild mouse embryo cells, were used for immunization of C3H/HeN mice. SC-1 cells were infected with one of the following MuLV: MuLV 1063c, MuLV AKR 13, MuLV 4070A or MuLV 247. To determine the degree of resistance produced by immunization, control and immunized mice were challenged with graded doses of tumor 1063 cells. The $\log_{10}$TD50 for control and immunized animals was calculated and compared with Pizzi formula; response to challenge was also evaluated with the Mantel-Haenszel test.

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 MuLV 4070A. 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.

Major Findings: Influence of MuLV 4070A infection in vitro on transplantability of two guinea pig fibrosarcomas. Two fibrosarcomas, syngeneic to strain 2 guinea pigs, were incubated in vitro with the amphotropic MuLV 4070A. Tumor cells exposed to MuLV 4070A expressed MuLV antigens and released infectious virus and reverse transcriptase into supernatant fluids; uninfected tumor cells did not release virus or express MuLV antigens. Inoculation of uninfected 104C1 cells or 107C3 cells into strain 2 guinea pigs led to the formation of progressively growing dermal tumors. In contrast, virus-infected 104C1 cells or 107C3 cells

did not grow progressively. There were no differences in viability, as determined by trypan blue dye exclusion of preparations of virus-infected and control, uninfected fibrosarcoma cells. Expression of viral antigens, release of infectious MuLV and growth and regression of infected cell lines have been stable characteristics of the cell lines for 6 months and 25 passages.

Histology of skin nodules in guinea pigs receiving murine leukemia virus-infected or uninfected fibrosarcoma cells. Few inflammatory cells were detected at sites of injection of uninfected tumor cells; in contrast, examination of sites of injection of virus-infected cells revealed the presence of numerous basophils among the tumor cells. Basophils were first detected on day 5 after tumor cell injection and formed a prominent part of the inflammatory cell infiltrate between day 5 and day 9.

Challenge of guinea pigs immunized with virus-infected tumor cells with uninfected tumor cells. Experiments were performed to determine whether guinea pigs immunized with MuLV 4070A-infected line 104C1 or 107C3 had resistance to challenge with uninfected tumor cells. There were no significant differences in tumor incidence in immunized animals compared to unimmunized control animals. While normal guinea pigs were able to reject greater than 10^6 virus-infected tumor cells, immunized guinea pigs failed to reject 100 fold fewer uninfected tumor cells.

Response of guinea pigs immunized with irradiated, uninfected tumor cells admixed with adjuvant to a challenge with uninfected tumor cells. Animals were immunized with a procedure found to produce strong immunity to the line 10 hepatoma of strain 2 guinea pigs. No intrinsic antigens were detected with this method of immunization.

Influence of chronic methotrexate treatment on growth and regression of MuLV-infected fibrosarcoma cells. Chronic methotrexate treatment has been shown to be a potent inhibitor of the development of contact sensitivity, of delayed hypersensitivity to protein antigens and of IgG formation. Recent studies by Shu have established that methotrexate treatment modified the host response to vaccination with mixtures of line 10 tumor cells and BCG cell walls. We tested whether the growth and regression of MuLV 4070A-infected tumor cells would be inhibited by methotrexate treatment. Chronic administration of methotrexate slightly delayed the regression of virus-infected tumor cells. Regression of virus-infected tumor cells occurred in the face of severe impairment in the ability of treated animals to form protein A fixing antibodies to virus-infected tumor cells and in the ability to develop immnunity to the line 10 hepatoma.

Influence of chronic cyclophosphamide treatment of the growth and regression of MuLV-infected tumor cells. Cyclophosphamide is a particularly potent inhibitor of antibody formation in guinea pigs and has been shown to prevent rejection of Moloney sarcoma virus-induced tumors in adult BALB/c mice. We tested whether cyclophosphamide administered according to schedules developed for inhibition of antibody formation would inhibit tumor regression. Cyclophosphamide treatment delayed the regression of MuLV 4070A-infected 104C1 cells. Regression of virus-infected tummor cells occurred in the face of impairment in ability to form protein A fixing antibodies to virus-infected tumor cells.

Kinetics of development of protein A-fixing antibodies to MuLV 4070A-infected fibrosarcoma cells. Antibodies to virus-infected tumor cells were detected by the method of Brown et al. Antibodies to virus-infected fibrosarcoma cells were detected on day 14 after tumor cell injection but not on day 7 after tumor cell injection. Animals that received methotrexate, 5mg, on alternate days, starting on the day of tumor cell injection were tested for the presence of antibodies to virus-infected tumor cells. A total of fifteen injections were given. Antibodies to MuLV-infected target cells were first detected in methotrexate-treated animals, 35 days after cell injection.

Suppression of growth of bystander targets as sites of rejection of murine leukemia virus-infected target cells. Infection of cavine fibrosarcoma cells with an amphotropic murine leukemia virus had a remarkable effect on tumor growth. Tumors which ordinarily grew progressively after intradermal injection, grew temporarily and regressed if the tumors had previously been infected in vitro. We tested whether the growth of uninfected hepatoma cells or uninfected fibrosarcoma cells would be modified by coinoculated virus-infected fibrosarcoma cells. The response to injection of mixtures of virus-infected and uninfected tumor cells could be divided into four stages: formation of a dermal tumor nodule between days 0 and 7; regression of the dermal tumor nodule between days 8 and 14; a tumor-free latent period of varying duration and reappearance, in some animals, of tumor nodules at sites of injection of cell mixtures. When 107C3 4070A cells and line 10 cells were mixed at a ratio of 10 virus-infected tumor cells to 1 uninfected tumor cells, complete regression of tumor was observed in 16 of 19 animals. Injection of virus-infected tumor cells at a separate dermal site had no influence on the growth of uninfected line 10 cells; there was some suggestion that i.p. injection of virus-infected tumor cells had an inhibitory effect of the growth of intradermally inoculated line 10 cells.

When 104C1 4070A tumor cells and 104C1 cells were mixed at a ratio of 1 virus-infected tumor cell to 1 uninfected tumor cells, complete regression of tumor was observed in 7 of 25 animals. Tumors which reappeared at sites of injection of mixtures of uninfected and infected 104C1 cells were free of infectious virus and did not express viral antigens.

Experiments were performed in an attempt to prevent reappearance of tumors at sites of injection of mixtures of infected and uninfected tumor cells. The data suggest that administration of cyclophosphamide, 10 mg daily, between day -2 and day 5, decreased the frequency of tumor reoccurrence. Chronic administration of cyclophosphamide delayed the rejection of tumors formed from injection of mixtures of 107C3 4070A cells and line 10 hepatoma cells. Administration of methotrexate delayed the rejection of tumors formed from injection of mixtures of 107C3 4070A cells and line 10; no decrease in frequency or reoccurence was evident.

Experiments were performed to compare the ability of MuLV 4070A-infected normal embryo cells, 104C1 cells or 107C3 cells to inhibit the growth of admixed 104C1 cells. Reciprocal experiments were performed to determine if MuLV-infected normal embryo cells, 104C1 cells or 107C3 cells would inhibit the growth of 107C3 cells. Of the three virus-infected cell lines, 107C3 appeared to be most effective in leading to suppression of growth of admixed tumor cell targets. Of the two target cell lines, 104C1 cells appeared to be the more sensitive to suppression of tumor growth.

Inhibition of growth of a transplantable chemically-induced murine fibrosarcoma by immunization with virus-infected embryo cells: correlation with the presence of epitopes defined by monoclonal antibodies to gp70 and p15(E). The phenotypic expression of murine leukemia virus-associated antigens by SC-1 cells infected with different MuLV was evaluated using a panel of monoclonal antibodies to gp70 and p15(E). SC-1 cells infected with MuLV 1063c expressed gp70 epitopes a,b,c and d and p15(E) epitopes a, b, and c. SC-1 cells infected with MuLV 247 expressed gp70 epitopes b and c and p15(E) epitopes a,b and c. SC-1 cells infected with MuLV AKR 13 or MuLV 4070A did not express the gp70 epitopes; p15(E) epitopes a, b, and c were detected on cells infected with MuLV AKR 13; p15(E) epitopes b and c were detected on cells infected with MuLV 4070A.

Increased resistance to challenge with tumor 1063 was observed in mice immunized with SC-1 cells infected with MuLV 1063c. Immunization of mice with SC-1 cells infected with MuLV 247, AKR 13 or 4070A did not increase resistance to tumor challenge. Increased resistance to challenge with tumor 1063 was correlated with the expression of gp70 epitopes a and d in the immunizing cell preparation. Immunization with MuLV 1063c-infected SC-1 cells consistently produced increased resistance to tumor 1063 challenge; the degree of resistance was relatively weak; a 10 fold increase in TD50 was usually observed.

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. One method that has been suggested for increasing host resistance to these tumors has been to introduce rejection antigens into these tumors with nonlytic, budding, murine leukemia viruses. The results of our experiments suggest that intrinsic tumor rejection antigens are a prerequisite for murine leukemia virus-mediated augmentation of immunity to uninfected tumor cells. Our results closely resemble those of Stephenson and Aaronson and those of Sobis et al. who studied the host immune response to tumors transformed by Moloney sarcoma virus, but not releasing MuLV. These nonproducer tumors lacked detectable rejection antigens. Superinfection of these nonproducer tumors led to the acquisition of rejection antigens; however, immunization with virus-infected transformed cells did not produce resistance to challenge with uninfected tumor cells. To date, the results of these experiments argue against the utility of immunization with MuLV-infected tumor cells in attempts to eradicate residual malignant disease remaining after surgery. In view of the dramatic alterations of transplantability of in vitro virus-infected tumor cells, superinfection of tumors in vivo may be a valuable experimental goal. Although we have observed complete suppression of uninfected tumor cells at sites of infection of infected tumor cells, we have been unable to obtain complete suppression of tumor growth after systemic administration of virus-infected tumor cells or virus alone.

The experiments which show a correlation between the the presence of gp70 epitopes a and d on the immunizing cell and the response to challenge with tumor 1063 cells have focused attention on particular portions of the gp70 molecule as potential weak tumor rejection antigens.

Proposed Course of the Project: We will continue our studies on modification of host response to weakly immunogenic tumors. Studies of superinfection of guinea pig tumors will be pursued with several murine leukemia viruses, and several additional guinea pig tumors. These studies are necessary to determine whether murine leukmia viruses other than MuLV 4070A would be able to stimulate host response to cryptic tumor rejection antigens of lines 104C1 and 107C3. Tumors with known transplantation rejection antigens will be superinfected in an attempt to develop knowledge of effective conditions for immunization with virus-infected tumors in the guinea pig. Attempts will be made to prevent reappearance of tumors at sites of injections of mixtures of virus-infected and uninfected tumor cells by administration of immune modifying drugs. When conditions have been developed which favor complete regression of uninfected tumor cells at sites of injection of virus-infected and uninfected tumor cells, attempts will be made to achieve systemic inhibition of tumor growth with murine leukemia virus alone and with murine leukemia virus-infected cells.

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., in press.

Zbar, B., Sugimoto, T., Manohar, V., and Ashley, M.P.: Immunoprophylaxis of transplantable methylcholanthrene-induced murine fibrosarcomas by immunization with syngeneic embryo cells infected with murine leukemia virus. In Israël, L., Lagrange, P., and Salomon, J.C. (Eds.): Les Colloques de l'INSERM, Cancer Immunology and Parasite Immunology. Paris, INSERM, 1980, Vol. 97, pp. 231-242.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08530-03 LIB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immune Eradication of Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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2.5	1.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chronic MTX treatment had no effect on established tumor immunity, but prevented the development of the primary immune response to tumor. Adoptive transfer of tumor immunity was insensitive to MTX treatment indicating that the primary cell-mediated response of the recipient was not required for effective transfer. The efficacy of adoptive immunity was evaluated in guinea pigs depleted of T cells. Despite demonstrable deficiency in T cell reactions, "B" guinea pigs were capable of rejecting tumor after adoptive transfer. These results suggest that expression of adoptive immunity was independent of recipient T cell function. Evidence for the participation of recipient components other than T cells was obtained using the inhibitors carrageenan and trypan blue.

PHS-6040

Project Description

Objectives: The principal goal of this project is to study the mechanisms of adoptive immunity to the guinea pig line-10 hepatoma. We have looked for evidence of 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. Animal were challenged with ascites line-10 cells intradermally. Growth of the inoculated tumor was monitored for evaluation of tumor immunity.

Production of T cell depleted "B" guinea pigs. "B" animals were produced by adult thymectomy, lethal whole body irradiation and bone marrow transplantation. Fifteen days after thymectomy, adult guinea pigs were lethally irradiated (1,000 rads) and reconstituted with 10^8 syngeneic bone marrow cells from age and sex matched donors. The efficacy of the T cell depletion procedure was evaluated by measurement of delayed cutaneous hypersensitivity reactions to purified protein derviative (PPD) and by measurement of the in vitro proliferative response to Con A, PPD and allogeneic cells.

Treatment of guinea pigs with methotrexate (MTX), carrageenan or trypan blue. MTX (5 mg) was administered by intraperitoneal injection every 48 hr. Each guinea pig usually received a total of 9-10 injections. Carrageenan was disolved in saline by boiling. Carrageenan (3mg/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 cavities 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: Last year we reported that quantitative analysis of adoptive immunity to the guinea pig line-10 hepatoma revealed that the number of tumor cells eradicated increased exponentially as a function of the number of immune lymphoid cells transferred. One hypothesis that might explain this observation in that the recipient's primary immune response to the tumor challenge contributes to tumor eradication following adoptive transfer. The efficacy of adoptive was

analyzed in guinea pigs that had been treated with MTX to selectively inhibit the induction of cell mediated immunity. Our results demonstrated that guinea pigs were capable of being adoptively immunized when they were treated with MTX at regimens which would block active immunization. Thus the rejection of tumors by adoptive immunity was independent of the primary immune response that might have occurred in the recipient.

The failure of immune effector cells to mediate tumor cytotoxicity in vitro, documented in our previous study and in other recent reports, suggests that transferred immune cells may serve helper or amplifying functions in the induction of cytolytic T lymphocytes. This possibility was investigated in T cell depleted guinea pigs. Our procedures for T cell depletion were verified by a number of in vivo and in vitro tests. Despite deficiency of T cells, "B" guinea pigs were able to reject the inoculated tumor when transfused with immune spleen cells. This observation affords direct evidence that T lymphocytes of the host origin were not involved in tumor rejection.

To investigate the contributions of the 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, adoptive transfer experiments were carried out in animals treated with carrageenan or trypan blue. Treatment of recipient guinea pigs with a total of 120 mg carrageenan starting one day prior to adoptive transfer and tumor inoculation blocked the expression of adoptively transferred immunity. Trypan blue treatment of recipient guinea pigs starting one day prior to adoptive transfer and tumor inoculation, on the other hand,, did not seem to affect the expression of immunity to line-10 tumor. Treatment with each chemical resulted in considerable death of the 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 indicates that trypan blue's effect on the adoptive immunity is 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 carragenan 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.

Significance to Biomedical Research and the Program of the Institute: Interest in the possibile 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 and resistant to 500 rad irradiation.

Proposed Course of Project: This research project was initiated to investigate the efficacy of immune lymphoid cells in the control of neoplastic disease. To overcome histocompatibility barriers to adoptive transfer of immunity, T cell growth factor (or interleukin 2) may provide a means to maintain and expand autologous immune effector cells. Our results afford a better understanding of the principle of adoptive immunity. Logical extension of this project includes: 1) production and biological characterization of guinea pig T cell growth factor. Since guinea pig T cells did not respond to T cell growth factors of human, rat or mouse origin, the preparation of guinea pig T cell growth factor needs to be pursued independently. 2) quantitative study of the efficacy of in vitro expanded immune T cells in the adoptive transfer of immunity to guinea pig line-10 tumor. 3) analysis, with the aid of T cell growth factor, of conditions for in vitro sensitization to tumor specific transplantation antigens. 4) cloning of T cell colonies with references to adoptive immunity against only inoculated skin tumor but also established metastatic tumors.

Publications

Shu, S., Steerenberg, P.A., Hunter, J.T., Evans, C.H., and Rapp, H.J.: Adoptive immunity to the guinea pig line-10 hepatoma and the nature of in vitro lymphoid-tumor cell interactions. Cancer Res. 41: 3499-3506, 1981.

Shu, S., Hunter, J.T., Rapp, H.J, and Fonseca, L.S.: Mechanisms of immunologic eradication of a syngeneic guinea pig tumor. I. Quantitative analysis of adoptive immunity. J. Natl. Cancer Inst. 68: 673-680, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08550-08 LIB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Modification of Tumor Cells and Immune Cytolysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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0.8	0.8	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

PHS-6040

Project Description

Objectives: 1) To study the mechanism whereby enzymes, metabolic inhibitors and anti-lipidemic agents increase the sensitivity of tumor cells to immune attack; 2) to study mechanisms whereby hormones decrease sensitivity of cells to immune attack; 3) to determine metabolic pathways and the physical and chemical properties 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 complement (C) 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}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-Forssman 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 correlated with their ability to synthesize complex lipids, but not DNA, RNA, protein, or complex carbohydrate.

Metabolic inhibitors (adriamycin and actinomycin D) that increase the sensitivity of the cells to antibody-C killing were examined for their effects on the ability of the cells to synthesize and incorporate specific lipids into plasma membrane and intracellular membrane fractions. Cells that had been rendered sensitive were inhibited in their incorporation of newly synthesized phosphatidylcholine and cholesteryl ester into the plasma membrane, as well as incorporation of phosphatidylcholine, cardiolipin, cholesteryl ester, and triglyceride into including mitochondria, endoplasmic reticulum, nuclear membrane, or microsomes. Drug-treated cells recultured in the absence of the drug regained their ability to resist antibody-C killing and to synthesize and incorporate lipids into plasma and intracellular membranes. These data suggested that agents modifying the sensitivity of the tumor cells to humoral immune killing have a concomitant effect on plasma membrane and intracellular lipid synthesis.

Line-10 cells that had been rendered resistant to killing by human C following incubation for 1 hr with insulin or hydrocortisone were enhanced in their incorporation of newly synthesized phosphatidylserine, phosphatidylcholine, and triglycerides into the plasma membrane as well as phosphatidylcholine, phosphatidylserine, and cholesteryl ester into mitochondria, endoplasmic reticulum, nuclear membrane, or microsomes; these cells were inhibited in cardiolipin synthesis. Cells cultured for 4 hr with hormone regained their sensitivity to C-mediated killing and reverted to control levels in their ability to synthesize and incorporate lipids into plasma and intracellular membranes. These data suggest that agents which increase the resistance of the tumor cells to humoral immune killing stimulate the synthesis and incorporation of specific complex lipids into plasma membrane and intracellular organelles; these effects generally were opposite those observed after treatment with metabolic inhibitors.

Drug treatment reversibly increases, whereas hormone treatment reversibly decreases the polyunsaturated fatty acid content of the cellular lipids. Hormone treatment also increases 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 decreases 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 suggest 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- or 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 recultured for 24 hr in serum-containing tissue culture medium; the cells regained controls levels of susceptibility to antibody-C killing at this time. These results suggest 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 absorption test. Cells isolated in G_1 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.

The sensitivity of the human cell lines, Raji and PY, and the mouse mastocytoma P815 to killing by antibody plus C can be increased following treatments with various metabolic inhibitors. The effectiveness of the drugs in rendering the human cells sensitive was dependent upon the growth phase of the cells in culture. Cells in early stationary phase were generally not rendered susceptible to killing whereas cells in lag or log phase of growth could be rendered more susceptible than untreated control cells. Similar results were observed with P815 cells while in addition cells in late stationary phase of growth could be rendered susceptible. The effectiveness of drug treatment was also dependent upon whether the cells were suspended in fresh or conditioned (i.e. medium collected from log or stationary phase of growth) medium. These results suggest that physiological properties of the cells in addition to physicochemical properties will influence susceptibility to immune killing.

Studies on the effects of lipids on C-mediated killing of line-1 and line-10 tumor cells showed that phosphatidylglycerol, cardiolipin, and phosphatidylethanolamine were effective in inhibiting the cytotoxic action by human, rabbit and goat C. Phosphatidylserine and phosphatidylglycerol were effective in inhibiting GPC activity while phosphatidylglycerol enhanced rat C activity against line-10 cells sensitized with antitumor antibody but not anti-Forssman antibody. The inhibitory effects of the various phospholipids are dependent upon antibody used to sensitize the cells, on concentration of lipids used to treat the C and on the time the lipids were added to C. The inhibitory effect of the lipids appeared to be at the stage of formation of T*. Addition of lipids to T* did not interfere with its transformation to dead cells. 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.

Under conditions of limiting C almost all lipids tested interfered with C activity. Additional studies indicated that the fatty acyl composition and subtle structural configuration of the lipids influenced C activity. Modification of C activity also occurred upon addition of free fatty acid to C. Enhancement or inhibition or no effect was observed and appeared to be dependent upon the fatty acid, fatty acid concentration, specificity of the antibody and concentration of C. Cells pretreated with selected fatty acids, but not more complex lipids, were increased in their sensitivity to killing by C.

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.

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.

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.: Synthesis of lipids or lipid-containing macromolecules in tumor cells: Relevance to host defense. Survey Immunol. Res., in press.

Ohanian, S.H., Borsos, T. and Schlager, S.I.: Enhancement of immune killing of tumors following treatment with chemotherapeutic drugs. In Israël, L., Lagrange, P. and Salomon, J.C. (Eds.): Les Colloques de l'INSERM, Cancer Immunology and Parasite Immunology. Paris, INSERM, 1980, Vol. 97, pp. 199-211.

Ohanian, S.H., Schlager, S.I. and Saha, S.: Effect of lipids, structural precursors of lipids and fatty acids on complement-mediated killing of antibody-sensitized nucleated cells. Mol. Immunol. 19: 535-542, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08551-07 LIB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Induction of Tumor Immunity by Chemotherapy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Ohanian	Research Microbiologist	LIB NCI
OTHER:	T. Borsos	Chief, Humoral Immunity Section	LIB NCI

COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Immunobiology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Selected chemotherapeutic agents injected into tumors growing in vivo cause the tumor to regress. Animals cured of their tumors are resistant to rechallenge with the same tumor. The purpose of this work is to analyze the mechanisms of intralesional chemotherapy and include histological techniques, and methods for analyzing humoral and cellular mediated immune mechanisms.

Project Description

Objectives: 1) To cure tumors and induce tumor immunity by direct treatment of established tumors with chemotherapeutic agents; 2) to study the development of cellular and humoral immunity in cured animals.

Methods Employed: Inhibition of tumor growth in animals cured of tumors by intratumoral chemotherapy is used to measure the development of tumor immunity. Intradermal or sucutaneous implantation of guinea pig and mouse tumors into syngeneic animals are used to determine malignancy. Immunological techniques employed to measure humoral and cellular immunity include complement fixation, antibody-complement-mediated killing and cell-mediated cytotoxicity. Histological and radioisotope procedures are used to determine the effect and distribution of chemotherapeutic agents.

Major Findings: Intralesional injection of selected chemotherapeutic drugs causes regression of the malignant guinea pig hepatoma line-10. Intralesional injections of actinomycin D, adriamycin, BCNU, cisplatinum, vincristine, and bleomycin were effective in causing tumor regression. Methotrexate, DTIC, and 5 FU are not effective in causing regression of tumor. Injections, starting 7 days after tumor implantation, were given 5 times a week for 3 weeks. At this time, metastasis to draining lymph nodes had already occurred. Subsequent experiments showed that single intralesional injection of drugs was as effective as multiple injections of the drugs in causing tumor regression. However, systemic injection of similar concentrations of the drugs into tumor-bearing animals were toxic. The selection of chemotherapeutic drugs has been expanded to include bleomycin. The effectiveness of the drugs was dependent upon the concentration of drug; high concentrations of all drugs effective in curing animals interfered with the development of tumor immunity. With selected drugs such as BCNU, vincristine, bleomycin, there was an optimal concentration which cured animals and did not interfere with development of tumor immunity. Various combinations of ineffective drugs were not effective in curing animals.

Histological studies following a single intralesional injection of adriamycin or DTIC into line-10 tumor showed little host cell infiltration into the tumor site following injection of adriamycin compared to DTIC or saline-injected controls. The regional draining node of tumor-bearing animals was tumor-free within 7 days following intralesional injection of adriamycin. Minimal systemic effects were detected in adriamycin-treated animals as determined by differential analysis of blood and development of immunity in animals cured of their tumors.

Analysis of the persistence of drug following intralesional injection of ^{57}Co-bleomycin into tumor-bearing mice indicated that no difference in the rate of elimination following i.v., i.p., i.m., s.c., or i.t. injection from tumor-free or tumor-bearing animals with 5 day old tumors. Significant retarded elimination was observed in animals bearing 12 day old tumors. Differences in distribution of labeled drug into various organs was observed following injection by the different routes. In general, there was less of a systemic distribution when the drug was injected i.t., i.m., or s.c. and tumor-bearing animals showed a lower concentration in the organs compared with that of tumor-free animals. Neither after i.v. nor i.t. injection of ^{57}Co-bleomycin in the mice bearing 5 day old tumors do the lymph nodes draining the tumor attain the levels of radioactivity detected in lymph nodes of normal mice or animals bearing 12 day old tumors.

Additional experiments have been performed in tumor-bearing mice and guinea pigs comparing the effectiveness of aqueous solutions of bleomycin with water-in-oil emulsions of bleomycin. The results indicate cures can be obtained with much lower concentrations of bleomycin in a water-in-oil emulsion than in water solution.

In attempts to determine if the host immune response influenced the effectiveness of chemotherapy tumor bearing guinea pigs were treated with rabbit anti GP ALS before and following intralesional chemotherapy with vincristine. ALS was found to prevent the cure of animals treated with low concentrations of vincristine. Animals treated with 10-fold higher concentrations of the drug were cured of their tumor but were not immune to challenge. Tumor-bearing animals treated with just the vincristine were cured and immune to challenge.

Significance to Biomedical Research and the Program of the Institute. These studies in a syngeneic tumor animal model system are designed for analyzing the mechanism of intralesional chemotherapy. The development of this sytem should make it possible to better utilize intratumoral therapy of human cancer.

Proposed Course of Project: Further studies on the effects of intratumoral chemotherapy into tumors growing for longer than 7 days. Additional histology of the tumor sites at various times after injection of chemotherapeutic agents will be studied. Studies will be performed to determine the effect of multiple small injections of drugs into tumors growing for longer than 7 days. Techniques of humoral and cellular immunity to the tumor will be employed to more critically define the immune status of the treated animals.

Publications

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Bier, J., Bier, H., Siegel, T., Ohanian, S.H., Borsos, T. and Rapp, H.J.: Intralesional chemotherapy of line-10 tumors in strain-2 guinea pigs. In Israel, L., Lagrange, P. and Salomon, J. C. (Eds.): Les Colloques de l'INSERM. Cancer Immunology and Parasite Immunology. Paris, INSERM, 1980, pp. 125-135.

Bier, J., Siegel, T., Bier, H. and Ohanian, S.H.: Intratumoral versus intravenöse chemotherapie in stamm 2-meerschweinen mit linie 10-tumoren. Dtsh. Z. Mund-Kiefer-Ghesichtschir. 5: 270-281, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08552-16 LIB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Mechanism of Complement Fixation and Action

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Borsos	Chief, Humoral Immunity Section	LIB NCI
OTHER:	J. J. Langone	Expert	LIB NCI
	R. Ejzemberg	Guest Worker	LIB NCI
	A. Circolo	Visiting Fellow	LIB NCI
	H. Kato	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

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Laboratory of Immunobiology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

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: It has been known for many years that IgG-C4 complexes are generated during the activation of the classical complement pathway. Information regarding binding of C4 by IgM was not available. The binding of C4 by IgM anti-methotrexate antibody was studied. MTX was coupled to the cell surface covalently. The specifically bound IgM was eluted from the cell surface by soluble MTX. By determining the amount of IgM eluted, the number of C4 molecules eluted and remaining on the cells and by reattaching the eluted IgM to cells it was found that no C4 was eluted from the cells, no C4 transferred from cell to cell and that the C4 remaining on the original cells were fully active. The data conclusively demonstrated that in contrast to IgG, IgM was not capable of binding C4.

Regarding the activation of C1 by Igs studies were initated 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 in which the density of IgG did not vary, the number of lytic sites remained approximately the same when the average distance between MTX molecules was between 5 and 7 nm, but decreased rapidly with an average distance less than 10 nm. These results were interpreted to mean that in addition to aggregation for IgG to acquire maximal complement fixing and activating properties, the angle of the Fab arms of the molecule must not be larger than about 50 to 60°.

The hemolytic efficiency of Igs and complement depends not only on the distribution of the hapten, on the binding of complement by the Igs or the cell surface but also on the source of complement components. With whole guinea pig serum as the source of complement only about 10% of the cell bound IgM molecules were capable of lysing the cell. However, when purified components were used all the IgM molecules were capable of inducing cell lysis. The block occurred at the C4 level and it was shown that homologous complement components tended to be inefficient.

Lysis of cells requires that all the components of complement react in the proper sequence at and in the cell surface. It was shown that the size of the complement lesion depended even when whole serum was used as the source of complement on the number of C9 molecules bound at the lesion. Depending on the conditions of the experiment, during the interaction of the cell bound Ig and complement a series of different sized lesions are formed which is reflected in the kinetics of lysis of the cells.

These observations reconcile discrepant reports from various laboratories concerning the kinetics of lysis of cells by different complement sources.

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 C1FT 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.

Publications

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Borsos, T. and Langone, J.J.: Detection of antigens and haptens by inhibition of passive immune hemolysis. In Langone, J.J. and Van Vunakis, H. (Eds.): Methods in Enzymology, Immunochemical Techniques, Part C. New York, Academic Press, 1981, Vol. 74, pp. 161-165.

Boyle, M.D.P., Gee, A.P. and Borsos, T.: Heterogeneity in the size and stability of transmembrane channels produced by whole complement. Clin. Immunol. Immunopathol. 20: 287-295, 1981.

Circolo, A. and Borsos, T.: Lysis of hapten-labeled cells by anti-hapten IgG and complement: effect of cell surface hapten density. J. Immunol. 128: 1118-1121, 1982.

Ejzemberg, R. and Borsos, T.: Hemolytic efficiency of cell-bound IgM: evidence that IgM-C1 complexes activate C4 molecules not hemolytic with homologous C2-C9. J. Immunol. 128: 1600-1602, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 CB 08557-04 LIB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immunoassay of Fluid-Phase and Cell-Bound Antibodies and Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	T. Borsos	Chief, Humoral Immunity Section	LIB NCI
	Y. Kato	Visiting Fellow	LIB NCI
	A. Paterson	Visiting Fellow	LIB NCI

COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Immunobiology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is the development and application of immunoassays for the quantitative determination of antibodies, antigens and haptens in physiological fluids or bound to the cell surface. Emphasis is placed on analysis of tumor-associated products and chemotherapeutic drugs. These assays also are used to study the interaction between antibodies and antigens and components of the complement system.

Project Description

Objectives: To develop sensitive and specific immunological assays which can be used to study the metabolism, disposition, and immune reactions of molecules of interest in cancer research and treatment.

Methods Employed: Immunological assays for fluid-phase or cell-bound antibodies and antigens are based on a general method developed in this laboratory in which ^{125}I-labeled protein A (^{125}I PA) (from Staphylococcus aureus) is used as a tracer for IgG antibody directed against the target molecule. Radioimmunoassay (RIA) and immunoradiometric assay, which utilize radiolabeled antigen or antibody, respectively, also are used. Other immunological and physicochemical methods including gel filtration, ion exchange and high performance liquid chromatography, thin layer chromatography, affinity chromatography (including immunoadsorption), complement fixation, electrophoresis and ultracentrifugation are used to prepare, purify, characterize, and quantify immunological and chemical products.

Major Findings: The mechanism of interaction between IgG and protein A has been studied further. We found that protein A forms high ($K_D = 3x10^{-11}M$) and low ($K_D = 1x10^{-9}M$) affinity complexes with rabit immunoglobulin G antibodies that are involved in immune binding to the immobilized monovalent hapten methotrexate. High affinity complexes are formed only when there is a relatively high density of ligand. The high affinity complexes constitute 7-12% of the total and involve two antibody molecules separated by 40-45Å and cross-linked by a single divalent protein A molecule. If more than one protein A can bind to each antibody molecule in the low affinity complexes, then binding at one site does not affect binding at the other.

To characterize this activity more rigorously we plan to use monoclonal antibodies against methotrexate. In the course of preparing these antibodies we have studied the effect of the immunogen composition on the efficiency of producing hybridomas secreting anti-methotrexate. In brief, hybridomas were generated by fusion of FO myeloma cells with splenocytes obtained from mice immunized with methotrexate covalently conjugated with keyhole limpet hemocyanin. When the methotrexate/hemocyanin molar ratio was 430-530/1, 395/817 (44%) of the hybridoma cultures synthesized antibodies to methotrexate, whereas 69/817 (9%) secreted antibodies to hemocyanin. When the molar ratio was 50/1, only 14/343 (4%) cultures synthesized anti-methotrexate and 93/343 (27%) synthesized antibodies to hemocyanin. Thus the frequency of hybridomas producing anti-hapten antibodies can be controlled by manipulating the ratio of methotrexate molecules to carrier immunogen.

Titers and binding constants of monoclonal antibodies in ascites fluid containing 2-3 mg/ml anti-methotrexate and obtained from mice injected with selected cell lines are higher than those of polyclonal antibodies made in rabbits, mice, and a goat. Competitive inhibition assays showed that the monoclonal antibodies were more specific than the conventional antibodies for methotrexate compared to structually related compounds. IgG2a and IgG2b antibodies will be used to study the mechanism and properties of the reactions between IgG and protein A or complement.

In related experiments aimed at studying the effects of plant lectins on antibody and complement activity, we found that native tetravalent Con A and the divalent

acetylated derivative increased the hemolytic titer (i.e., the reciprocal of the antibody dilution required to give an average of one lytic site per sheep erythrocyte) of IgG antibodies against Forssman antigen by up to 225% with guinea pig and human complement. Although the average number of lytic sites generated at each antibody concentration increased, the slope of the titration curve did not change. Other lectins with the same or different sugar specificity either augmented or inhibited hemolysis but were less potent than Con A. Augmentation by Con A was consistent with the ability of lectin on the cell surface to bind but not activate guinea pig C1. Thus it appears that cell-bound Con A and IgG yield a complex that behaves like a doublet of IgG antibody molecules in its ability to fix and activate C1, where activation is dependent on the IgG component. In contrast, the highest dose of Con A inhibited by at least 50% the hemolytic activity of IgG antibodies against either a sugar-free protein (HSA) or a protein reactive with Con A (human myeloma IgE) using cells to which these antigens were coupled using chromic chloride. This suggests that the identity, density, and/or mode of presentation of the antigen on the cell surface may be important determinants of lectin-induced augmentation. Although both the enhancement or inhibition by Con A in the presence of whole C correlated with the number of C1 molecules bound and activated, there was no correlation with the ability of the lectin to agglutinate the cells.

Significance to Biomedical Research and the Program of the Institute: Quantitative determination of antibodies and antigens is basic to the understanding of their role in immunological defense against cancer. Similarly, quantitation of bio-active agents, either endogenous compounds or chemotherapeutic drugs and their metabolic products, is fundamental to our understanding of how these agents work and may lead to a more rational basis for their clifnical use. Sensitive and specific analytical methods should help achieve these goals.

Proposed Course of Project: Improved immunological and related physical and chemical methods for the quantitative determination of tumor-associated products, cancer chemotherapeutic agents and other compounds of interest in cancer research will be developed and used to study the mechanism of antibody-antigen reactions and antibody-complement-mediated processes.

Publications

Langone, J.J.: Protein A of Staphylococcus aureus and related immunoglobulin receptors produced by Streptococci and Pneumococci. In Dixon, F.J. and Kunkel, H.G. (Eds.): Advances in Immunology. New York, Academic Press, Vol. 32, pp. 157-252.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08575-10 LIB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	E. Alteri	Visiting Fellow	LIB	NCI

COOPERATING UNITS (if any)

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SECTION
Immunopathology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to study the cells that participate in the effector arm of the immune response. The current emphasis is on chemotaxis, which is a mechanism by which cells can be attracted to inflammatory sites, delayed hypersensitivity reactions and growing tumors. The project includes chemistry of lymphocyte derived chemotactic factors, identification of substances that modulate chemotactic and phagocytic responses, definition and separation of functional subpopulations of leukocytes, alterations of leukocyte chemotaxis in cancer patients.

Project Description

Objectives: To develop quantitative measures of reactions occurring in the effector limb of the immune response; to study chemotaxis and phagocytosis of inflammatory cells in relation to this objective.

Methods Employed: Complement-derived and lymphocyte-derived chemotactic factors were generated. Human peripheral blood leukocytes were separated on Ficoll-Hypaque gradients to obtain monocyte- and basophil-rich fractions. Dextran sedimentation was used to obtain peripheral blood neutrophils. Peritoneal macrophages were harvested from normal and BCG-infected mice. Protein fractionation methods were employed to characterize chemotactic factors and serum factors that modulate mouse macrophage chemotactic responses. Radiolabeled chemotactic peptides were used for binding studies.

Major Findings: A. Definition and characteristics of migrating and non-migrating leukocyte subpopulations. It was previously shown that the number of human blood monocytes responding to chemotactic stimuli under optimal condtions in vitro was less in cancer patients than in normal controls or hospitalized patients without cancer. Questions about the nature of this defect have led to studies of monocyte subpopulations, chemotaxin receptors, maturation dynamics and factors affecting chemotactic response. Studies were facilitated by development of a multiwell chamber for measuring chemotaxis, use of an image analyzer for counting migrated cells, and construction of a separation chamber that allows for collection of migrated and non-migrating cells.

Among normal human blood monocytes, 20-40% respond to chemoattractants. In the case of the peptide attractant, f-met-leu-phe (FMLP), the non-migrating monocytes lack the receptor for the attractant. This laboratory is now determining whether absence of FMLP binding by non-migrating monocytes is an isolated difference or whether these cells differ in many respects from the morphologically similar migrators. Populations of migrating and non-migrating monocytes were obtained by use of the chemotaxis separation chamber. Phagocytic capacity for IgG coated sheep erythrocytes was the same for the two populations. Respiratory burst activity was also studied by continuous measurement of superoxide anion production in response to phorbol myristate acetate (PMA) or chemotactic peptides. Migrating monocytes exhibited a respiratory burst to FMLP, whereas the non-migrators had little or no response. Relative to the migrators, the non-migrators had a much lower response to PMA.

In an approach to the question of how maturation might affect the proportion of migrating and non-migrating monocytes, chemotaxis assays were made on young monocytes that repopulated the circulation during leukopheresis of normal human donors. Over a period of 2 hours, donors were depleted of approximately 10^9 monocytes, equivalent to the total in the circulating blood. New monocytes entered the circulation, since monocyte counts were normal at the end of the procedure. Blood samples were drawn at the start and end of leukopheresis and 3 hours later. The number of monocytes migrating to optimal chemotaxin concentrations was decreased to about half at the end of leukopheresis, with recovery 3

hours later. Since these changes were not observed in 3 sham donors who went through the same procedure without withdrawal of cells, they reflect functional characteristics of newly circulating monocytes. Chemotaxin bindings studies of these cells are in progress.

B. Chemoattractants and the differential accumulation of inflammatory cells. What mediators cause accumulation of inflammatory cells and how are differential patterns of inflammatory cell foci achieved? Studies from this laboratory on delayed cutaneous hypersensitivity in the guinea pig state the problem: simultaneous challenge with tumor cells, PPD and KLH at separate sites elicited strikingly different reactions -- the KLH site, for example, comprising about 80% basophils, whereas the PPD site had none.

The chemotactic peptide FMLP was oxidized at the methionine residue to the sulfoxide or sulfone form. Both oxidized forms of the molecule failed to attract human neutrophils, but remained chemotactic for human monocytes despite lower binding affinity for both monocytes and neutrophils. This is an interesting model for differential accumulation of leukocytes at inflammatory sites.

C5a, derived by activation of the fifth component of complement, is the major chemoattractant that can be produced from serum. It was found that modification of C5 in a process that does not involve complement activation results in potent chemoattractant activity. This was done by ultracentrifugation of mouse serum and collection of the concentrated high MW residuum remaining in the bottom of the tube. Chemoattractant activity of this material was detected at dilutions relative to serum as high as 1/900 - comparable to the potency of serum-derived C5a. The attractant was heat-stable. The precursor, or one of the reactants involved in attractant formation, was heat-labile. Neither classical nor alternative complement pathways appear involved, since attractant was formed during centrifugation in the presence of 0.01M EDTA. These findings suggest that at local inflammatory sites enzymatic modification of C5 may generate chemotactic activity without complement activation.

Significance to Biomedical Research and 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 the serum factors affecting function of macrophages and neutrophils. Characterization of monocyte and neutrophil subpopulations. Analysis of the chemotaxis abnormality in patients with cancer.

Publications

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Falk, W., Harvath, L., and Leonard, E.J.: Functionally distinct populations of human monocytes: Receptors for F-met-leu-phe are expressed only on the chemotactically responsive cells. 9th International RES Congress, 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08576-10 LIB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immunological Mechanisms of Tumor Rejection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

PHS-6040

Project Description

Objectives: To determine what immunological mechanisms occur in tumor cell killing in order to provide a rational basis for elimination of tumor cells by immunological means.

Methods Employed: Chemically-induced murine tumors, maintained in tissue culture or by passage in syngeneic hosts were used. Effector immune responses were measured by cytotoxicity assays based on the release of radioactive label from damaged target cells. Protein isolation techniques were used for purification of macrophage activation factors.

Major Findings: (1.) Macrophage activation by LK for nonspecific tumor cytotoxicity requires multiple reactions. Stable, noncytotoxic cellular intermediates between immature mononuclear phagocytes and fully activated tumoricidal macrophages can be identified. For example, LPS at ng/ml concentrations synergistically increase cytotoxicity by macrophages treated with low LK concentrations (1/500). That cells can be activated by LK alone at high concentrations (1/20), however, suggests an LPS-like signal may be present in LK. Macrophages cultured 4 hours in low concentrations of LK (1/500) developed cytotoxic activity only after subsequent exposure to LPS or 1/20 LK for 1 hour; macrophages treated with LPS or LK alone were not cytotoxic. Thus, cells exposed to one signal (low concentrations of LK) enter into a receptive, primed state in which they are not yet active but can then be triggered by another signal (LPS or LK) to develop full cytotoxic activity. Trigger signals in LK were not contaminating LPS: unlike LPS, LK trigger signals were heat-labile (60°C/30 min) and not affected by polymyxin B. Activation by LK signals required a defined treatment sequence: optimal cytotoxicity occurred with cells treated (primed) 4 hours with low concentrations of LK and then triggered 1 hour with high concentrations. The reverse was ineffective. Macrophages cultured continuously for 36 hours in 1/320 LK show no cytotoxic activity at any time. Yet cells cultured for various times in 1/320 LK, then exposed to 1/10 LK for 1 hour behave comparably, in terms of time course and levels of cytotoxicity, to cells continuously incubated in 1/10 LK. The optimal time for LK priming was 4 hours; LK triggering required only 20 min. Both LK effects were temperature dependent. LK priming and trigger signals form the basis of a regulatory system that sets the threshold and determines the onset of macrophage effector function.

(2.) Macrophages from C3H/HeJ mice (Lps^d) fail to develop tumoricidal activity after any of several in vivo or in vitro treatments that induce cytotoxicity with cells from responsive C3H/HeN (Lps^n) mice. Under certain conditions, however, C3H/HeJ macrophages were cytotoxic: cells from in vivo immune reactions such as those induced by BCG infection, but not cells from irritant-induced peritoneal exudates, developed full cytotoxic capability after in vitro exposure to LPS, LK and any of a variety of plant lectins. Thus, activation of C3H/HeJ macrophages required 2 stages: cells primed during immune responses to BCG in vivo develop cytotoxicity only after in vitro exposure to a variety of apparently unrelated trigger signals. Either treatment alone was ineffective. A similar 2-stage activation process occurs with cells from responsive C3H/HeN mice: macrophages exposed 4 hours to low LK concentrations (1/500) enter into a primed stage in which they are not yet cytotoxic, but can then respond to trigger signals to develop tumoricidal activity. The number of different trigger signals

able to induce cytotoxicity with LK-primed C3H/HeN macrophages was considerably less than that able to activate BCG-primed C3H/HeJ cells.

was considerably less than that able to activate BCG-primed C3H/HeJ cells. Induction of cytotoxic activity in LK-primed C3H/HeN macrophages was most effective with high concentrations (1/20) LK; LPS was less effective and a variety of lectins (effective with BCG-primed C3H/HeJ cells) were either weakly active or inactive. These data suggest that within the multistep reaction for induction of cytotoxic activity, BCG-primed C3H/HeJ macrophages and LK-primed C3H/HeN cells may be at different reaction stages.

(3.) Supernatant culture fluids from a phorbol myristate acetate (PMA)stimulated subline of the murine EL4 thymoma activated inflammatory macrophages for non-specific tumoricidal activity in vitro; active supernatants from PMA-stimulated EL4 were not directly toxic to tumor target cells in the absence of macrophages. Additionally, macrophages treated with supernatant fluids of PMA-stimulated EL-4 also developed potent microbicidal activity against the protozoan, Leishmania tropica. Macrophages treated with supernatant fluids of unstimulated EL-4, concanavalin A-stimulated EL-4 or PMA-stimulated BFS cultures (an IL-2 independent T cell line unrelated to EL-4) were not cytotoxic. Supernatant fluids from 10 other continuous T cell lines stimulated with PMA were also inactive. The titer of activity in cytokines from PMA-stimulated EL-4 was similar to that found in LK. The time courses for development of macrophage cytotoxic activity with EL-4 or LK activities were identical. Both EL-4 and LK activities were nondialyzable, destroyed at 100°C for 10 minutes and could be separated from IL-2, macrophage-granulocyte colony stimulating or B cell growth factors. Activities in the EL-4 fluids but not in LK were stable at pH 2. Further physicochemical characterization of the EL-4 cytokines showed 2 distinct activities that activated macorphages for cytotoxicity: one was indistinguishable from gamma interferon (50,000 mw, pI 5.2 to 6.0, pH 2 labile and neutralized by anti-gamma interferon sera); the other was clearly distinct (23,000 mw, pI 4.4 to 5.2, pH 2 stable and unaffected by anti-gamma interferon sera). These results suggest that the EL-4 cell line may produce one or more of the macrophage activation factors present in LK.

(4.) Macrophages continuously exposed to LK and target cells throughout the 48 hour cytotoxicity assay exhibit 3-fold more tumoricidal activity than do cells optimally treated with LK before addition of tumor cells. Increased cytotoxic activity induced by continuous LK treatment was not due to direct toxic effects of LK on tumor target cells or to alterations in target cell susceptibility to cytopathic effects of LK-activated macrophages. Moreover, sensitivities of responsive macrophages to LK activation signals and time courses for onset and loss of tumorcidial activity during continuous exposure or LK pulse were identical. Analysis of macrophage or or LK dose-responses and time courses for development of cytotoxicity each suggest that differences in tumoricidal activity between macrophages continuously exposed or pulsed with LK were quantitative: the number of cytotoxic events was increased 3-fold during continuous LK treatment. Optimal levels of macrophage tumoricidal activity then occur only if effector cells, target cells and activation stimuli are simultaneously present for a defined time interval: tumor cells need not be present during the

initial 2 to 3 hours of culture; LK can be removed after 8 hours with little or no loss of cytotoxic activity. However, removal of LK or target cells during the critical 4 to 8 hour interval decreased levels of cytotoxicity 3-fold. Thus, nonspecific effector function by LK-activated macrophages is controlled by both the physicochemical nature of the LK mediator and the time interval effector and target cells are exposed to LK.

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: Studies on the mechanisms of the killing of tumor cells by macrophages.

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Annual Report of the Laboratory of Cell Biology

National Cancer Institute

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The following are selected highlights of the research efforts of the staff and collaborators of the Laboratory of Cell Biology (without reference to specific investigators; see individual reports for details).

Studies of mouse plasmacytomas, the immunoglobulins they synthesize, and the genes that control this synthesis reveal the following: Two mouse strains, BALB/c and NZB, uniquely develop plasmacytomas after intraperitoneal injections of mineral oil. Studies of the mechanisms of this plasmacytomagenesis indicate that the mineral oil (pristane) has at least two biological effects: 1) it stimulates B-lymphocyte proliferation, and 2) it creates a microenvironment of oil granulomas that secrete diffusible substance(s) that support the growth of transformed B cells such as plasmacytomas. Karyotypic analysis shows that these plasmacytomas frequently have a translocation of a fragment of chromosome 15 onto one of the chromosomes that contains the structural genes for immunoglobulin light or heavy chains. This suggests that chromosome 15 contains an oncogene specifically associated with lymphocyte malignancies which may be activated when it is translocated next to an active promotor for the Ig genes. The pristane injections also induce chronic arthritis in BALB/c mice. Genetic crosses of BALB/c mice with strains resistant to these effects of pristane are under way to identify the gene(s) responsible for susceptibility to arthritis and plasmacytomas.

Three-dimensional structure of the antigen-binding region of myeloma proteins is being studied by amino acid sequence analysis, reaction with monoclonal antiidiotypic antibodies, X-ray diffraction and computerized model building. These studies have determined that anti-phosphocholine and anti-galactan antibodies have very different shaped binding sites which determine their immunological specificities. Similar approaches are being utilized to compare the structure of the binding sites for a protein antigen, egg white lysozyme, to those that bind the carbohydrate antigens mentioned above. Studies of the amino acid sequence of proteins of mutant plasmacytomas which no longer bind antigen have shown that single amino acid substitutions in the framework portion of the variable region as well as those in the complementarity determining region can affect antigen binding.

Studies of phosphocholine binding Ig heavy chains in BALB/c and C3H mice have demonstrated that there are two allelic V_H genes that can be used in this response. The V_H sequence of a hybridoma protein that binds phosphocholine has been shown to differ from these two allelic genes in a way that suggests that a novel process, not previously demonstrated in mammalian cells, "gene conversion", may play a role in generation of antibody diversity. The distribution of these allelic forms of phosphocholine binding V_H and V_L genes as well as the families of V_H and V_L genes for antigalactan antibodies are

being determined in many strains of inbred and wild mice using cDNA probes we and others have developed. While there are at least 4 V_H genes for phosphocholine binding, galactan binding seems to be encoded in a single V_H gene. The differences in stability of these two gene families during interdependent evolution in wild mouse populations will provide important insights into the genetic mechanisms that conserve the function of these biologically important molecules.

A cDNA probe for IgCλ has been prepared and has been used to study differences in λ genes in inbred and wild mice. In contrast to the 4 Cλ genes present in all inbred mouse strains, several stocks of wild mice have been shown to have 8-12 Cλ genes. Isolation and cloning of these genes from one of these wild mouse stocks has been started and is expected to reveal how this apparent duplication of all or a portion of the Cλ genome has occurred.

Wild mice from our colony have been screened by Southern blotting for the presence of the genes of mammary tumor virus. Interestingly, many different wild mice were found to be completely free of these genes, while some have only a single provirus in the genome and others just the long terminal repeated sequence of this virus. These stocks of mice are being studied to determine their incidence of mammary tumors, and they will be used to test the promotor-insertion theory of mammary tumorigenesis.

The organization and expression of the constant region genes of the Ig heavy chains has focused on the mouse IgD δ heavy chains. We have cloned δ cDNA and genomic δ and determined the structure of the introns, exons and 3' gene segments encoding secreted and membrane forms of δ chain. The 2 exons (δ_{m1} and δ_{m2}) encoding the membrane binding portion are strikingly similar in sequence and exon structure to those of μ chains except that δ_{m2} has a much longer 3' untranslated region. In plasmacytomas and normal spleen RNA we found an unexpected multiplicity of membrane δ mRNAs. These 3 δ_m mRNAs appear to be the result of alternative splicings of a long common transcript from the δ gene or the μ plus δ genes. IgD appears to be an important multifunctional B cell receptor, and the multiple δ_m mRNAs may indicate that there are multiple forms of δ_m protein, each capable of a different receptor function. Similar studies on RNA from an IgA secreting plasmacytoma discovered two α chain mRNAs encoding membrane-bound α chains. Using these RNAs to probe germline genomic DNA clones we located the size for a gene segment encoding the membrane binding portion of α chains and determined its DNA sequence.

Purification and Properties of H-2^b

Purified H-2^b incorporated into liposomes has been used to stimulate secondary cytotoxic responses in vitro. The CTL stimulated by H-2 containing liposomes are identical in every way to CTL stimulated by allogeneic cells. The density of H-2^b on the liposome seems to be important since higher protein to lipid ratios in the liposome result in more efficient stimulation. There is also a dependence on the lipid composition of the liposomes. Didodecanoyl lecithin is markedly superior to egg lecithin, ditetraecanoyl lecithin or distearoyl lecithin. In agreement with results from other laboratories, inclusion of membrane matrix proteins in liposomes improves

their ability to stimulate CTL. During the course of these studies we discovered that, due to our purification procedures, $H\text{-}2^b$ slowly dissociates from β_2 microglobulin and is no longer an effective immunogen. We are currently altering our purification procedures to prevent this problem.

A first successful isolation of amphotropic type C retroviruses from sources other than wild mice was accomplished. One of these two new isolates proved to be a recombinant virus which derived its gag gene from AKR-like MuLV and env gene from the endogenous amphotropic virus, as shown by competitive radioimmunoassays and tryptic peptide maps. The fact that this virus emerged from a Rauscher-MuLV induced tumor, RBL-5, makes it more intriguing as to the role of Rauscher-MuLV in the viral activation and recombination processes.

The regulation of expression of H-2 and beta-2-microglobulin in trophoblast cell lines established in this laboratory from murine placentas was examined. We found that the very low expression and synthesis of H-2 and beta-2-microglobulin in these cells is due to the transcriptional control as is the case with embryonal carcinoma (EC) cells. However, the presence of Endo A and Endo B cytoskeletal proteins and absence of SSEA-1 antigen in trophoblast cells indicates that their differentiation status is different from that of EC cells. Natural Killer (NK) cells, but not alloimmune T-cells, were able to kill trophoblast cells. The effects of interferon and tumor promotor on the NK-sensitivity of trophoblast cells were analyzed.

We were able to isolate 6 strains of polyoma virus mutants (PyTr) that can replicate in trophoblast cells and 2 mutants (PyEC) that can replicate in EC cells. Although the PyEC mutants can replicate in EC, and trophoblast as well as other differentiated cells, the PyTr mutants can replicate only in the latter two groups of cells. This differentiation stage-restricted growth capability of polyoma virus mutants should provide a tool for analyzing gene control mechanisms associated with differentiation. By restriction endonuclease analysis and marker rescue experiments, the mutation site was localized to the late, non-coding region near the origin of virus DNA replication.

Our cooperative study with Dr. Nancy Hopkins (MIT Cancer Center), concerning the phenotypic expression of Meth A specific tumor antigen continues and has yielded interesting results. BALB/c 3T3 (f7) subline (specifically cloned and selected) has been transfected with high molecular weight DNA from Meth A sarcoma using appropriate controls. The following assays done blindly have been accomplished from transformed foci:

1) assay for malignancy in the syngeneic BALB/c host

2) presence of TSSA by absorption of the specific cytotoxicity of a Meth A syngeneic antiserum, and

3) tumor rejection and specificity (TSTA)

We have observed a high frequency of co-transfection of transformation and TSTA at least among the primary transfectants. Most transfected lines (from Meth A DNA) grew progressively in BALB/c mice; 5 of 8 foci selected

from one experiment possessed Meth A TSTA. DNA extracted from several of these lines was used in secondary rounds of transfection; 5 of 8 of II transfectants contained Meth A-specific TSTA. These results suggest that in the Meth A sarcoma a transforming gene and a genetic determinant of TSTA are intimately related since one would expect the frequency of a transfer of the 2 phenotypes to be only 0.01 to 0.001 if specified by unlinked genes.

Several of the transfected lines have been passaged continuously in vivo and assayed through 15 generations for immunizing capacity against Meth A. Results show some instability of the TSTA phenotype. Not all have as yet been assayed for specificity.

Meth A DNA cleaved with restriction enzymes retains transforming capacity (Hopkins and Vande Woude); these transfectants are now being assayed for the presence of Meth A TSTA. This DNA fragment appears to be of a size (15 Kb) appropriate for molecular cloning. The frequency of co-transfer is much lower (20% or less) and not all immunogenic clones have been assayed for specificity.

Two transfectants produced by the DNA fragment (cloned 15 Kb determinants) have been assayed for malignancy - that is progressive growth in syngeneic BALB/c mice and for the Meth A-specific TSTA. Both grew as undifferentiated neoplasms (probably of endothelial origin) and one (MAL-210) in preliminary tests appears positive for Meth A immunogenicity. These findings are only of a preliminary nature and more tests are in progress.

It will be of interest to determine if this isolated determinant is related to viral onc genes and the relationship of this determinant to our 75K TSTA.

Attempts are being made to isolate and purify the TSTA of RBL-5, an RMu-LV induced T cell leukemia. Higher yields of TSTA are now available through use of cytosol-derived antigen without resort to cell membrane isolation and solubilization (with detergents). Purification is proceeding through the usual gel filtration and affinity chromatography. Two major bands (~ 75K and 65K) on SDS-PAGE have been visualized. These appear to be related. These materials along with their dimers are very immunogenic (particularly the 75K material) against other FMR-induced lymphomas but not against G-MuLV-induced or radiation induced leukemia. The 75K material is not related to the 75K Meth A isolate immunologically. Also, although the RBL-5 TSTA isolate has FMR group specificity it appears not related to gp70 or any other viral proteins.

The major transplantation antigens of the mouse are a family of cell surface glycoproteins which serve as recognition signals in many immunological reactions. They are detected on the plasma membrane of virtually all somatic cells. We have reported previously that F9 teratocarcinoma stem cells do not express any major histocompatibility antigens. Evidence has been obtained that this regulation of major histocompatibility antigen expression is due to transcriptional control of both H-2 and β_2 microglobulin. In order to assess if this control is more general and may serve as an example of the controls that function in normal embryonal development, we have examined two cloned cell

lines derived from the whole placenta of two strains of mice. Very low levels of both H-2 and β_2 microglobulin were detected on the cell surface. These data correlate with the presence of low levels of mRNA for these proteins. The cloned cells have been shown to be trophoblast, which in the embryo are located at the interface between fetal and maternal circulations. The low level of histocompatibility antigens in this particular region may be an important factor in the survival of the fetus as an intrauterine semi-allograft.

A serum protein which has been shown to lack H-2 alloantigenic determinants and binds β_2 microglobulin has been described. In order to understand its biological function, it is imperative to know its biochemical properties and structural relationship to H-2 antigens. We have obtained data on the molecular properties of this protein, e.g., its molecular weight, amino acid composition, pI value and peptide maps. The primary structural analysis of this protein is under study in order to compare its relationship to the histocompatibility antigens.

One of the more perplexing problems in cellular immunology is the mechanism by which T-lymphocytes recognize antigen only in association with macrophages of a particular H-2 type. An approach to the investigation of this question is to examine in detail the specificity of T lymphocyte antigen recognition using a family of peptide antigens. Our initial observation was that the same rank order or relative antigenicity was observed within a set of peptide antigens from cytochrome of different species and the acetimidyl derivatives of this same group even though the two sets stimulated different T-lymphocyte clones. This finding suggested the possibility that antigen recognition proceeded by utilizing two different sites; one site to account for the invariant rank order and the second site to account for distinguishing between the derivatized native peptides. The possibility that the MHC product and the antigen may interact directly has long been discussed. Such an interaction would appear as a second site when the specificity of antigen recognition was investigated. However, the specificity of such a second site would be resident in the macrophage, not the T cell. In other words, if a T cell clone would be found which reacted with antigen in association with two different H-2 types, the rank order of antigen reactivity would depend not only on the T cell clone but also on the H-2 type of the macrophage. This was found to be the case when the specificity of T-lymphocyte hybridomas were examined. Thus we have shown that there is specific interaction between the macrophage and antigen. Currently investigations are underway to define the sites of T cell-antigen and MHC-antigen interaction. Secondly, we are investigating whether a particular MHC-antigen interaction site can cooperate with a large variety of T cell clones.

Methylcholanthrene-induced sarcomas are known to carry a distinctive antigen (TATA) which can impart transplantation immunity to the tumor of origin by prior immunization with that tumor or a soluble form of the antigen derived from it. A TATA with an apparent molecular weight of 75,000

has been isolated from the cytosol of the BALB/c sarcoma Meth A. The antigen was purified by immunoaffinity chromatography following hexylamine agarose, gel filtration and hydroxylapatite chromatography. A second protein having a molecular weight of 75,000 was isolated from the cytosol of the recently derived methylcholanthrene induced sarcoma, CI-4, by essentially the same procedure. Both 75 kilodalton proteins effectively primed BALB/c mice to reject only their tumors of origin and afforded no protection against challenge by other independently derived sarcomas of BALB/c origin. The antigens purified from Meth A and CI-4 sarcomas appear to be closely related but non-identical proteins. The two antigens can be recognized by a rabbit antiserum prepared against the Meth A 75 kilodalton protein and have identical molecular weights but differ in their chromatographic behavior on hexylamine agarose and hydroxylapatite. Amino acid compositions of both antigens are similar but not identical. We are currently undertaking a study of the primary structures of these antigens to determine the relationship between individual TATAs from different sarcomas. In addition, studies are now in progress to determine the molecular organization of the genes involved in the expression of these antigens. By the use of nucleic acid probes we hope to answer a critical question: Are these antigens derived from a multigene family or are they products of somatic mutation of a single gene?

Fisher Rat Thyroid cells grown in low or no serum (FRTL) have continued to provide a powerful resource; they are the first and possibly only example of a karyotypically normal, long term cell strain that has retained complex differentiated functions including biosynthesis of physiological amounts of thyroglobulin (TG), concentration of 100-fold or more of iodide, and apparently normal response to the hormone thyrotropin (TSH). We have sent these cells out to laboratories around the world and they have provided the basis for numerous studies involving hormone mechanisms especially the response of the cyclic AMP system to TSH stimulation.

Last year we found that clones of TG producing, FRTL cells could be efficiently screened for TG secretion by overlaying the cells with a layer of agarose + 6H medium containing added rabbit anti-rat-TG serum. As the TG is secreted by the cells in the colonies it diffuses into the agarose layer and precipitates when it encounters the rabbit antibodies. The precipitate is readily visualized using low power stereo microscopes. We have used this method of visualization of TG secretion as a means for selecting rare non-producers, i.e., variants or mutants (spontaneous or induced) that fail to secrete TG. We have found that following EMS mutagenization two kinds of colonies are produced: those with large amounts of TG-anti-TG precipitate around them and a minority of colonies with much less precipitate. (Control plates without EMS treatment show only the heavy precipitate colonies). Very few or no colonies have been found that produce no precipitate at all. We interpret these under-producer colonies to be single hit mutants what one would expect if one of two diploid genes had been inactivated. If we subculture

one of these under producer clones and mutagenize it again - we predict (but have not yet found) there will again be two classes of colonies: those that produce small amounts of precipitate and a minority population of colonies that produce no precipitate. These will be the mutants that have hit a second allele and resulted in a null producing mutant strain. Because we conceive that there must be several mutation sensitive steps in the synthetic pathway for TG synthesis and secretion, we anticipate that these mutants will fall into several complementation groups. The complementation groups should be easily testable by cell fusion and either agarose-overlay or indirect FITC anti-TG visualization procedures. Using cloned probe DNA (supplied by the Naples group) we can tell which mutants transcribe and ultimately hope to characterize several types of mutants useful in the classic molecular genetic types of experiments.

Work has continued to investigate the mechanism by which cells generate slow rhythmic pacemaker potential (10-40mV fluctuation with period 5-10 min). Input resistance does not change during the pacemaker cycle, therefore, it is not due to change in membrane ion permeability. Slow pacemaker can be induced by intracellular injection of H^+ or Na^+. These facts suggested that the pacemaker is due to an electrogenic ion pump regulated by intracellular Na^+ and/or H^+. Na^+/H^+ exchange pumps move H^+ out and Na^+ into cells and are blocked by amiloride. Amiloride blocked pacemaker potentials initiated by H^+. This suggests that activity of Na^+/H^+ exchange is necessary for H^+ to initiate pacemaker potential. However the pacemaker potential is not due to cyclic activity of the Na^+/H^+ pump since amiloride does not abolish spontaneously active pacemaker potentials. Intracellular Na^+ seems to be necessary for initiation of the pacemaker because:

(1) Injection of Na^+ initiated pacemaker potentials
(2) Amiloride did not block pacemaker potentials initiated by injection of Na^+
(3) Pacemaker potentials were initiated by cessation of amiloride which allowed reestablishment of Na^+/H^+ exchange (i.e., Na^+ into cell for H^+ out)

Studies continue on the effects of interferon on murine retrovirus. When chronically infected SC-1 cells were treated with highly purified interferon for 24 to 48 hours, there was approximately a 100-fold reduction of infectious virus post interferon treatment, but the reverse transcriptase assay indicated only a ten-fold reduction in the number of particles, indicating a significant production of noninfectious virus. Furthermore, there was a 2.5 fold increase in the number of virions accumulating along the cell surface following interferon treatment, suggesting that release is slowed. After a 24 hour exposure to interferon, the virions contained an 85 K dalton glycoprotein of non-viral origin which was present in excess of the amount of the viral envelope glycoprotein, gp70. Virus particles produced from cells treated with interferon from 32-48 hours were nearly devoid of gp70 and contained measurably lower quantities of p30. These results are consistent with

previous observations that gp70 is essential for infectivity. Although interferon did not alter the intracellular processing of viral polyproteins to mature structural proteins, there was an accumulation of viral p30 and p12, consistent with the observed increase in cell associated virions. In contrast, the levels of gp70 released into the tissue culture medium were the same in the presence and absence of interferon, but little gp70 was found associated with virions produced from interferon-treated cells. This may be related to the finding that no gp70-p12E complex was detected in either virions or tissue culture fluids following interferon treatment. It is probable that gp70 is not assembled into particles and is released from p15E/p12E following proteolytic cleavage of pPr 85^{env}. While the molecular basis for this failure of incorporation of pg70 into the MCF class of retroviruses is not known, there are two principal possibilities which have to be considered. There could be a steric interference phenomenon whereby an altered carbohydrate side chian in gp70 would prevent the formation of stable disulfide bonds between gp70 and p15E/p12E. Alternatively, interferon could produce alterations in the plasma membrane itself, also leading to an unstable gp70-p15E/p12E complex. Given the current results, it seems quite possible that restriction in virus assembly is not exclusively localized to virus maturation sites, but may occur at other locations in the plasma membrane of the host cell, while leading to the same end result, the failure of incorporation of gp70 into virions.

In studies of the mechanisms for tumor cell induced immunosuppression, specific cytotoxic T cells against tumor associated antigens were generated in mixed lymphocyte tumor cell cultures. By employing this technique, we have demonstrated a unique suppressor mechanism which was triggered by the tumor cells and was mediated through two populations of macrophages (splenic and peritoneal). Preexposure of splenic macrophages to tumor cells initiated the suppressor event, and this was followed by the later involvement of peritoneal macrophages to complete the suppressor circuit. Therefore, three major cellular components were involved in this suppressor mechanism: tumor cells, splenic macrophages, and peritoneal macrophages.

Modern trends in genetic mechanisms of carcinogenesis pointed to the frequency that murine type-C leukemia virus of low pathogenicity, when recombined with a subset of host cellular nucleotide sequences (c-src), gave rise to competent oncogenic viruses. In a continuing project investigating the genetic mechanisms of carcinogenesis we have established four recombinant DNA clones of rat endogenous leukemia helper virus (RaLV) genomic DNAs. Restriction maps were deduced for two molecularly cloned RaLV DNAs of laboratory and feral origins. Functional organizations of both these RaLV DNAs were established by hybrid-select cell-free translations of RaLV mRNA and by microinjection studies of the cloned DNAs, total or of subgenomic fragments. By heteroduplex analysis, nucleotide sequences of these RaLVs, when compared to various rat sarcoma viral and

c-src DNA sequences, that are of highly conservative nature or divergent in evolution, were identified. Divergent nucleotide sequences were generally synonomous with viral DNA sequences essential for DNA recombination with c-src sequences, attributing towards oncogenic potential.

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 (NRK^{153}). Release of rat C-type leukemia helper viruses by the microinjected cells was examined by superinfection on Kirsten transformed non-producer cells (K-NRK). Immediate release of helper leukemia viruses at a very low level was observed only in the $NRK^{153}_{m3.5/cir}$ 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^{153}_{m} cells carrying various linear RHHV DNAs, en toto or of subgenomic sizes, with N-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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 03200-13 LCBGY

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October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Factors Influencing the Induction, Growth and Repression of Neoplasms

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	Nancy Hopkins	Staff Member	MIT Cancer Res. Ctr.
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: Major emphasis is placed upon the study of tumor antigens of the transplantation rejection type (TSTA), and of tumor antigens (TA) assayed by in vitro techniques and of the immune responses they evoke. As a corollary to this study the biologic properties in vitro and in vivo of histocompatibility (H-2) antigens are under study. Solubilization and methods of purification of both TSTA and H-2 are under investigation with the ultimate purpose of defining these membrane antigens after purification in physico-chemical, biologic and molecular terms. The role of a B-tropic retrovirus as a vector in the transmission of genetic information of a specific tumor antigen (TSTA of Meth A) is under study as well as the use of TSTA markers (Meth A) in the hope of defining more clearly the molecular events in transfection studies using DNA of chemically-induced sarcomas.

I. Tumor Antigens

1. Studies of purification and biological effects of TSTA

A. Attempts to purify to homogeneity the tumor rejection antigen(s) of the Meth A sarcoma CI-4 sarcoma and RBL-5 leukemia. These are collaborative studies (DuBois, Appella and Law, LCB)(Rogers and Law LCB) and DeLeo and Old (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 of Meth A and also of a recently derived MC-induced sarcoma, CI-4. 3) The active fraction following S-200, lectin and AcA54 chromatography is in the 75K range and 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 AcA54 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 75K 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.

The most enriched material has been used to develop hybridoma anti-Meth A specific cytotoxic and also precipitating antisera which are being used in further purification. These monoclonal antibodies interestingly are not highly specific.

2. Phenotypic studies of cells transfected with high molecular weight DNA from Meth A sarcoma cells

Our cooperative study with Dr. Nancy Hopkins (MIT Cancer Center), concerning the phenotypic expression of Meth A specific tumor antigen continues and has yielded interesting results. BALB/c 3T3 (f7) subline, (specifically cloned and selected) has been transfected with high molecular weight DNA from Meth A sarcoma using appropriate controls. The following assays done blindly have been accomplished from transformed foci:

1) assay for malignancy in the syngeneic BALB/c host

2) presence of TSSA by absorption of the specific cytotoxicity of a Meth A syngeneic antiserum, and

3) tumor rejection and specificity (TSTA).

We have observed a high frequency of co-transfection of transformation and TSTA at least among the primary transfectants. Most transfected lines (from Meth A DNA) grew progressively in BALB/c mice; 5 of 8 foci selected from one experiment possessed Meth A TSTA. DNA extracted from several of these lines was used in secondary rounds of transfection; 5 of 8 of II transfectants contained Meth A-specific TSTA. These results suggest that in the Meth A sarcoma a transforming gene and a genetic determinant of TSTA are intimately related since one would expect the frequency of a transfer of the 2 phenotypes to be only 0.01 to 0.001 if specified by unlinked genes.

Several of the transfected lines have been passaged continuously in vivo and assayed through 15 generations for immunizing capacity against Meth A. Results show some instability of the TSTA phenotype. Not all have as yet been assayed for specificity.

Meth A DNA cleaved with restriction enzymes retains transforming capacity (Hopkins and Vande Woude); these transferants are now being assayed for the presence of Meth A TSTA. This DNA fragment appears to be of a size (15 Kb) appropriate for molecular cloning. The frequency of co-transfer is much lower (20% or less) and not all immunogenic clones have been assayed for specificity.

Two transfectants produced by the DNA fragment (cloned 15Kb determinants) have been assayed for malignancy - that is progressive growth in syngeneic BALB/c mice and for the Meth A-specific TSTA. Both grew as undifferentiated neoplasms (probably of endothelial origin) and one (MAL-210) in preliminary tests appears positive for Meth A immunogenicity. These findings are only of a preliminary nature and more tests are in progress.

It will be of interest to determine if this isolated determinant is related to viral onc genes and the relationship of this determinant to our 75K TSTA.

3. Tumor Antigens of the RBL-5 Lymphoma

With the departure of Dr. O. Alaba, collaborative efforts have begun with Dr. Michael Rogers in attempts to isolate and purify the RSTA of RBL-5, an RMu-

LV-induced T cell leukemia. Higher yields of TSTA are now available through use of cytosol-derived antigen without resort to cell membrane isolation and solubilization (with detergents). Purification is proceeding through the usual gel filration and affinity chromatography. Two major bands (~ 75K ξ 65K) on SDS-PAGE have been visualized. These appear to be related. These materials along with their dimers are very immunogenic (particularly the 75K material) against other FMR-induced lymphomas but not against G-MuLV-induced or radiation induced leukemia. The 75K material is not related to the 75K Meth A isolate immunologically. Also, although the RBL-5 TSTA isolate has FMR group specificity it appears not related to gp70 or any other viral proteins.

3. Association of retrovirus and expression of the Meth A antigen

We continue our studies of the mechanisms involved in the specific Meth A immunogenicity found in the cells of an M-MSV (MuLV)-induced BALB/c sarcoma (Appella, Law, Chang, Wivel, LCB and DeLeo and Old, Sloan-Kettering). It was found originally that a tissue-culture passaged variant of the neoplasm, 11A, was capable of absorbing cytotoxicity of the specific α-Meth A antiserum, the only one (except Meth A) of more than 100 neoplasms, normal tissues and viruses capable of absorption. The 11A variant was found to immunize BALB/c mice in a specific manner against Meth A only. The 11A variant is a virus producer line but in contrast to the original 11A tumor line (carried in vivo) does not contain rescuable MSV.

An endogenous B-tropic retrovirus has been isolated from the 11A variant line. This uncloned virus (11A-MuLV) when infecting SC-1 cells was found to transmit specific immunity to Meth A. 11A-MuLV was cloned by twice limiting dilutions but upon infectivity of SC-1 cells was negative in specific immunogenicity assays against Meth A suggesting that 11A-MuLV represents a minority among the 11A-MuLV population of viruses. Then, SC-1 cells were infected with a higher multiplicity of virus (sucrose-banded virus). The infected SC-1 cells were cloned and assayed for XC and RT reactivities and for their ability to absorb cytotoxic α-Meth A antibody. Among the many clones assayed and those showing XC (+) reactivity, about 20% (9 clones) were positive for absorption. These (+) clones were assayed for Meth A immunogenicity and only 2 clones (#s 36, 46) were immunogenic against Meth A. Recently however, 2 11A-infected clones (#s 17 and 22) were found to be XC-, RT+ and absorbed Meth A cytotoxicity. These clones produced virus particles and immunized against Meth A but only in the early passage (not late passage) generations. If virus and TSTA are coordinately expressed, these results suggest that virus replication is inefficient and that successive cell passages reduce the number of progeny virus. These observations are consistent with other data indicating defective virus. Nonetheless early passages of virus in SC-1 cells may provide the source for isolation of the 11A defective virus and studies to this end are being pursued.

Recently the Meth A antigen detected serologically was detected on another Mo-MUSV transformed cell, SAC(-), a non-producer neoplasm of STU strain origin. This neoplasm and its control sublines are not being assayed for Meth A TSTA and preliminary data suggest the presence of this tumor specific antigen. We continue our collaborative efforts (DeLeo and Old, Sloan-Kettering) on attempts to define the mechanism responsible for the association of transformation and Meth A antigen expression.

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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TITLE OF PROJECT (80 characters or less)
Structure and Cloning of Histocompatibility and Tumor Antigens

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Structural studies of histocompatibility antigens, tumor antigens, transformation related antigen (p53) and epoxide hydrase. It is the purpose of this project to study the chemistry and structure of different molecules. Biochemical isolation of a transformation related protein (p53), tumor antigens and epoxide hydrase is carried out. The chemical structure of these proteins is being approached both by micro methods and nucleic acid sequence and the relationship of the amino acid sequence to other known structures is being analyzed. Cloning of tumor antigens and the p53 protein is being approached in order to study the molecular organization of these genes and permit a better understanding of the mechanism involved in the regulation and expression of different genes in relation to the molecular events taking place during transformation.

Histocompatibility Antigens

The study of the organization and expression of the H-2 gene family has been carried out recently by the cloning methods of recombinant DNA. Of 35 genomic bacteria phage clones isolated from BALB/c ($H\text{-}2^d$) colonies which cross hybridize to mouse cDNA probes specific for H-2, two have been identified which encode $H\text{-}2D^d$ and $H\text{-}2L^d$. To define precisely these assignments, these clones were introduced into mouse L cells by cotransformation with the herpes virus thymidine kinase gene by the calcium phosphate method. Stable transformant cloned cell lines express usual levels of $H\text{-}2D^d$ and $H\text{-}2L^d$. In addition these transformant cell clones serve as specific targets for cytotoxic T lymphocytes. This approach represents a novel and powerful way to study the expression and function of the genes of this and other families.

The major transplantation antigens of the mouse are a family of cell surface glycoproteins which serve as recognition signals in many immunological reactions. They are detected on the plasma membrane of virtually all somatic cells. We have reported previously that F9 teratocarcinoma stem cells do not express any major histocompatibility antigens. Evidence has been obtained that this regulation of major histocompatibility antigen expression is due to transcriptional control of both H-2 and $\beta 2$ microglobulin. In order to assess if this control is more general and may serve as an example of the controls that function in normal embryonal development, we have examined two cloned cell lines derived from the whole placenta of two strains of mice. Very low levels of both H-2 and $\beta 2$ microglobulin were detected on the cell surface. These data correlate with the presence of low levels of mRNA for these proteins. The cloned cells have been shown to be trophoblast, which in the embryo are located at the interface between fetal and maternal cirulations. The low level of histocompatibility antigens in this particular region may be an important factor in the survival of the fetus as an intrauterine semi-allograft.

A serum protein which has been shown to lack H-2 alloantigenic determinants and binds $\beta 2$ microglobulin has been described. In order to understand its biological function, it is imperative to know its biochemical properties and structural relationship to H-2 antigens. We have obtained data on the molecular properties of this protein, e.g. its molecular weight, amino acid composition, pI value and peptide maps. The primary structural analysis of this protein is under study in order to compare its relationship to the histocompatibility antigens.

One of the more perplexing problems in cellular immunology is the mechanism by which T-lymphocytes recognize antigen only in association with macrophages of a particular H-2 type. An approach to the investigation of this question is to examine in detail the specificity of T-lymphocyte antigen recognition using a family of peptide antigens. Our initial observation was that the same rank order or relative antigenicity was observed within a set of peptide antigens from cytochrome of different species and the acetimidyl derivatives of this same group even though the two sets stimulated different T-lymphocyte clones. This finding suggested the possibility that antigen recognition proceeded by utilizing two different sites; one site to account for the invariant rank order and the second site to account for distinguishing between the derivatized native peptides. The possibility that the MHC product and the antigen may interact directly has long been discussed. Such an interaction would appear as a second site when the

specificity of antigen recognition was investigated. However, the specificity of such a second site would be resident in the macrophage, not the T cell. In other words, if a T cell clone would be found which reacted with antigen in association with two different H-2 types, the rank order of antigen reactivity would depend not only on the T cell clone but also on the H-2 type of the macrophage. This was found to be the case when the specificity of T-lymphocyte hybridomas were examined. Thus we have shown that there is specific interaction between the macrophage and antigen. Currently investigations are underway to define the sites of T cell-antigen and MHC-antigen interaction. Secondly, we are investigating whether a particular MHC-antigen interaction site can cooperate with a large variety of T cell clones.

TUMOR ANTIGENS

Methylcholanthrene-induced sarcomas are known to carry a distinctive antigen (TATA) which can impart transplantation immunity to the tumor of origin by prior immunization with that tumor or a soluble form of the antigen derived from it. A TATA with an apparent molecular weight of 75,000 has been isolated from the cytosol of the BALB/c sarcoma Meth A. The antigen was purified by immunoaffinity chromotography following hexylamine agarose, gel filtration and hydroxylapatite chromatography. A second protein having a molecular weight of 75,000 was isolated from the cytosol of the recently derived methylcholanthrene induced sarcoma, CI-4, by essentially the same procedure. Both 75 kilodalton proteins effectively primed BALB/c mice to reject only their tumors of origin and afforded no protection against challenge by other independently derived sarcoma of BALB/c origin. The antigens purified from Meth A and C1-4 sarcomas appear to be closely related but non-identical proteins. The two antigens can be recognized by a rabbit antiserum prepared against the Meth A 75 kilodalton protein and have identical molecular weights but differ in their chromatographic behavior on hexylamine agarose and hydroxylapatite. Amino acid compositions of both antigens are similar but not identical. We are currently undertaking a study of the primary structures of these antigens to determine the relationship between individual TATAs from different sarcomas. In addition, studies are now in progress to determine the molecular organization of the genes involved in the expression of these antigens. By the use of nucleic acid probes we hope to answer a critical question: Are these antigens derived from a multigene family or are they products of somatic mutation of a single gene?

Recently, a transformation-related protein with a molecular weight of aproximately 53,000 daltons has been described by various laboratories. This protein, termed p53, can be immunoprecipitated from many different tumor cell lines, but is absent (or present in very low titers) in normal resting cells. An isolation procedure for p53 which results in an 800 fold purification to apparent homogeneity, with a recovery of 20% has been described. The protein isolated has been identified as the transformation-related p53 by immunologic methods. We have also reported its amino acid composition and the first 20 amino acid residues of the amino terminal sequence. Further work is now in progress to clone by recombinant DNA the gene encoding this protein and to establish its role in transformation.

PROTEIN CHEMISTRY

Epoxide hydrase is a critical enzyme involved in the metabolism of many endogenous substrates and exogenous substance such as drugs, mutagens, carcinogens and other toxic compounds. Epoxide hydrase has also been identified as a preneoplastic antigen. We have recently shown that the human liver enzyme and the rat liver enzyme are structurally similar and share a common evolutionary origin. The rat liver enzyme, labeled with an active site inhibitor, 2-bromo-4'-nitroacetophenone, has been specifically cleaved into two large peptides of 33,000 and 17,000 daltons. The larger peptide contains the active site radiolabel and can be cleaved into 9 peptides by cyanogen bromide cleavage at methionine residues. Again a single peptide retains all of the radiolabel. This peptide has a molecular weight of approximately 6,500. It should now be possible to ascertain the amino acid sequence of this active site peptide and design inhibitors to control the activity of this enzyme.

Four cloned cDNAs from mouse lens were shown to be derived from mRNAs encoding α-crystallin polypeptides with similar but non-identical NH_2-terminal ends. The data suggest the existence of a closely related family of γ-crystallin genes.

A lactoprotein clone, p-W52 , was found to encode the complete sequence of rat whey phosphoprotein. Clone p-Wp52 has allowed the study of the expression of whey phosphoprotein mRNA during functional differentiation of rat mammary gland and in mammary tumors. In the tumors, the mRNA was barely detected and, in correlation with the reduced expression, the gene was found to be hypermethylated.

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TITLE OF PROJECT (80 characters or less)
Biological Studies of Various Normal, Virus-Infected, and Malignant Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	S.S. Yang	Chemist	LCBGY	NCI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Cell Biology

SECTION
Ultrastructural Biology Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	1.00	0.75

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the primary purpose of this project to study some of the pertinent factors which influence cell differentiation and malignant transformation, using techniques and approaches which range from the microscopic to the molecular level. Particular emphasis is given to those systems in which murine RNA tumor viruses or their precursors or chemical carcinogens may be the transforming agent. A variety of mouse model systems are used, including plasma cell tumors, mammary tumors, neuroblastomas, and methylcholanthrene-induced sarcomas. Current projects include: 1) effects of interferon on the assembly and maturation of murine retroviruses with special emphasis on the study of mechanisms whereby whole virions are rendered noninfectious; 2) effects of interferon on methylcholanthrene-induced sarcomas of the BALB/c mouse with the aim of defining anticellular and/or immunological activity; 3) relationship of retroviruses to the expression of certain phenotypic changes in chemical carcinogen-induced murine sarcomas; 4) study of transforming genes in methylcholanthrene-induced sarcomas.

Major Findings:

1. Effects of interferon on murine retroviruses. Most of the studies in this system are being done with Dr.Paula Pitha and reflect a continuing interest in the molecular mechanisms which account for the effect of interferon on retroviruses. In the case of most viruses, interferon causes an impaired mRNA translation with resulting defects in viral structural proteins. However, the effects of interferon on retroviruses occur after the synthesis of viral RNA and most of the structural proteins. Our work and that of other laboratories has demonstrated a mode of action involving the late stages of virus assembly and maturation. However, the precise type of change observed seems to vary among the different classes of retroviruses. In the case of 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 for propagation. Even in the systems where there is a release of virions from interferon-treated cels, the released particules have reduced infectivity. It must be emphasized that this interference with assembly is 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 within 18-24 hours.

Our most recent experiments have been done with a newer class of murine retrovirus, MCF, which is apparently an env gene recombinant of ecotropic and xenotropic viruses endogenous to the AKR mouse, and which seems to accelerate the development of leukemia. One of the characteristics of MCF virus is a slow rate of processing of the pr76 env precursor to gp71, as compared to the ecotropic viruses. Since previous studies with ecotropic viruses indicated altered rates of proteolytic cleavage of the env gene products, the strategy in using MCF was to exploit an assembly stage in which the kinetics of cleavage are already slower than average. An additional advantage derives from the fact that MCF viruses can be titrated by focus assay on mink cells which are insensitive to interferon; thus the infectivity assay is not affected by the possible presence of residual mouse interferon in the system.

When chronically infected SC-1 cells were treated with highly purified interferon for 24 to 48 hours, there was approximately a 100-fold reduction of infectious virus post interferon treatment, but the reverse transcriptase assay indicated only a ten-fold reduction in the number of particles, indicating a significant production of noninfectious virus. Furthermore, there was a 2.5 fold increase in the number of virions accumulating along the cell surface following interferon treatment, suggesting that release is slowed. After a 24 hour exposure to interferon, the virions contained an 85 K dalton glycoprotein of non-viral origin which was present in excess of the amount of the viral envelope glycoprotein, gp70. Virus particles produced from cells treated with interferon from 32-48 hours were nearly devoid of gp70 and contained measurably lower quantities of p30. These results are consistent with previous observations that gp70 is essential for infectivity. Although interferon did not alter the intracellular processing of viral polyproteins to mature structural proteins, there was an accumulation of viral p30 and p12, consistent with the observed increase in cellassociated virions. In contrast, the levels of gp70 released into the tissue culture medium were the same in the presence and absence of interferon, but little gp70 was found associated with virions produced from interferon-treated cells. This may be related to the finding that no gp70-p12E complex was detected

in either virions or tissue culture fluids following interferon treatment. It is probable that gp70 is not assembled into particles and is released from p15E/p12E following proteolytic cleavage of gPr 85^{env}. While the molecular basis for this failure of incorporation of pg70 into the MCF class of retroviruses is not known, there are two principle possibilities which have to be considered. There could be a steric interference phenomenon whereby an altered carbohydrate side chain in gp70 would prevent the formation of stable disulfide bonds between gp70 and p15E/p12E. Alternatively, interferon could produce alterations in the plasma membrane itself, also leading to an unstable gp70-p15E/p12E complex. Given the current results, it seems quite possible that restriction in virus assembly is not exclusively localized to virus maturation sites, but may occur at other locations in the plasma membrane of the host cell, while leading to the same end result, the failure of incorporation of gp70 into virions.

2. 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 the aforementioned requirements; additionally it can be serially 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 effects of interferon from the antiviral ones. It is also of importance that there are a number of stable membrane markers such as the tumor associated transplantation antigen (TSTA) and the tumor specific surface antigen (TSSA) which are potentially susceptible to the action of interferon.

Our first series of experiments established that in vitro treatment with 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 ^{3}H-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 the 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%. The number of cells required to consistently produce tumors in 100% of nude mice is 10-fold lower than in BALB/c mice. 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.

Since most of our experiments have involved treatment of Meth A cells for 72 hours, we elected to study the effects of prolonged exposure. Cells were treated with interferon for a period of 25 days and again no marked cytotoxic effects were observed. However, when mice were challenged with 5×10^6 cells, 100% of the controls developed tumors and none of the mice receiving interferon-treated cells developed neoplasms. In order to determine if the primary effect of interferon is mediated through the immune system, a number of experiments are underway to study immunosuppressed mice given Meth A cells after 25 days of in vitro exposure to interferon.

3. Transforming genes in methylcholathrene-induced sarcomas. A recent project involves the extraction of high molecular weight DNA from different methylcholathrene-induced (MCA) sarcomas, use of this DNA to transfect BALB/3T3 cells, observation of such cells for the development of transformed foci and isolation of such foci for later challenge of BALB/c mice. The goal of these experiments is to determine if transforming genes can be demonstrated in a number of different sarcomas, and to determine if TSTA co-transfers with transforming activity as it has been shown to do for the Meth A sarcoma. One unltimate aim of such work would be to ascertain the role of TSTA in the process of transformation; it could simply be a marker characteristic of MCA tumors or it could have a more fundamental role.

Publications:

Bilello, J.A., Wivel, N.A., and Pitha, P.M.: Effect of interferon on the replication of MCF virus in murine cells: Synthesis, processing, assembly and release of viral proteins. J. of Virol., in press, 1982.

DeLeo, A.B., Chang, K.S.S., Wivel, N.A., Appella, E., Old, L.J., and Law, L.W.: Possible role of a retrovirus in the expression of tumor specific antigens of the Meth A sarcoma. Int. J. of Cancer, in press, 1982.

Wivel, N.A., and Pitha, P.M.: Interferon treatment of murine Meth A sarcoma cells: Effects on the malignant phenotype and expression of tumor-specific and H-2 antigens. Int. J. of Cancer, in press, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 04834-06 LCBGY

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Genetic Mechanism of Carcinogenesis and Biological Modifier as a Defense Mechanism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. S. Yang	Chemist	LCBGY	NCI

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Ultrastructural Biology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.00	1.00	1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major thrust of this study is to elucidate the molecular genetics of neoplastic transformation of normal tissues and the action of a biological modifier in cellular defense mechanism. Two experimental systems were used: 1) Two rat leukemia helper viruses - a) KSV(RHHV) originally isolated in this laboratory and b) WR-RaLV, a wild rat tumor virus, and 2) Interleukin 2, a rat spleen cell product. Multidisciplinary approaches involving nucleic acid and protein chemistry, recombinant DNA, and tissue culture were employed. We have earlier completed extensive morphological, biological, biochemical and immunological characterizations of these retroviruses. Our current interests focus on the molecular mechanisms involved in the evolution of a transforming DNA sequence through the recombination between a RaLV DNA sequence and rat endogeneous DNA sequence c-src. Our total research efforts are concentrated on: 1) Molecular cloning and recombinant DNA analysis, 2) Restriction endonuclease map, 3) Functional organization, 4) Heteroduplex analysis of conservation and divergency in nucleotide sequences, 5) Microinjection of RaLV total and subgenomic DNA sequences for DNA transduction analysis and 6) Immediate and long term effects of interleukin 2 on T-lymphocytes and natural killer cells in cellular defense mechanism.

Major Findings:

1. Molecular cloning and recombinant DNA analysis of RHHV and of WR-RaLV DNA sequences via a plasmid vector pBR 322 in E. coli RRI host.

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 functional organization with respect to the c-src sequences in the genome of a transforming rat virus.

Relatively little is known of the genomic organization of the endogenous rat leukemia helper viruses. Recently we have identified the DNA sequences specific for both the endogenous RHHV and WR-RaLV as 8.0-8.8 kilobases in length. Among the endogenous ecotropic MuLV of the AKR type and the Mo-MuLV, genomic DNA also shows a larger size relative to that of the murine sarcoma viruses; it varies from 8.2 to 8.8 kilobases. Both the integrated and the proviral DNA sequences of the RHHV and WR-RaLV have been isolated and purified from their respective host cells using a combination of chemical extractions, CsCl density gradient isopycnic centrifugation and Reverse Phase V(RPC-V) high pressure liquid chromatography of the restricted DNA by EcoR1 endonuclease. The viral genomic DNAs of both the RHHV and WR-RaLV were then successfully ligated onto the pBR 322 DNA at the restriction site of endonuclease BamH1 and molecularly cloned in E. coli RRI (EK-1) host. Plasmid pBR 322 carried both the ampicillin resistance and the tetracycline resistance genes. Since the ligation site of the RaLV DNA onto the pBR 322 DNA was at the Bam H1 site located within the tetracycline resistance locus, the E. coli RRI cells transformed by the recombinant DNA were therefore first tested for ampicillin resistance and tetracycline sensitivity. After screening approximately 16,000 ampicillin resistant clones 38 were found persistently tetracycline sensitive. These ampicillin resistant, tetracycline sensitive clones were then subjected to colony hybridization using high specific activity ^{32}PcDNA probe synthesized in an endogenous reverse transcriptase reaction of purified RHHV or WR-RaLV. (These were reported in publications 1, 2 and 3.) Two of these RaLV clones, 8/23 of RHHV and WR-RaLV 13, were analyzed extensively in the aspects of their genomic and functional organizations, conservation and divergency of RaLV DNA sequences in evolution, conservative and vital DNA sequences essential for DNA recombination with c-src during DNA transduction.

2A. Structural organization of RHHV 8.8 kb DNA

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 DPNI 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 instrumental 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. The length of LTR for retroviruses among various species varied greatly, ranging from 400 bp to 1,500 bp. At least one to three copies of LTR had been reported for each viral genomic DNA. In this rat helper leukemia virus genomic DNA, at least two copies of the 600-800 bp LTR were detected per 8.8 kb DNA. Currently, the nucleotide sequence of this candidate LTR is in the process of being resolved so that its length and characteristic may be more precisely defined. Molecular cloning and the structural organization of RHHV DNA were reported in publications 2, 3, and 4.

2B. A restriction endonuclease map of WR-RaLV genomic DNA

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 amendable 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. Molecular cloning and restriction map of WR-RaLV was published in publication 5.

3. Functional organization of RHHV DNA.

The functional organization of RHHV DNA, i.e. the gene order along the 8.8 kb DNA was examined by cell-free translations of RHHV specific mRNA selected by hybridization to the restricted RHHV DNA fragments in a rabbit reticulocyte lysate

system. Recombinant DNA, blot-transferred onto nitrocellulose membrane, total or isolated 8.8 kb DNA, was cut out for hybridization selection of RHHV specific mRNA. The mRNA eluted off the filter at the end of 48 hours of hybridization was was used in cell-free translation with ^{35}S-methionine as label. Prominent cell-free translated protein bands immunoprecipitated by anti-RaLV serum and readily discernible were the viral precursor polyproteins, P180-200, P120, and P85, and individual proteins, P70, P29-30, 30, and P15. Based on the polyproteins and individual proteins encoded by the various restricted DNA fragments along the 8.8 kb DNA in a series of such hybrid-select cell-free translation experiments, a functional scheme of the RHHV genomic DNA has been constructed. (See publications 3 and 4)

4. 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 both 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 exexamine 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 approxiately 5.5 to 7.0 kb, which corresponded in part with the env gene of the leukemia helper virus genome. WR-RaLV formed perfect heteroduplex with the subgenomic 1.0 kb DNA of the KiSV P21 coding sequence. 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.

5. 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 (NRK^{153}). Release of rat C-type leukemia helper viruses by the microinjected cells was examined by superinfection on Kirsten transformed nonproducer cells (KNRK). Immediate release of helper leukemia viruses at a very low level was observed only in the $NRK^{153}_{m3.5/cir}$ 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^{153}_{m} 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. (See publications 3 and 4.)

6. Immediate and long term effects of interleukin 2 on T-lymphocytes and natural killer cells in cellular defense mechanism

Interleukin (IL2), a biological modifier, is produced by spleen cells when freshly cultured from W/Fu rat spleen, in the presence of Concanavalin A. IL2 was purified 50-100 fold by ammonium sulfate fractionation and sephadex G100 column molecular filtration technique. It was found to possess short-term growth-promoting effect on preformed cytotoxic T-lymohocytes for 3-5 weeks. It augmented the cytotoxic activity with the appropriate antigenic specificity. IL2 also promoted the selective growth of T cells isolated from tumor site and these T cells showed augmented in vitro and in vivo anti-tumor activity. This was submitted to Int. J. Cancer (publication 7).

As a continuing project and in collaboration with Dr. C.C. Ting, 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. 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. This was submitted to J. Cell. Immunol. (publication 8).

Relationship of Research to National Cancer Plan

Project Area - 10; Approach Elements - 2; Approach - 4

Publications

1. Yang, S.S., Chen, C., Taub, J., and Ting, R.C.: Viral specific DNA sequences and appearance of aneuploidy and marker chromosomes in fisher rat tumors and embryonic cell transformation by KSV(RHHV). In Yohn, D.S., and Blakeslee, J.R. (Ed.): Advances in Comparative Leukemia Research. New York, NY, Elsevier/North-Holland Biomedical Press, 1982, pp. 347-350.

2. Yang, S.S., Taub, J., Modali, R., and Gardner, M.: Molecular cloning of the

restricted DNA fragments. In Yohn, D.S., and Blakeslee, J.R. (Ed.): Advances in Comparative Leukemia Research. New York, NY, Elsevier/North Holland Biomedical Press, 1982, pp. 351-352.

3. Yang, S.S., Modali, R., Wu, R., and Gardner, M.: Molecular cloning of the endogenous rat C-type helper virus DNA sequence: Structural organization and functional analysis of some restricted DNA fragments. J. Gen. Virol. (In press, Sept. 1982)

4. Yang, S.S., Modali, R., and Murphy, E., Jr.: Cloning of the rat endogenous helper leukemia virus DNA sequence and expression of the helper activity encoded by the cloned DNA sequence in normal rat kidney cells by microinjection. In Acton, R. (Ed.): Cell Culture in Support of Bioscience. Proceedings of the Cell Culture Conference. New York, NY, Plenum Press, (In press, 1982)

5. Yang, S.S., Taub, J., Modali, R., Brown, D., and Murphy, E., Jr.: Biological activity of cloned rat endogenous C-type viral DNA transferred by microinjection. J. Gen. Virol. (In press, Sept. 1982)

6. Yang, S.S. and Modali, R.: Genomic complexity and molecularly cloning of a proviral DNA specific for a feral rat endogenous C-type virus, originated from a 3-methyl cholanthrene induced fibrosarcoma. In Cohn, E.W. (Ed.): Progress in Nucleic Acid Research and Molecular Biology. Vol. 27, New York, NY, Academic Press. (In press, June 1982)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05550-13 LCBGY

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Role of Retroviruses in Oncogenesis and Tumor Immunogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	2.50	1.50

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 neoplasia, the use of viral mutants to analyze the regulation of gene expression associated with cell differentiation and oncogenesis, and the immunologic responses of the host to tumor associated antigens.

The topics of current interest are: 1) Further characterization of amphotropic viruses isolated in this laboratory. 2) Molecular cloning of 11A-MuLV and amphotropic virus DNA by recombinant DNA techniques. 3) Expression of histocompatibility antigens and other differentiation antigens in oncogenic murine trophoblast cell lines. 4) Isolation and characterization of polyoma virus mutants that can grow in trophoblast and/or embryonal carcinoma cells. 5) Further studies on 11A-MuLV-induced Meth A-transplantation rejection antigen. 6) Natural killer cell susceptibility of trophoblast cells.

Project Descriptions:

I. Further characterization of amphotropic viruses isolated in this laboratory.

Two strains of amphotropic viruses, R5NX and YACNX, which were isolated from RBL-5 and YAC cell cultures respectively, have been subjected to further study. Since this is a first successful isolation of amphotropic viruses from sources other than wild mice, and since they were isolated from Rauscher-MuLV-induced, and Moloney-MuLV-induced leukemia cells of inbred mice (C57BL/6 and A strain, respectively), it is essential to compare with the R-MuLV, M-MuLV, AVR-MuLV and the prototype amphotropic virus (4070A) in order to elucidate the possible mechanism of emergence of these viruses from these neoplastic cells in culture. The ability of these new isolates to induce leukemia or other pathogenic changes has been investigated by neonatal infection of C3Hf/He mice. The results so far (6 months) have been negative.

Competitive radioimmunoassays, using specific rabbit antisera against p12 and gp70 of these and other type C retroviruses, have been completed. The results of tryptic digest mapping of p12, p30 amd gp70 of these viruses also corroborated the conclusion that the R5NX virus is a recombinant virus deriving its gag gene from the endogenous AKR like MuLV and env gene from the amphotropic virus while the YACNX virus is more related to the prototype amphotropic virus (4070A) but not identical. The origin of the pol gene of R5NX and YACNX is not known yet, and the possible contribution of R-MuLV and M-MuLV in the generation processes of these amphotropic viruses needs further investigation.

II. Molecular cloning of 11A-MuLV and amphotropic virus DNA by recombinant DNA techniques:

In order to study the role of a B-tropic virus (11A-MuLV) in inducing transplantation immunity against Meth A tumor, and to study the amphotropic virus gene expression in RBL-5 and YAC cells which generated the newer isolates of amphotropic viruses mentioned above, a plasmid was constructed by ligating the linearized pBR322 (cut by restriction enzyme Pst I) with the double stranded viral DNA which was derived from 11A-MuLV or prototype amphotropic virus (4070A). E. Coli MC1061 was then transfected with each of the recombinant plasmids and selected for the tetracycline resistant and ampicillin-sensitive colonies on agar plates. After screening 1000 colonies for each virus by replica plate method, we could obtain colonies of bacteria containing the plasmid with recombinant DNA. The recombinant DNA for 11A-MuLV and 4070A virus was extracted, digested with EcoR I, BamH I, and Hind III enzymes, gel-electrophoresed, and hybridized with [^{32}p]70S RNA of 11A-MuLV and 4070A virus after Southern blot transfer to nitrocellulose filter paper. A DNA fragment of approximately 6 Kilobase pair (Kbp) and 7 Kbp respectively from these plasmids was found to specifically hybridize with the [^{32}p]70S RNA of 11A-MuLV and 4070A virus. Further work is in progress to characterize these cloned viral DNAs. These will be valuable for studying the following problems of interest: The role of 11A-MuLV provirus present in the XC(-) clones of SC-1 cells which were found to induce transplantation immunity against Meth A cells; the amphotropic virus genome status in RBL-5 and YAC cells; and the blast cells (vide infra) which do not produce infectious viruses after exogenous infection; etc.

III. Expression of histocompatibilty antigen and other differentiation antigens in oncogenic, murine trophoblast cell lines:

Since the major histocompatibility complex (MHC) plays an important role in the recognition of viral and T-cell dependent, antigens, as well as in transplantation immunity, the regulation of MHC gene expression in embryogenesis is an important step in cell differentiation. We have established trophoblast cell lines from murine placentas and reported their tumorigenicity across histocompatibility barriers among various strains of mice. We have reexamined the surface expression of H-2 antigens and beta-2-microglobulin with antibody-mediated cytotoxicity and flow microfluorometry techniques employing defined monoclonal antibodies. The level of $H\text{-}2^d$ antigen present in trophoblast cells (of BALB/c origin) was about 50 times less than that of BALB/3T3 cells. By ^{35}S-methionine labelling and specific immunoprecipitation followed by SDS-PAGE analysis, we examined the in vitro synthesis of H-2 and beta-2-microclobulin of these cells. As compared with spleen cells of BALB/3T3 cells, much lower levels of synthesis of H-2 and beta-2-microglobulin were observed with trophoblast cells. The total mRNA preparations from these cells were electrophoresed, blot-transferred to nitrocellulose and hybridized with [^{32}p] labelled H-2 and beta-2-microglobulin probes. Since very low levels of this mRNA were found in trophoblast cells, it was concluded that the regulation of expression of these antigens is at the level of transcriptional control.

Two cytoskeletal proteins, Endo A (MW 55K) and Endo B (MW 50K) which were reported to be present in extraembryonic endoderm and trophectoderm cells (Oshima, 1981) were found to be present also in our trophoblast cells but not in embryonal carcinoma (EC) or 3T3 cells. However, our trophoblast cell lines were devoid of stage-specific embryonal antigen, SSEA-1, which were found in EC cells, early fertilized egg of 8-cell to morula stages, and inner cell mass (Solter and Knowles, 1978). The presence of these differentiation antigens would indicate that the differentiation status and gene control of our trophoblast cells are more like those of trophectoderm cells than EC cells. The relationship of these differentiation properties of trophoblast cells to their oncogencity would be an interesting subject for further study.

IV. Isolation and characterization of polyoma virus mutants that can grow in trophoblast and/or embryonal carcinoma (EC) cells:

As reported previously, both trophoblast and EC cell lines are unable to support the growth of type C retroviruses, and wild-type polyoma viruses. Many attempts to isolate type C retrovirus mutants that can grow in these cells have not been successful. However, we were able to isolate six strains of polyoma virus mutants (PyTr) that can replicate in trophoblast cells and two mutants (PyEC) that can replicate in EC cells. Interestingly, the former (PyTr) cannot replicate in EC cells, but the latter (PyEC) can replicate in trophoblast as well as in EC cells. All of these mutants as well as wild-type virus can grow in differentiated cells such as teratoma or 3T3 cells. Such a undirectional, differentiation-stage-restricted growth capability of polyoma virus host-range mutants may provide a tool for analyzing the gene control mechanisms exhibited by trophoblast and EC cells in their differentiation processes.

Restriction endonuclease analyses revealed that the late, non-coding region near the origin of viral DNA replication contained nucleotide changes

necessary for the growth of these PyTr mutants in trophoblast cells. A 347 base pair (bp) fragment obtained by double digestion by two single-cut enzymes, Bgl I, and Bcl I (map units 65.4-72.2), covers this region. The following marker rescue experiments confirmed this contention.

Hybrid molecules containing a larger Bgl I/Bcl I fragment of wild-type or a mutant, and smaller Bgl I/Bcl I fragment of a mutant, or wild-type, respectively, were contructed, and their ability to replicate in trophoblast cells was tested. The results indicated that hybrid molecules containing the smaller Bgl I/Bcl I fragment of the mutant can replicate in trophoblast cells whereas the reciprocal hybrid molecules containing the smaller Bgl I/Bcl I fragment of the wild-type can not. Further work is in progress to compare the nucleotide sequence of these mutants to draw conclusions as to the essential structural changes in this region of viral DNA which enable the cells at various differentiation stages to recognize and process for viral replication.

The trophoblast cells replicating the PyTr mutants did not show cytopathic effects or increase in their oncogenicity when inoculated into mice. The tumor specific transplantation antigen (if present) of these cells particularly in relation to the lack of H-2 antigen expression in these cells would be an interesting subject for further study.

V. Further studies on 11A-MuLV-induced Meth A-transplantation rejection antigen:

Further work on the role of 11A-MuLV on Meth A transplantation immunity was conducted. The cloned 11A-MuLV was passaged in CII-10, another methylcholanthrene-induced tumor cell line which has no Meth A transplantation antigen, and then infected on SC-1 cells. Immunization of BALB/c mice with these SC-1 cells induced resistance against challenge of Meth A but not against CII-10 tumor cells. Control SC-1 cells uninfected or infected with Stansly-MuLV passaged in CII-10 cells did not show similar effects. These experiments are being repeated and broadened to include viruses passaged in CII-7 cell line, which is distinct from CII-10 or Meth A, in order to confirm the ability of 11A-MuLV to induce Meth A transplantation antigen. Among various possible explanations for this phenomenon, if it is confirmed , it may be hypothesized that the 11A-MuLV DNA can integrate into cellular DNA at a certain locus where the dormant (unexpressed) Meth A gene (the gene that codes for Meth A specific tumor transplantation antigen) may be activated as a result of the insertion (Insertion-promotion hypothesis), and subsequently picked up by 11A-MuLV which acts as a vector for transfer of this gene into SC-1 cells. Further experiments to test this hypothesis are in progress.

VI. Natural Killer (NK) cell susceptibility of trophoblast cells:

The significance of the high susceptibility of trophoblast cells to NK cells has been pointed out in previous reports, especially in view of the fact that they show little H-2 antigen expression and that they play important roles in feto-maternal relationships. Their NK-sensitivity was decreased either by short-term treatment with interferon (IFN) or by culture for more than 4 days in the presence of tumor promotor, 12-O-tetradecanoylphorbol-13-acetate (TPA). IFN-treatment of trophoblast cells cultured previously in TPA medium resulted in restoration of NK-sensitivity to the original level

exhibited by cells untreated with IFN or TPA. The decrease in NK-sensitivity of IFN or TPA-treated trophoblast cells was not due to the loss of NK recognition membrane structures, or to induction of prostaglandin which elevates cyclic-AMP levels. Inhibition of protein synthesis by cycloheximide restored the NK-sensitivity of TPA-treated trophoblast cells, indicating the possible contribution of de novo synthesized protein(s) which could confer NK-resistance. The TPA-treated trophoblast cells became more tumorigenic, producing larger tumors and a higher mortality than the untreated controls when inoculated into N:NIH(s)-nu or conventional adult mice. The fact that the T-cell deficient N:NIH(s)-nu mice did not easily allow the untreated trophoblast cells to form a tumor may suggest the important role of NK cells in tumor rejection. By contrast, L and $S^{+}L^{-}$ mink cells with or without TPA treatment were able to grow as tumors although both were as NK-sensitive as untreated trophoblast cells. Therefore, the decreased NK-sensitivity of trophoblast cells as a result of TPA treatment may be a necessary but insufficient condition for these cells to produce tumors in N:NIH(s)-nu mice. Thus, these H-2 deficient trophoblast cell tumors may provide a tool for analysis of the roles of immune T-cells as well as NK cells in tumor-host relationships.

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PERIOD COVERED October 1, 1981 through September 30, 1982

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Mammalian Cellular Genetics and Cell Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to analyze and develop new and difficult cell systems in culture. We have developed and are attempting to exploit applications of normal rat thyroid cell cultures. These cells are hormone dependent. They synthesize and secrete a very large protein product, thyroglobulin. They concentrate iodide 100-fold from the medium. They offer a unique opportunity to study secretion, ion uptake and cAMP response. These are being studied in our lab and in other labs, however, our approach is primarily to use electrophysiological techniques. We are attempting to study long term regulation of membrane potential and its relationship to secretion and hormone levels. We are also studying neurons and neuroblasts in cell culture. There are too few mammalian cell systems where "blast" cells can be observed in transition to mature, differentiated cells. We have tried this in nerve cells using cellular hybridization and cellular transformation (with ts SV40 viruses) and by using little known cell systems in which blast cells persist throughout life (olfactory epithelium). We are especially interested in the development of electrophysiological competence and in the development of chemical identity (specificity).

I. Introduction:

During the past year we have expanded the work with our normal, hormone dependent rat thyroid strains (FRTL, FRTL-5, and T-78) to include the cellular genetics of the regulation of thyroglobulin (TG) synthesis and secretion as well as the ionic and electrophysiology of TG secretion. We have enlarged and extended our collaboration with the Naples thyroid group to cover cellular genetics and molecular biology of the regulation of TG biosynthesis and secretion. Secondly, we have continued to try to understand the neuroblast cell strains that have been obtained by ts SV40 transformation of cells from the cerebellar anlage of rat embryos, 13 to 15 days in term. Thirdly, our interest in refining and further developing the techniques for studying cultures of differentiated cells has advanced with what could turn out to be an important observation related to the current wide spread interest in cells grown in hormonally defined media. When cells are grown under an agarose overlay such that macromolecules are inhibited from diffusing away from the cell surface they have very different physiological characteristics from identical cultures grown in the usual "ocean" of liquid medium.

Projects begun last year and the year before, cloning of malarial parasites and testing the effects of microinjection of insulin into individual cells, have been completed. The cloning of malarial parasites by micromanipulation of individual, infected red cells has been established and several clones and subclones are being investigated. Microinjection of insulin proved to have no observable effect on cells that survived the injection. In particular, there was no increase in mitosis conferred by large doses of intracellular insulin. Our participation in both these projects has come to an end.

Our plans to work with clonal strains of the neuroblasts from the olfactory epithelium has been temporarily abandoned until we can bring a student or postdoc on board to devote the time needed to accomplish the large job. The problem is exciting and the techniques are at hand; what is needed is budgetary support for a person.

Drs. Ron Magnusson and Anna Shekhtman left our group for jobs in Texas and the Immunology Branch, respectively. Dr. Shigeru Yasumoto from Japan has joined us as a Visiting Fellow. Our perennial problem of having more to do than people to do it with remains unchanged.

II. Culture of differentiated:

A. Thyroid cells:

Our Fisher Rat Thyroid cells grown in low or no serum (FRTL) have continued to provide a powerful resource; they are the first and possibly only example of a karyotypically normal, long term cell strain that has retained complex differentiated functions including biosynthesis of physiological amounts of thyroglobulin (TG), concentration of 100-fold or more of iodide, and apparently normal response to the hormone thyrotropin (TSH). We have sent these cells out

to laboratories around the world and they have provided the basis for numerous studies involving hormone mechanisms especially the response of the cyclic AMP system to TSH stimulation.

1. Human thyroid cell strains:

However useful these rat cells may be there is always a premium paid (especially by the physicians) on having a human cell strain available. After Dr. Ambesi-Impiombato and I succeeded in developing strategies that were successful in getting rat cell strains we thought that surely there would be no difficulty in getting human cell strains in the same way. That was not to be. Human cells have been supplied by Dr. William Valenti from surgical procedures on diseased thyroids which necessitated removal of small amounts of apparently normal tissue as well as from the "instant autopsy" program of the University of Maryland. In collaboration with Drs. Ambesi-Impiombato, Valenti, and L. Kohn we have made many attempts to culture human thyroid cells without success. Recently, during his visit from Naples, Dr. Ambesi has succeeded in overcoming the "human thyroid problem". The same media and hormone supplements used for the rat cell lines are used but no serum (fetal calf, calf, horse, human, etc.) can be tolerated by the human cells at any time. Furthermore, they require self-conditioned medium for long term survival. It has been known for some time that certain tumor cells and transformed cells may produce growth simulating factors in the culture medium and recent evidence suggests that even normal cells may produce growth factors such as insulin (albeit in very small quantities). Apparently the human thyroid cells require a factor that they themselves synthesize. This sort of "autocrine" phenomenon may be related to the chalones and other similar growth regulating substances that are vital to maintaining the integrity of groups of cells in tissues and may very well have important implications for the future understanding of the cancer problem. At any rate,. the availability of human cell strains with thyroid function appears near reality. Such cell strains, if they are like the rat cell lines, will prove important developments. Whenever we learn something about how to grow normal, differentiated cells in culture we are getting closer to understanding how these cells are integrated into tissues and organs and are therefore, closer to understanding how those mechanisms can go astray.

2. Genetic approach to the regulation of TG synthesis and secretion:

Last year we found that clones of TG producing, FRTL cells could be efficiently screened for TG secretion by overlaying the cells with a layer of agarose + 6H medium containing added rabbit anti-rat-TG serum. As the TG is secreted by the cells in the colonies it diffuses into the agarose layer and precipitates when it encounters the rabbit antibodies. The precipitate is readily visualized using low power stereo microscopes. We have used this method of visualization of TG secretion as a means for selecting rare non-producers, i.e., variants or mutants (spontaneous or induced) that fail to secrete TG. We have found that following EMS mutagenization two kinds of colonies are produced: those with large amounts of TG-anti-TG precipitate around them and a minority of colonies with much less precipitate. (Control plates without EMS treatment show only the heavy precipitate colonies.) Very few or no colonies have been found that

produce no precipitate at all. We interpret these under-producer colonies to be single hit mutants what one would expect if one of two diploid genes had been inactiviated. If we subculture one of these under producer clones and mutagenize it again - we predict (but have not yet found) there will again be two classes of colonies: those that produce small amounts of precipitate and a minority population of colonies that produce no precipitate. These will be the mutants that have hit a second allele and resulted in a null producing mutant strain. Because we conceive that there must be several mutation sensitive steps in the synthetic pathway for TG synthesis and secretion, we anticipate that these mutnats will fall into several complementation groups. The complementation groups should be easily testable by cell fusion and either agarose-overlay or indirect FITC anti-TG visualization procedures. Using cloned probe DNA (supplied by the Naples group) we can tell which mutants transcribe and ultimately hope to characterize several types of mutants useful in the classic molecular genetic types of experiments.

a. Insertion mutants in the TG system:

It has been widely accepted among the community of virologists that cells transformed by viruses like SV40 should show defects in the expression of the differentiated characteristics of the parent cell strain. Our findings with SV40 transformed FRTL cells have shown that the FRTL differentiated functions of hormone dependence, TG secretion, and iodide concentration remain unchanged. Unchanged, that is, until after 20-40 population doublings when one can find heteroploid substrains emerge that often, but not not always, show loss of one or more of the characters studied. The presence of the "T" antigen itself appears not to interfere with the differentiated functions. Dr. Yasumoto has extended these observations in the past year and it is now clear that primary transformed colonies exhibiting "T" antigen synthesis (as detected by fluorescent antibody) always produce TG.

There must be at least one class of transformant that would prevent TG synthesis or secretion: that class where viral DNA has intergrated in the TG gene itself (or into some other required gene) in such a way as to prevent its effective expression. These would be insertion mutants in which the viral DNA intergration was the mutagen. Fortunately, the TG gene alone is gigantic - the MW of TG is ~600,000 so it would appear to be a reasonable target. We are adopting techniques of high frequency transfection and/or infection to test whether such intergration mutants can be isolated.

If we can produce insertion mutants with viral genomes or other insertion sequences we shall have a very powerful method for locating and isolating genes that perform regulatory roles. Let us suppose we found that a second site mutation could knock out TG transcription. If the mutation were obtained by ordinary mutagenesis, then there would be no way to locate the gene. However, if the mutation were produced by insertion of a sequence that could be probed, then it is marked and can be isolated, cloned and sequenced. By using a two step protocol and a second virus or insertion sequence both the diploid state of the genome and the possibility of multiple integration sites can be overcome. This method or something to grow out of it may prove the only way to approach the problem of studying genes that are crucial for synthetic sequences or

developmental pathways but that are not a dominant part of the mRNA population of the cells.

b. Agarose anti-TG overlay and clonal heterogeneity:

When we first made our assays on clones of FRTL cells for TG secretion we were scoring the density of TG-anti-TG precipitate that appeared over the colonies after a few days and applying a rough (+/-/++) quantitation scheme familiar to immunologists. If these plates were left in the incubator for 10 to 15 days, however, an unanticipated further heterogeneity appeared. Some colonies had scattered, dense, aggregated clumps over them, others showed a very dense homogeneous halo around them while still others showed striking patterns of concentric rings around them. Several examples of cell strains exhibiting different precipitate morphology patterns have been isolated and are being studied using 2-D gel electrophoresis of immunoprecipitated media and isolated agarose plugs from the region surrounding the colonies. At present the most likely explanation of the "Saternian" colonies seems to be the action of restriction-like specific proteases degrading TG. Perhaps this process is not unlike the proteolysis of TG that occurs on resorbtion of the colloid and results in the liberation of T3 and T4. At minimum we are presented with an underlying richness of fabric that we hope will open up a new genetics of the secretion process.

c. Physiology of thyroid cells and other cell cultures under agarose:

Much attention has been paid in recent years to the substrate presented to the "basal" portion of the cell in cultures. Extravagant claims have been made for the efficacy of one or another extract used as a coating on cell culture dishes. The thrust of this interest is to provide a cell attachment as much like those in tissues as possible. Thus laminin, collagen, fibronectin are used as substrates for cultures of epithelial cells. At the same time all that has been done for the apical portion of the cell (part in contact with the medium) is to insist on defining the hormone status of the medium. But whatever is done, the apical end of all cell cultures today swim in a very untissue-like ocean of medium.

Usually cell cultures are fed twice weekly. Even at that, more dense cultures may deteriorate unless more frequent feeding is done. We were suprised to note that FRTL colonies remain synthetically active and maintain a lower but appreciable mitotic rate for 4 to 8 weeks without further feeding when they are under the agarose/medium overlays used in our screening procedures. Normally, in liquid medium these cells would require renewal of TSH at least weekly to remain alive. Under agarose they show no such requirement. This amazing difference between the two modes of cell culture must be followed up. Judging from the TG and its visualization (when anti-TG is in the agarose) we know that one difference between liquid and agarose culture would be the maintenance of high concentrations of macromolecular cell products near the apical surface resulting in a much more tissue-like environment for the cells.

We are looking at the effects of agarose overlays on thyroid and neuron/neuroblast cultures. We plan to try other strategies that might make more convenient refeeding of cultures but still maintain an enriched macromolecular

environment for cells. We will test the effects of Sephadex and other microbeads as well as still other overlaying substances.

d) FRTL and tissue reconstruction:

Ultimately we would like to use clonal populations of normal cells to help us reconstruct tissues and to learn how component cells interact in complex tissues. We would like to be able to check the normality of our cultured cell strains. Can they reintegrate into tissues in vivo and function normally? An obvious way to check the FRTL cell would seem to be to inject them into thyroidectomized animals and to see if in that situation FRTL's would supply T3 and T4 and whatever else was supplied from the missing thyroid gland. This is not as, simple an experiment as it sounds. We are aware of some of the complexities such as "rests" of thyroid cells and the need for parathyroid support. To date one experiment has been tried without success, i.e. no difference in T3, T4, or weight gain was seen in animals injected with FRTL cells and those injected with thyroid fibroblasts.

This experiment will be continued in two ways: 1) we shall repeat the obvious protocols using a larger number of cells. 2) We have initiated a collaboration with Dr. Eugene Bell at MIT in which we shall try to make a partially reconstructed tissue before transplanting it into thyroidectomized rats. Bell is combing thyroid fibroblasts with the epithelial gland cells (FRTL) in a collagen matrix. These organ cultures have shown promising restoration of lobular structures and will soon be tested for function. We may have to add capillary endothelial cells to the mix before rapid and effective integration in the host animal is achieved.

3) Electrophysiological properties of FRTL:

a. Slow pacemaker potential.

During the last two years Dr. Sinback found a slow pacemaker potential (P.P.) of 10-40mv, with 5-10 min period in single FRTL cells. This is an endogenous property of FRTL cells which occurs without TSH fluctuations and even in absence of TSH. Also it is interesting since it is the first evidence for slow P.P. in an animal cell. We do not know the physiological function of the P.P. Just the fluctuation in membrane potential would reduce the electrochemical gradient for I^- transport. Also blood levels of thyroid hormones fluctuate with periods of 10 min thus it is also possible that the P.P. is important for hormone secretion.

b. Electrophysiology of FRTL:

Dr. Sinback has continued to investigate the mechanism by which cells generate slow rhythmic pacemaker potential (10-40mv fluctuation with period 5-10 min). Input resistance does not change during the pacemaker cycle, therefore, it is not due to change in membrane ion permeability. Slow pacemaker can be induced by intracellular injection of H^+ or Na^+. These facts suggested that the pacemaker is due to an electrogenic ion pump regulated by intracellular Na^+ and/or H^+. Na^+/H^+ exchange pumps move H^+ out and Na^+ into cells and are

blocked by amiloride. Amiloride blocked pacemaker potentials initiated by H^+. This suggests that activity of Na^+/H^+ exchange is necessary for H^+ to initiate pacemaker potential. However the pacemaker potential is not due to cyclic activity of the Na^+/H^+ pump since amiloride does not abolish spontaneously active pacemaker potentials. Intracellular Na^+ seems to be necessary for initiation of the pacemaker because:

(1) Injection of Na^+ initiated pacemaker potentials.
(2) Amiloride did not block pacemaker potentials initiated by injection of Na^+.
(3) Pacemaker potentials were initiated by cessation of amiloride which allowed reestablishment of Na^+/H^+ exchange (i.e. Na^+ into cell for H^+ out)

This year Dr. Sinback will determine the ionic mechanism for maintainence of the pacemaker after it is initiated by intracellular Na^+. He will test the hypothesis that the rhythm is maintained by two mechanisms (1) a constant increased ion permeability causing "leak" of intracellular ions which initiates P.P. and (2) an increased electrogenic pumping (probably Na^+/H^+ pump). Dr. Sinback has shown that Na^+ injection causes prolonged (up to 20 min) decreased input resistance. He will determine which ion permeabilities are increased by intracellular Na^+. The ion pumps then investigated will be determined by the ion which is leaking. If successful this will be the first time such a slow pacemaker has been explained at this level. Also, if his hypothesis is correct this is the first explanation for how a cell regulates cyclic activity in one of its electrogenic pumps.

c. Response of FRTL to nonadrenaline:

It is believed that noradrenaline causes secretion of thyroid hormones by increasing cAMP. This belief is largely based on the fact that dibutryl cAMP stimulates thyroxine secretion.

We have shown that noradrenaline increases release of intracellular Ca^{++} which causes a depolarization by increasing Na^+ and Cl^- permeability. There is no reason to believe that cAMP is necessary to Ca^{++} release. Injection of cAMP does not release intracellular Ca^{++}. Injection of cAMP reduces the efficiency of Ca^{++} in opening Na^+ and Cl^- channels. However it is possible that extracellular dibutryl cAMP causes release of intracellular Ca^{++} by activating an extracellular adenosine receptor. Iontophoresis of ATP causes release of intracellular Ca^{++}. In the coming year he will investigate the adenosine receptor as an explanation for the effect of dibutryl cAMP.

He will investigate the mechanism by which Ca^{++} regulates membrane Na^+ and Cl^- permeability using an extracellular patch clamp amplifier he built to measure conductance and open time of single Na^+ and Cl^- channels. He will determine the effects of Ca^{++} on channel characteristics. If he can do this in FRTL, he can expand the use of this technique to see if transformed cells have Na^+ channels in which the Ca^{++} control mechanism is unusual. This will be investigated because both Ca^{++} and Na^+ have been shown to be involved in the control of differentiation.

B. Neurons and neuroblasts:

1. CBSVIA, a neuroblast cell line.

As noted in last year's annual report (and manuscript in preparation) we have attempted to study the cell type that has proven the most refractory to cell culture methodology, the uncommitted embryonic or "blast" cell by using a temperature sensitive SV40 mutant and transforming these cells. Cell lines have been produced by infecting suspensions of cells from the embryonic cerebellum. It was hoped that such cell lines could be propagated at 33°C and caused to return to a more normal state - perhaps to differentiate - at the non-permissive temperature of 40°C. We found that most commonly an intermediate result is attained. The neuroblasts continued to propagate (and to produce the "T" antigen) at 33°C but that as the cultures became crowded, cells that looked like differentiated neurons would appear. It developed that these neuron-like cells had concomitantly lost the "T" antigen (by FITC-anti-T fluorescent staining). These differentiated cells apparently fail to accumulate detectable quantities of "T" in their nuclei in spite of the fact that the culture is kept at the permissive temperature of 33°. They do not divide; they do not resynthesize T antigen. If the cultures are shifted up to 40°, all cells become T-negative in a few days but no new "neuron-like" cells appear. As long as they are incubated at 40° the neuron-like cells that have already differentiated at 33° may continue to improve (as measured by accumulation of neuronspecific enolase, 14-3-2, and/or decreased resting membrane potentials). But new neurons probably do not form.

It has proven possible to get nearly pure populations of cells in both the neuroblast-like state as well as in the differentiated state. Cultures growing at 33° if fed FUdR in thymidine free medium become progressively enriched (to over 95%) in the differentiated form of the cells. The dividing neuroblast-like cells die after incorporation of FUdR. If cultures of dividing neuroblast-like cells are maintained in log phase by rapid passage at low densities relatively fewer of the differentiated cells appear and if either the 0.5% calf serum and/or the Ca^{++} concentration is lowered to 0.1lm equiv. then no differentiated cells are formed. When serum and Ca^{++} are restored neurons reappear in time. Thus highly purified populations of both the blast cell and of the differentiated form are available for study. This situation is probably unique among cell systems showing differentiation in vitro. Certainly it is unique for nerve cells.

a. The developmental switch extends to the SV40 "T" antigen.

Perhaps the most mysterious aspect of CBSVIA's differentiation is that concomitantly with the acquisition of neuronal differentiation, the "T" antigen is "turned off". This in spite of the fact that the cells are continuously grown at 33° the permissive temperature for the ts mutant. By preparing >95% pure cultures of the differentiated cell (SF) we have found by hybridization that the entire SV40 genome is still present in integrated form in the neurons. It just is not functioning there. Our hypothesis now is that the integration sites(s) of SV40 DNA are in regions of the cellular DNA that are active (or at least not shut down) in the neuroblast but that are shut down during the differentiation process. The SV40 sequences thus provide a probe for one or

more such portion(s) of the genome. While not a completely unique situation (cf. fetal/adult hemoglobin switch) it would certainly be among the first mammalian developmentally regulated systems to become available. A simple experimental approach would be to ask whether the intergrated SV40 sequences are equally sensitive to S1 micrococcal nuclease in differentiated and neuroblast cell populations. Is the degree of methylation the same in the two cells? etc., etc.

b. Further characterization of the SF cells.

In collaboration with Dr. Suzanne Beckner (A, LNE) we have been looking at further characterization of the SF cells. Work has begun to look at tetanus toxin binding (a neuron-specific marker among brain cells) and for neuron specific phospholipid profiles. At this time both efforts look very promising but the results are not yet definative.

c. Efforts to speed up or force the neuroblast to neuron shift.

Also in collaboration with Dr. Beckner, we have begun to look at various methods of increasing the intracellular cAMP of flat cells in order to see if the factors that cause spontaneous neuron differentiation are mediated by cAMP. Apparently some of them are but not ones that commit the cells to neural differentiation because removal of the agents that cause cAMP increase results in a loss of acquired neuron-like morphology and the cells revert to dividing flat cells rather then progressing to SF cells. Among the differentiated functions we believe are initiated are increased tetanus toxin binding and the change in phospholipid profiles. These subjects will be investigated further in the coming year.

d. CBSVIA as antigens.

We have long used cultures of hybrid neuron cell strains and now viral transformed neural cell strains as antigens in an effort to generate neuron-specific and possibly region or cell specific antisera. Together with Dr. G. David Trisler (H, IR, BG) we are attempting to develop monoclonal antibodies specific to the two forms of CBSVIA cells: the flat, neuroblasts, and the rounded, neuron-like, SF cells.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 05553-13 LCBGY

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Immunoglobulin Structure and Diversity
Characterization of cell membrane proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Immunoglobulin structure and diversity: 1) Amino acid sequence analysis of a series of hybridomas with specificity for β(1,6) galactan is in progress to evaluate structure, function and idiotype. Nucleic acid probes are being developed to characterize the gene structures coding for these proteins. 2) Amino acid sequence analysis of antigen binding variants of a phosphorycholine binding myeloma has demonstrated that single amino acid substitutions in framework regions as well as complementarity determining regions may alter antigen binding specificity providing a potential model for somatic mutation in the generation of antibody diversity.

II. Cell membrane proteins: Papain fragments have been prepared and purified from the major transplantation antigens obtained from three inbred herds of miniature swine. Amino terminal sequences have been obtained for the heavy chains as well as the associated β2-microglobulin. DNA probes have been used to characterize the genes coding for these antigens by Southern blot analysis and to isolate genomic clones containing transplantation antigen sequences. Characterization of genomic clones is currently in progress.

I. Immunoglobulin structure and diversity

One of the major ongoing programs in this laboratory is a study of the structure of antibody molecules and the mechanisms by which the diversity in the large immunogloblin repertoire is generated. We have previously approached this problem by protein sequence analysis of myelomas and hybridomas with defined antigen binding specificity. More recently we have begun to employ recombinant DNA techonolgy to examine the structure and organization of genes involved in these particular responses.

a) In our previous report we described the complete amino acid sequences of the κ light chains from 6 myeloma proteins with specificity for β(1,6) galactan containing antigens. It was noted that among these proteins all four J (joining) region segments were used in conjunction with a single κ variable region and that no changes in specificity or affinity were observed in spite of these structural differences. Furthermore, position 96 at the junction of V and J was not encoded in either V or J but was generated by the recombination event and in 5/6 proteins was Ile. To assess the fidelity of this unusual V-J recombination and the diversity among anti-galactan antibodies we have begun amino acid sequence analysis of 11 hybridomas demonstrating the same specificity. Complete sequence analysis of these hybridomas will permit an evaluation of structural diversity, functional diversity, idiotypy and the mechanisms involved in the generation of these molecules. We have now completed light chain sequences from 10 of these monoclonal antibodies and found that the unusual V-J recombination is faithfully reproduced in all molecules. We have simultaneously prepared and characterized c-DNA clones encoding both the light and heavy chain variable regions and are now beginning to assess the genetic basis of this recombination event. The light chain sequences from these proteins show only minimal variation. Thus, functional and antigenic variation among these molecules must reside in the corresponding heavy chains. Heavy chain sequence determinations are now in progress.

b) It is now clear that there exist a large number of immunoglobulin germ line genes and that the respective light and heavy chains are encoded in multiple gene segments. It is not known, however, whether the structural diversity created by recombination of these gene segments as well as potentially subsequent somatic mutation are effective means of generating functional antibody diversity. To determine the effect of a small number of amino acid substitutions as a potential model for somatic mutation we are examining antigen binding variants of the phosphorylcholine (PC) binding myeloma protein S107. An S107 cell line has been adapted to tissue culture and variants with altered antigen binding have been selected. A number of studies have indicated that the alterations in antigen binding are associated with the heavy chain and we have therefore undertaken amino acid sequence analysis of these chains. Previous determination of the heavy chain structure from two variants revealed a single identical substitution in the J segment of these molecules while the complementarity determining regions were found to be identical. Thus it appears possible that sustitutions in the framework portion of the molecule may be able to effect antigen binding. We have now determined the heavy chain structure from an additional molecule which has completely lost the ability to bind antigen and observed a single amino acid substitution in the first complementarity determining region. Based on the known structure of the PC binding site derived

from X-ray analysis, the substitution we have observed provided a clear structural basis for alteration in the binding site configuration and subsequent loss of activity. Recently we have begun an analysis of a series of independent phenotypic revertants arising from one of the J region mutants. The first two of these molecules have been found to revert to the parental sequence at the original mutation site. In addition each has a second alteration at position 82a in the framework. The 82a substitution differs in the two molecules. These results suggest that the J region position represents a mutational 'hot-spot' and the apparent linkage of a second mutational event is a previously unobserved and novel phenomena. Additional revertants are currently being characterized.

We are further using the PC system to examine the evolution of specific germ line structures. A number of hybridomas have been generated in inbred strains expressing allelic forms of the PC heavy chain which we have previously identified. We have now determined complete heavy chain sequences from three CBA/J and four C57BL PC binding hybridomas. This analysis has confirmed the allelic nature of the PC heavy chain gene and defined the occurrence of point mutations during the evolution of this gene family. We have further demonstrated that a second V_H gene can be used in the anti-PC response. The gene expressed in the particular hybridoma appears to have undergone 'gene conversion' thus introducing an entirely new process which may contribute both to the generation of antibody diversity and the conservation of related multigene families such as the immunoglobulins. We are currently cloning the expressed gene as well as the CBA/J germ line genes to define the molecular basis of the postulated 'conversion.'

II. Characterization of cell membrane proteins

a) In the Annual Report, 1980 we described pertinent features of a large animal transplantation biology model in miniature swine and initial experiments aimed at the isolation of transplantation antigens for chemical and biological experiments. We have now prepared and purified papain fragments of the major transplantation antigens (SLA) from each of our three herds. The SLA heavy chains have been separated from non-covalently associated β_2-microglobulin and NH_2-terminal sequences determined. Appropriate comparisons have been made from other species. Chemical and enzymatic fragmentation studies are now in progress in order to isolate peptides and obtain primary sequence data from other portions of the molecule.

Concomitantly, we have begun an analysis of the genes encoding porcine MHC antigens. A porcine genomic clone expressing an MHC antigen has been isolated and characterized. Using DNA mediated gene transfer, this clone has been introduced into mouse L cells and expression demonstrated both serologically and biochemically. Nucleotide sequencing of the coding region is currently in progress. Characterization of other sequences in this genomic clone is also being undertaken in an attempt to analyze the presence and role of repetitive sequences adjacent to coding regions.

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Rudikoff, S., and Potter, M.: Immunoglobulin heavy chains from anti-inulin myeloma proteins: evidence for a new heavy chain joining segment. J. Immunol. 127: 191-194, 1981.

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October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Pathogenesis of plasma cell neoplasia: characterization of antigen-binding proteins

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SUMMARY OF WORK (200 words or less - underline keywords)

Identification of genes in BALB/c mice controlling susceptibility to induction of plasmacytomas by pristane is approached by construction of BALB/c congenic carrying marked loci, and chromosomal translocations from Resistant strains and determining plasmacytomagenesis susceptibility in these congenics. Characterization of the binding sites of monoclonal antibodies to protein antigens (galactan binding myeloma proteins; avian lysozymes) is being determined by structural analysis of monoclonal antibodies, and the specificity of the antibodies for closely related antigens. Unusual immunoglobulin genes in wild mice, are being characterized by classical and recombinant DNA methodologies.

Plasmacytomagenesis

Using the LBI-closed colony environment we have been able to carry out plasmacytomagenesis experiments that involve watching mice for one year after the first injection of oil to quantitatively determine plasmacytoma incidence. Success depends upon the freedom from intercurrent infections. We have refined the methods of induction to provide more rapid assays. The standard treatment of three 0.5 ml doses of pristane at days 0, 60 and 120 gives a 60% yield of plasmacytomas. A single 1 ml dose, gives a 35-44% yeild of plasmacytomas. Another series of experiments have been done with two 0.5 ml doses given at different intervals. A single 0.5 ml dose produces a low incidence of plasmacytomas with 20% or less. When a second dose is given at 30 to 120 days the incidence increases to 40 to 60%. We have now completed three experiments with a day 0-180, 2 dose schedule and found that the additive effect can be remembered for 180 days. This result suggests the target cell in plasmacytomagenesis is probably the B-lymphocyte since it would not be expected that a plasma cell would persist for this long in the organism. Thus, plasmacytomagenesis could begin in a cell that does not express the neoplastic trait, and only when the key differentiation step supervenes is the neoplastic state attained. Pristane probably has at least two biological effects on plasmacytomagenesis. First, it may stimulate B-lymphocyte proliferation, and second it creates a more permanent granulomatous microenvironment that is involved in the selection of abnormal cells for growth. Proliferation can activate endogenous type C RNA retroviruses as shown in collaborative experiments with Dr. Janet Hartley, NIAID. Retrovirus activation can lead to the formation of new recombinant virus forms, that are more efficient in re-integration or recombination with host genes. In collaboration with E. Premkumar Reddy and S. Bauer we are looking for oncogene activation as another consequence of retroviral proliferation.

Arthritis

In addition to inducing plasmacytomas pristane also induces the formation of a chronic arthritis in BALB/c mice. This affects the ankle joints, and feet but can involve other joints. Some mice develop ankylosis. In any given experiment from 10 to 30% of the mice develop arthritis. The interesting feature of this arthritis is the long latent period beginning 4 to 5 months and lasting throughout the time when plasmacytomas develop and lack of evidence of mycoplasma infection. A possible mechanism is an autoimmune process beginning with a destructive tissue change in the peritoneum. The evidence to date indicates the development of arthritis is dependent on the amount of pristane, i.e., 0.5 ml induces a very low incidence 1.0 to 1.5 ml of oil produces relatively high incidence.

We are attempting to find genes that control susceptibility to arthritis. C57BL/6 and DBA/2 and (BALB/c x DBA/2)F1 are resistant. We are nearing completion of the Bailey RI strains and BALB/c.DBA/2 congenic lines to find genes that determine susceptibility of arthritis.

Karyotypic analysis of plasmacytomas.

In collaboration with Dr. G. Klein of the Karolinska Institute, a karyotypic analysis of primary and early transfer generation plasmacytomas has been carried out using G-banding to identify specific chromosomes. Among 18 tumors analyzed several characteristic translocations have been identified: T6;15, T12;15, deleted 15. Sixteen of the 18 tumors have the T12;15 and del 15 abnormality and 3 have the rcpT6;15. All of the latter are kappa chain producers. In a series of 8 lambda tumors none has rcpT6;15. Since chr. 6 carries the K-chain genes and chr. 12 carries the heavy chain genes, there seems to be some association with Ig-gene complex loci. This is further strengthened by the observation that the translocations (like the allelic exclusion phenomenon) involves only 1 haplotype in a cell and in tetraploid cells only 2 of the 4 chromosomes are involved. The break in chr. 15, however, is consistent throughout and appears to occur in the same band site. Because trisomy of chr. 15 is also implicated in T-cell leukemias in the mouse, it is possible that chromosome 15 may contain a lymphocyte associated onc. gene.

Continuing our collaboration with G. Klein we are attempting to induce plasmacytomas in BALB/c hybrids that carry chr. 15 translocations from a plasmacytoma resistant strain e.g. T6(T14; 15+ del 15) on a CBA/H background; Rb6;15 which is on an AKR background; and Rk21 which is a paracentric inversion of chr. 15. We backcrossed T6 onto BALB/c and tested N1(to BALB/c) and obtained 6/24 plasmacytomas (an unexpected high yeild). 5 PCT's occurred in mice carrying 2 normal chr. 15, one in a heterozygote. The tumor had a non-random, associated T12;15 that involved the BALB/c chr. 15. The high yield of tumors has encouraged us to try (CBA/T6 x BALB/c)F1. We have tested hybrids carrying one AKRRb6;15 and one BALB/c chr. 15 and have again obtained tumors. We are systematically studying parental strains, and F1 hybrids.

We are testing conventional chr 15 markers from resistant strains Ca and bt (from C57BL/6), and Gdc-1 (from DBA/2) for susceptibility to plasmacytomagenesis. NAD-α-glycerolphosphate dehydrogenase from BALB/c and DBA/2 is being isolated in attempts to produce alloantisera to this enzyme to facilitate following Gdc-1.

We are further pursuing the karyotypic analysis of plasmacytomas with Abelson induced plasmacytomas (ABPCT) and lymphosarcomas (ABLS). S. Ohno of Kanazawa University, Japan is continuing this study. ABLS are balanced diploids. Two of five (ABPCTs) are either diploid or tetraploid and do not contain translocations. Others have rcp T6;15. Very few T12;15 + del have been found suggesting Abelson virus can transform plasma cells without producing a translocation. This may permit a greater chance for rcp T6;15 to occur.

Identification of genes confering susceptibility to mineral oil plasmacytomagenesis in BALB/c mice. Most inbred strains of mice are not susceptible to peritoneal plasmacytomagenesis. The remarkable susceptibility of strains BALB/c and NZB suggests susceptibility is determined genetically. We are attempting to identify by linkage tests loci involved in susceptibility of the BALB/c An strain. The approach is at first indirect, and is concerned with identifying resistance (R) genes in other strains. Two R strains are being studied in detail DBA/2 and C57BL/6.

F1 hybrids of BALB/c and the resistant DBA/2 strains are resistant to plasmacytomagenesis suggesting resistance genes are dominant. We are attempting to identify resistance (R) genes in DBA/2 by backcrossing a number of DBA/2 loci onto the BALB/c genotype, to produce BALB/c congenics carrying R genes (C·D2).

The following C·D2 (congenics listed by genotype) have been tested and found sucsceptible. CCAA; CCaa; Idh-1^b-Pep-3^b; Pgm-1^b; Es-3^c; and Igh^c. In first test Fv-1^{nn} was partially resistant, and is now being repeated at N6, and N10. The following C·D2 congenics are under study: $Rmcf^R$-cv-1^-; Lyt2·1; CCaadd; and the following are under development Hba^a-Es-3^c; $Qa2^+$; X; and Tol-1;. We have acquired DAG. We are working out methods to construct Gdc-1(chr 15); Lym-21; Pgm-2^m; Mod-1^m(obtainable from S. Lewis). In addition we hope to be able to use DNA hybridization to detect new markers. We hope also to develop markers for revelant onc genes this year, using flanking regions as markers.

To include larger regions of DBA/2 chromosomes, we have at the suggestion of Dr. Eva Eicher of the Jackson Lab, begun making BALB/c congenic carrying x-ray or mutagen induced paracentric inversions. Dr. Thomas Roderick has developed these stocks. The large inversions prevent or reduce crossing over, thus allowing the introduction of chromosome segments that could be as large as 60 or 70%. Many of these inversion stocks were induced in DBA/2 thus, we are able to extend our list to include many new linkage groups including chromosome 15.

A study is being completed on C3H/HeJ and C3H/HeN, both of which are resistant to developing plasmacytomas. This will provide a new genomic source of resistance genes. The LPS-sensitivity gene of C3H/HeN origin has been backcrossed onto BALB/c to N6 but this gene is not associated with resistance. The CBA/N x linked gene that controls the development of a subpopulation of B-lymphocytes was backcrossed onto BALB/c An by Dr. Carl Hanson has been tested and this stock is resistant to plasmacytomagenesis.

A second approach to finding genes that are associated with susceptibility and resistance is to compare the susceptibility of BALB/c sublines. We have obtained BALB/c Boy, BALB/c Jax, BALB/c ORNL, BALB/c Arg, and BALB/c wt. The JAX, ORNL, and wt lines were separated from BALB/c An over 40 years ago. Of these mice BALB/c Jax develops only 10-20% placsmacytomas i.e. is partially resistant. The others except BALB/c Wt (now being tested) are highly susceptible.

The partial resistance of BALB/c J provides a model system for studying the genetics of plasmacytoma susceptibility. A survey of genetic differences including over 50 genes (Rodericks' Alpha gene survey) only 1 difference (Qa-2 and Qa-3). It is also known that BALB/c An and BALB/c J have differences in genes controlling alphafetoprotein biosynthesis. We are attempting to study the role of these 2 genes on susceptibility.

A working hypothesis is that BALB/c is susceptible to developing plasmacytomas because (differentiation specific) genes controlling plasma cell proliferation are regulated in a special way. Dysregulation could be caused by genetic events that disturb these putative genes, such as retroviral gene insertion, or consequences of retroviral gene recombination with host genes.

Oil granuloma-growth promotion. The peritoneal oil granuloma provides an essential microenvironment for the growth of primary (early) plasmacytomas. This phenomenon resembles the growth dependency of certain endocrine tumors, e.g., estrogen dependent interstitial cell tumors of the testis. Attempts are being made to identify the factors involved in growth dependency.

In studies with Mr. R. Nordan primary plasmacytoma cells (PCT-C) have been propagated in millipore diffusion chambers (MDC). It has been possible to make preparations of primary plasmacytomas that contain a very few macrophages, and these have grown in MDC in normal and pristane conditioned mice. There has been a consistent increase in the proliferation of granuloma dependent primary PCT in MDC implanted in pristane conditioned mice as compared with normals strongly suggesting that diffusable substances influence primary plasmacytoma growth. Alternatively it is possible that normal peritoneal exudate cells produce inhibitors for primary plasmacytoma growth and that the injection of pristane in some way inhibits these cells. This possibility has not been ruled out. Further studies will attempt to better characterize these effects with the hope of determining the biochemical nature of the growth stimulating substance. Mr. Nordan has further shown that when primary plasmacytomas and certain long term transplantable plasmacytomas are put in millipore diffusion chambers and implanted into pristane conditioned mice treated with indomethacin growth is severely inhibited.

Immunochemistry and genetics of immunoglobulins

1. Vk-isotypes. The Vk structures produced in the BALB/c mouse have a diversity of structures in the sequence to the first invariant tryptophan (Trp35). Amino acid sequence analysis of this segment can potentially provide data on the number of Vk-genes in the BALB/c genome. Seventeen new Vk-partial sequences have been completed by S. Rudikoff. We have sent these sequences to Drs. E. Haber and John Newell, Massachusetts General Hospital, who have analysed them for relatedness and constructed a dendrogram based on the number of differences.

2. Structure of galactan binding myeloma proteins (GALBMP). Complete V-region structures of four galactan binding myeloma proteins have been determined by S. Rudikoff. Some progress has been made by Dr. D.R. Davies and his group in solving the structure of one of these J539. Hypothetical model building offers new approaches to determining 3 dimensional structures. Models are made by modifying segments of V-regions utilizing when possible known secondary structures. A new approach is being attempted in studies with Richard Feldmann using a computerized program, in which 3 dimensional space filling models can be graphically depicted from α-carbon coordinates To derive a new Ig-V structure from a determined structure requires an analysis of the amino acid side chain contacts, in the substituted molecule Richard Feldmann has worked out a computer program for this process and has analyzed J539, and found a minimal number of contacts with all substitutions in place. The first J539 model has been published. We have refined the model by correcting all of the angles at rejoined sites, and also corrected the backbone for all substitutions involving proline.
These methods were worked out by C. Mainhart and R. Feldmann. In collaboration

with C.P.J. Glaudemans, we are attempting to define the contact amino acids in the galactin binding site.

Models of the other GALBMP X24, X44, and T601, have been constructed and using a monoclonal antiidiotypic antibody to X24 (HyX24-14) prepared by Dr. M. Pawlita and Ms. E.B. Mushinski, we are currently mapping the idiotope. This monoclonal antibody has unusual properties. It not only recognizes an epitopic site that is unique to X24 but in addition a second cross-reactive site that is distributed on several other GALBMP. These two sites are about 15 Å apart and provide the basis for defining the binding site on HyX24-14. We plan this year to begin sequencing HyX24-14 with Dr. S. Rudikoff and to construct a model of it.

In collaborative studies with Dr. S. Smith-Gill who monoclonal antibodies have been prepared to hen egg white lysozyme (HEL). Using a battery of sequenced avian lysozymes from various species, it has been possible to localize the region on HEL contacted by the monoclonal antibody HyHEL-5, using 3-dimensional space filled models. We plan to extend this work by sequencing the V-regions of HyHEL-5 in collaboration with Dr. S. Rudikoff.

These two systems, the anti X24 idiotype and the anti-lysozyme system should provide models of antibody-protein interactions.

Ms. C. Scott has prepared a probe for mouse IgC λ and is studying differences in λ-genes in wild mice. The Cλ genes in many stocks of wild mice have multiple Cλ genes(e.g. 8 to 12) in contrast to 4 in inbred strains. Using these Cλ markers, and Akv-2 probes provided by W. Rowe we are attempting to map the lambda locus in the mouse.

In collaborative studies with R. Lieberman we have found mice trapped from Centreville, Maryland, have defective immune response to phosphorylcholine. This rare phenotype is of unusual interest, and we (R. Leiberman, L. D'Hoostelaere) are attempting to identify the genes controlling nonresponsiveness. Preliminary studies indicate there are 3 different genes involved.

Mammary tumorigenesis in wild mice

In collaborative studies with Dr. R. Callahan, NCI, wild mice in our colony were studied for mammary tumor virus genes. We have found mice from many different wild stocks are free of MTV, and have established breeding stocks of these mice. Further it has been found that a number of wild mice from Maryland have only a single MTV provirus. These proviruses are highly polymorphic and we are trying to determine if this polymorphism is due to reinsertion of MTV, or polymorphism of a a single common locus or both. Dr. Callahan has shown that M. musculus domesticus (Centreville Lights) from the Eastern Shore of Maryland, carry only a partial copy of an MTV genome that includes the LTR regions. These mice can be used to study the promoter-insertion theory in mammary tumorigenesis. Accordingly, the Centreville Lights are being expanded and mammary tumor incidence will be determined in virgin and breeding females.

Through various crosses with inbred mice the chromosomal localization of of the LTR-fragment in Centreville Lights will be sought.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08726-05 LCBGY

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Biological and Biochemical Characterization of Transplantation Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. J. Rogers	Research Chemist	LCBGY	NCI
OTHER:	L. W. Law	Chief	LCBGY	NCI
	D. Siwarski	Bio. Lab Technician	LCBGY	NCI
	L. Gooding	Professor	Emory University	
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COOPERATING UNITS (if any) Litton Bionetics, Dept. Molecular Toxicology, Immunotoxicology Branch; Emory University, Dept. of Immunology

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to examine the chemical and biological properties of murine histocompatibility antigens (H-2) and tumor associated transplantation antigens (TATA) assayed by their ability to elicit tumor rejection in vivo. The approach is to purify the molecules expressing these antigens and determine their: 1) ability to induce in vivo immune responses such as tissue rejection and humoral response, 2) ability to stimulate in vitro immunity such as cytotoxic lymphocytes or lymphocyte proliferation, 3) ability to function as a restriction element, 4) chemical properties with particular emphasis on how the molecules interact with the plasma membrane. Once these antigens are purified, monoclonal antibodies will be prepared to assist in the characterization of the molecules and their expression on tumor cells. Moreover, the availability of purified molecules and high titered antibodies will permit the preparation of suitable probes to study the organization and expression of the genes encoding these molecules. In the case of TATA it is hoped that this knowledge Results will contribute to a rational basis for in vivo therapeutic protocols.

Purification and Properties of H-2^b

Purified H-2^b incorporated into liposomes has been used to stimulate secondary cytotoxic responses in vitro. The CTL stimulated by H-2 containing liposomes are identical in every way to CTL stimulated by allogeneic cells. The density of H-2^b on the liposome seems to be important since higher protein to lipid ratios in the liposome result in more efficient stimulation. There is also a dependence on the lipid composition of the liposomes. Didodecanoyl lecithin is markedly superior to egg lecithin, ditetraecanoyl lecithin or distearoyl lecithin. In agreement with results from other laboratories, inclusion of membrane matrix proteins in liposomes improves their ability to stimulate CTL.

During the course of these studies we discovered that, due to our purification procedures, H-2^b slowly dissociates from B_2 microglobulin and is no longer an effective immunogen. We are currently altering our purification procedures to prevent this problem.

The ability of H-2^b to function as a restriction element in liposomes is also under investigation. Mice are immunized with purified cytochrome b_5 and spleen or lymphnode cells are restimulated in vitro with liposomes containing cytochrome b_5 and H-2^b. CTL are then tested against tumor cells that have cytochrome b_5 bound to the plasma membrane. So far only low levels of cytotoxicity have been observed, and the system is being systematically altered to improve the response. Once CTL have been obtained, the system may be used to study the role of H-2^b as a restriction element. Moreover, the sequence and 3 dimensional structure of cytochrome b_5 are known and there are small sequence differences among species. Therefore, it is possible that cytochrome b_5 may be used to map the CTL receptor in much the same way that cytochrome c and albumin have been used to map the T-helper cell receptor and antibody binding site respectively.

In other work associated with the H-2 project, Dr. Linda Gooding, while selecting for SV40 transformed cells of C3H origin that would grow in vivo, isolated several H-$2K^k$ loss mutants. Using a DNA probe specific for the N-terminal portion of the H-$2L^d$ gene we have established that a genetic alteration has occurred in the H-2 genes of this mutant. Although this mutation does not appear to be due to insertion of SV40 sequences into the H-2 region, it has allowed preliminary identification of the H-$2K^k$ band in a southern blot hybridization of endonuclease digested C3H DNA. Experiments are in progress to confirm this result with the idea of eventually cloning the H-$2K^k$ gene. Dr. Gooding is also isolating other mutants to study this interesting phenomenon in more detail.

Purification and properties of TATA

Recent observations revealing the complexity of the H-2 genes and artifacts associated with tumors arising in non-pedigreed mice have led us to abandon, for the present, most of our studies on alien H-2 antigens expressed by tumor cells. Studies on the adjuvant effects of liposomes have also been discontinued temporarily.

All of our efforts have now been directed toward the purification of the TATA from RBL-5, a leukemia of B/6 origin. Earlier studies indicated that, although crossreacting with all FMR tumors, RBL-5 expressed a TATA which was unrelated to known viral structural antigens. We have now purified 75 Kd and 65

Kd glycopeptides which appear to be related and are highly immunogenic in vitro (<1μg provides complete protection). These peptides do not react with goat anti R-MuLV sera suggesting, but not proving, a non-viral origin. Antisera prepared against these proteins precipitate 135 Kd and 95 Kd peptides from RBL-5 cells suggesting that the purified proteins may be fragments.

Future studies will involve: 1) determining the relationships between the purified peptides and those precipitated from labeled cells, 2) sequencing the purified peptides to determine relationships, if any, to virally encoded proteins and to another 75Kd peptide isolated form Meth A, 3) constructing artificial DNA probes based on the sequence and using them to study the organization of the genes encoding these proteins.

Dose responses for tumor rejection using the purified peptides show a clear maximum at about 1μg with very little immunity at higher or lower doses. This is particularly interesting since irradiated tumor cells and very impure subcellular fractions do not show lowered immunity at high doses. Studies are underway, both in vivo and in vitro to establish the basis for this abrogation of immunity at high doses.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08727-05 LCBGY

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Organization and Control of material in immunoglobulin-secreting plasmacytomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	M. Potter	Chief, Immunochemistry Section	LCBGY NCI
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	P.W. Tucker	Associate Professor	Univ. of Texas
	F.R. Blattner	Associate Professor	Univ. of Wisconsin
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LAB/BRANCH

Laboratory of Cell Biology

SECTION

Immunochemistry Section

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NCI,NIH, Bethesda, MD 20205

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SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range purpose of this project to study the control mechanisms important in regulating protein synthesis in normal and malignant lymphoid cells. The orgainization of immunoglobulin light and heavy chain genes and the control mechanisms that selecting those being transcribed and those which are expressed in protein synthesis are being studied. Particular attention is being given to the molecular genetics of IgD, since this molecule, along with IgM appears to be a very important surface component of most B lymphocytes. We have prepared a cDNA clone for mouse IgD heavy chain and are attempting to do the same for human IgD. We have discovered that mouse IgD has multiple gene segments coding for alternative carboxyl termini for IgD that either is secreted or membrane bound. At least 5 mRNAs for different form of IdG heavy chain have been identified and their structures and functions are being studied. We have prepared cDNA clones for V_L and V_H of galactoside-binding antibody and are characterizing its representation in the genomes of laboratory and wild mice using these cDNA clones as probes.

Methods Employed:

These studies were done using the following tissues: BALB/c liver and spleens; BALB/c plasmacytomas and lymphomas; human chronic lymphocytic leukemia cells and cultured lymphoma and plasmacytoma cells. RNA was isolated using guanidinium thiocyanate or LiCl-urea, and DNA was isolated using SDS and proteinase K. Double stranded cDNA was prepared from RNA fractions enriched for particular messengers using reverse transcriptase, DNA polymerase I, and S1 nuclease. This DNA was inserted into the Pst I site of pBR322 by oligo dC-oligo dG tailing and annealing, and the recombinant DNAs were used to transform calcium shocked E. coli. Colony hybridization with ^{32}P labeled mRNA or nick translated DNA fragments identified likely candidates for desired clones. Genomic clones were identified in Charon 4A and Charon 28 libraries of genomic DNA fragments using cDNA plasmid clones. DNAs were mapped using restriction endonuclease digestion, and DNA sequence determination was performed using the Maxam-Gilbert techniques. mRNAs from cytoplasms and nuclei were studied by electrophoresis on methyl mercury hydroxide agarose gels, transfer to diazotized paper and hybridization to ^{32}P labeled probes. R loop and heteroduplex formations are studied using electron microscopy.

Major Findings:

A. Like other immunoglobulins, IgD exists in membrane and secreted forms. We have found that normal mouse spleen contains a major 2.9 kb and a minor 2.1 kb RNA encoding membrane δ chains (δ_m) and that TEPC 1017 and TEPC 1033 contain similar δ_m RNAs plus 1.75 kb RNA encoding secreted δ chains (δ_s). We also observed less abundant δ RNA species (2.65 and 3.2 kb). Different gene segments or combinations of segments are used in the 3' termini of these multiple δ RNAs. We have located and sequenced the secreted and membrane terminal exons of the murine immunoglobulin δ chain gene. The putative transmembranal exon is strikingly similar to that of the μ_m chain and the internal cytoplasmic exon codes for the same two amino acids, valine and lysine, that are found in μ_m. Two exons were found which could code for hydrophilic carboxyl termini of secreted δ. Different combinations of these exons probably result in the 5 different δ mRNAs we have reported.

We have also found 3 forms of μ chain mRNA in the XRPC 24 plasmacytoma. The smallest translates into the secreted form of μ chain with MW 53,000. The two larger mRNAs encode at least one large μ chain with MW 59,000. We have located by R looping and sequenced the DNA encoding the membrane terminus of the μ chain. It encodes a hydrophobic region suitable for anchoring the μ chains in the cell membrane.

B. We have prepared cDNA clones encoding the variable regions of the kappa light chain and alpha heavy chain of XRPC 24, a mouse plasmacytoma that secretes IgA that specificially binds Bl →6 galactosides. We have sequenced these clones and proved that they match the protein sequences already known for these chains. These clones have been used for hybridizing Southern blots of genomic DNA from tumors and normal tissues of laboratory and wild mice. The hybridizations suggest that there is only one gene in most wild mice for this V_H but many genes for V_L. Both V_H and V_L genes are found in all inbred strains and in nearly all wild mouse strains.

Significance and Proposed Course:

IgM and IgD constant region genes are located closer to one another than are any other two Ig heavy chain genes. It appears likely that the entire μ +δ region may normally be transcribed into one very large RNA which is a precursor from which secreted μ, membrane μ, secreted δ or membrane δ mRNAs an be produced by different RNA splices. This process will be studied in normal spleen cells, in plasmacytomas, and in μ and δ containing B-cell tumors. The RNA processing pathways in the IgD secreting plasmacytomas will be studied further to elucidate how the different δ_mmRNAs are created. We will also determine whether there are more than one from of δ_m polypeptide encoded by the multiple δ_m mRNAs by hybridization selection of spleen δ_m mRNAs and isolation of cDNA clones of spleen δ_m mRNAs.

BALB/c δ chains seem to lack the second constant region domain that appears to be present in human δ chains. We plan to study the human δ gene by making cDNA clones from human plasmacytoma in tissue culture and from leukemia cells which bear surface IgD. It will be important to see whether this Cδ2 is present in human DNA and other mouse genomic DNAs. In addition the IgD leukemia cells we have collected are form a family in which 4 out of 5 siblings contracted chronic lymphocytic leukemia, generally expressing surface IgD. We hope to see if the genomic DNA of these patients shows any characteristic defects or rearrangements in the DNA near the δ chain gene.

We intend to study V_h gene and multiple V_L genes for galactoside binding antibodies have changed as inbred mouse strains have been created. We wish to contrast this with the way these genes appear in different populations of wild mice collected from all corners of the globe. It is of interest to see to what extent the sequence of these interrelated genes has been preserved in natural populations of mice which have been genetically and geographically isolated for long periods of time. This will be done by cloning the genomic representation of the V_H, V_L and C_α from representative wild and inbred mice and studying the DNA sequence of the coding regions and the flanking regions.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 32555-07 LCBGY

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immune Response to Tumor Associated Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.C. Ting	Medical Officer	LCBGY NCI
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COOPERATING UNITS (if any)

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Laboratory of Cell Biology

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SUMMARY OF WORK (200 words or less - underline keywords)

1. Development of specific tumor immunity in tumor bearing hosts: Using C57BL/6 mice bearing syngeneic, progressively growing FBL-3 leukemia, a population of highly reactive cytotoxic T cells could be isolated from the tumor site. These T cells gave specific cytotoxicity against the target tumor FBL-3. In studying their in vivo effect, it was found that T cells isolated from tumor site only gave transient protection against tumor challenge, whereas T cells obtained from immunized donors gave long lasting protection. These findings indicate that the functions of sensitized T cells in tumor bearing hosts are impaired by the presence of tumor growth.

2. Mechanisms for the tumor cells induced suppression. Due to the fact that tumor cells may impair the function of immune cells, we have further studied the mechanisms for the tumor cell induced suppression. It was found that in the syngeneic system, tumor cells could trigger a macrophage-mediated suppressor mechanism. These macrophages acted by the increased production of prostaglandin which in turn switch on the generation of suppressor T cells, thus completed the suppressor circuit. These results strongly suggest that prostaglandin may play

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an essential role in immune regulation.

Project Description:

Objectives: 1) Development of specific tumor immunity in tumor bearing hosts; 2) Mechanisms for tumor cell induced suppression; 3) Prostaglandin regulation of immune response.

Methods Employed: Tumor cell transformed by oncogenic viruses, chemicals or unknown agents are kept in tissue cultures or as transplanted tumors in mice. The ^{125}IUdR 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 velocity 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.

Major Findings:

1) Development of specific tumor immunity bearing hosts: Using a Friend virus-induced leukemia in syngeneic C57BL/6 mice, we could isolate a population of highly cytotoxic T cells amd macrophages from the tumor site (ascites growth). This was achieved by separation of various cell populations by lg velocity sedimentation. The T cells gave specific cytotoxicity against tumor cells of FMR (Friend, Moloney, Rauscher virus-induced) origin, whereas the macrophages gave nonspecific cytotoxicity. The levels of cytotoxicity of these T cells were usually higher that the cytotoxic T cells obtained from immunized hosts. When testing in vivo, the lymphocytes isolated from tumor site only gave transient protection against tumor challenge, whereas the lymphocytes obtained from immune donors could confer long lasting immunity. It was later found that in tumor bearing hosts, there was a population of adherent suppressor cells which interfered with the function of immune T cells. After removing the suppressor cells, the T cells isolated from tumor sites were fully capable of conferring long lasting anti-tumor immunity.

2) Mechanisms for tumor cell induced immunosuppression: Specific cytotoxic T cells against tumor associated antigens were generated in mixed lymphocyte tumor cell cultures. By employing this technique, we have demonstrated an unique suppressor mechanism which was triggered by the tumor cells and was mediated through two populations of macrophages (splenic and peritoneal). Preexposure of splenic macrophages to tumor cells initiated the suppressor event, and this was followed by the later involvement of peritoneal macrophages to complete the suppressor circuit. Therefore, three major cellular components were involved in this suppressor mechanism: tumor cells, splenic macrophages, and peritoneal macrophages.

3) Prostaglandin regulation of immune response: Prostaglandin was found to play a central role in the tumor cell induced suppression. The reasons are twofold. First, addition of prostaglandin E (PEG) completely suppressed the generation of cytotoxic T cells in the syngeneic, mixed lymphocyte tumor cell cultures. Second, indomethacin, a prostaglandin synthetase inhibitor, blocked the tumor cell-induced suppression. Further studies revealed that the effect of PGE was probably mediated by the generation of suppressor T cells.

From these studies, it is clear that tumor cell-induced suppression is mediated by a chain of events. The tumor cells first triggered the macrophages to produce prostaglandins which in turn switched on the production of suppressor T cells. It was these suppressor T cells which shut off the generation of cytotoxic T cells.

Significance of biomedical research and the program of the National Cancer Institute.

From our studies, it is clearly shown that specific tumor immunity is developed in hosts with progressively growing tumors. However, the presence of various immunosuppressive factors may interfere with the function of the immune cells. Therefore, in order to counter the tumor cell induced suppression, one has to understand the nature of these immunosuppressive factors and the mechanisms of their actions. We have identified macrophages and prostaglandins as two major elements for inducing suppression. Further studies on their mode of action on immune surveillance are needed

Proposed Course:

1) Further studies on the mechanisms for tumor induced immunosuppression.
2) Prostaglandin regulation of T-cell response.
3) Effect of Interleukin 2 on T-cell response.
4) T-cell clonal selection and expansion.

Publications:

Ting, C.C., Rodrigues, D., and Nordan, R.: Studies of the mechanisms for the induction of in vivo tumor immunity. VI. Induction of specific and non-specific cell-mediated immunity in tumor bearing hosts and its correlation with transplantation tumor immunity. Cell. Immunol. 66: 45-48, 1982.

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