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STUDIES IN DEVELOPMENTAL IMMUNOGENETICS

Annual Progress Report

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ABSTRACT

This contract provides the sole research support, external to the Institution, for a research group concerned with a relatively large range of problems. The integrating thread that runs through it is that of an interest in development and its genetic regulation, mainly in complex organisms and with an emphasis on the immune system as a model for developmental analysis and as a tool for following the development of other systems, especially the brain. It includes studies of biochemical genetics, primarily from a developmental viewpoint and with particular regard to defense mechanisms, and cellular aspects of the development of the immune system. In several contexts it extends into the area of cancer immunology, especially with regard to the identification of tumors of thymic origin following radiation or carcinogen treatment, and the specificities of certain brain tumors grown in culture.

Introduction

Immunology is currently perhaps the most dynamic discipline in biomedical science. Impetus for the great activity and enthusiasm in this field comes from both the "basic science" and the "human relevance" directions. In a basic science context, the developments in molecular biology in recent years have come to particularly fruitful expression with regard to the genetic control of the elaboration of antibodies and the immune response. As molecular biology moves toward more complex problems--the events of cellular differentiation in development, cell interactions and the emergence of systems--the immune system offers some of the most precise tools and materials available for the elucidation of these complicated and challenging problems. From the viewpoint of human relevance, the involvement of the immune system in recovery from and resistance to disease, in the potential immunotherapy of cancer, in graft rejection, and in autoimmune states exemplify the deep and wide significance of this subject. And the utility of immunological tools in advancing the understanding of specificities and cell interactions, for example in the nervous system, further extends the potential of our discipline.

Our research group, relatively small as it is, samples rather widely from this moving field, with emphases on its interactions with genetics on one side, with biochemistry on another, with neurobiology on another, and with the central core of an interest in developmental processes.

Model Systems for Defining the Nature of the Antigen Reactive Site on Thymus-Dependent Lymphocytes

One of the central problems in immunology today deals with the nature of the antigen reactive site on thymus immunocompetent cells. Although little is known about the chemistry of these sites, biologically it has been observed that antigen interacting with these cells initiates a series of events, the end result being cellular-mediated type of immunity. The fact that this arm of the immune mechanism is so important in cancer and transplantation immunity, as well as in autoimmune disease and immunity against intracellular microbial infections, places additional emphasis on deciphering the series of events that lead to activation of thymic-dependent immune cells after binding with antigen. Therefore we have attempted to set up a model system for studying these cells. The first requirement is a large number of homogeneous cells with reactive sites for a specific antigen. The major obstacle to this requirement is that there is an almost infinite number of antigens that one has to deal with during a lifetime and the probability of isolating enough cells with activity against a particular antigen is rather small. In surveying the situation we have looked for a system that mimics the condition that occurs in multiple myeloma, a malignancy of immunoglobulin-producing cells. With this disease, which occurs in mice and man, homogeneous immunoglobulins are produced in great quantities and there have been found several antigens which bind specifically to these proteins. Therefore we selected various malignant states in mice where thymic leukemias are known to occur. These thymic-dependent leukemias can be initiated in various strains of mice by viruses, X-ray and chemical carcinogens. The thymic lymphomas are being assayed for populations of cells that will bind with

various antigens known to combine with several multiple myeloma proteins. These antigens are various haptens such as 2,4-dinitrophenol, histocompatibility antigens, lipoproteins and bacterial polysaccharides. In addition these tumor cells are being assayed for various thymic cell alloantigens to assure that we are dealing with a thymic-derived population of cells. Hopefully this system will yield a thymic tumor with cells that readily bind with one of the assay antigens. If our analogy with multiple myeloma is correct, then we should be able to demonstrate such binding, because the thymic cell has also been shown to cooperate in and be necessary for the induction of various humoral antibodies. Once binding has been demonstrated, these tumor cells can be grown in great quantities by passage in mice and should provide a source for chemically deducing the nature of the antigen reactive site as well as the way in which combination with antigen leads to cellular immunity.

A Search for Antigenic Markers on the Cell Surface Membrane of Human Thymocytes

Knowledge concerning the role of various lymphocyte populations in the immune response has been enhanced by the demonstration of antigenic cell surface markers on different cell types. Most notable is the theta (θ) alloantigen which appears on mouse thymic-dependent lymphocytes. Alloantigens such as θ have been utilized as markers in sorting out the series of events that takes place during differentiation of immunocompetent cells as well as defining the cell types involved in various malignant lymphomas. Unfortunately these observations have in most part been limited to mice, because suitable antisera against differentiation antigens have not been developed for other animals or for man. Therefore we have attempted to define a specific serological marker for human thymocytes. Although studies of this nature using random populations of individuals are often hampered by genetic polymorphism, there is evidence to suggest that histocompatibility antigens in mice and man may be chemically homologous. Thus our working supposition is that differentiation alloantigens analogous to θ in mice may also be found in man and be limited to a small number of allelic alternatives.

In our search for human thymic-specific antisera we have taken two approaches. One of these involves the immunization of rabbits with human fetal thymic cells for a source of antisera. Here we have been able to raise an antiserum against the thymic cells of a therapeutically aborted fetus obtained from the USC Medical Center, through the collaboration of Drs. Murray Gardner, John Parker and Nancy Warner. This antiserum has been shown to react with > 95% of the thymocytes obtained from each of several other fetuses representing different periods of gestation. Absorption with human myelogenous leukemic cells did not affect the titer of this antiserum, and the possibility is good that we may have a thymus-specific heteroantiserum. However, whether the antiserum is reacting against an antigen analogous to θ remains to be demonstrated.

Our second approach has been to use sera collected by Drs. J. Claude Bennett (University of Alabama in Birmingham, The Medical Center), Dr. Ray Teplitz

(City of Hope National Hospital), Dr. Christian Herrman (UCLA School of Medicine) and Dr. Joshua Levy (UCLA School of Medicine), from patients with various autoimmune diseases in which thymic lesions have often been implicated. The sera of patients with such pathological states as myasthenia gravis and lupus erythematosus have been shown to contain antibodies against various thymic cell constituents. Thus there is a good possibility that antibody against thymic alloantigens can be found in some of these patients. Our preliminary observations have suggested that about 5% of a battery of serum samples from patients with myasthenia gravis are reactive with about 15% of the cells from fetal thymic tissue. This approach therefore appears to be potentially fruitful.

In the event that an antiserum for human thymic cell alloantigens can be found, then a marker would be available to aid in following the differentiation events of human lymphocytes. Also this serological marker could be used diagnostically in determining the lymphocyte type of malignant lymphomas.

Amino Acid Sequence Analysis of Avian Immunoglobulin Heavy and Light Chains

In a 1971 report it has been demonstrated that the chicken and the turkey (order Galliformes) have one predominant light chain type which represents more than 95% of the light chain population. Sequence analysis at the N-terminus of these chains revealed a remarkable degree of homogeneity as compared with mammalian counterparts. In addition, amino terminal sequences of turkey and chicken light chains were shown to be closely homologous to mammalian λ chains.

These studies have been extended to ascertain the predominant light chain type for other avian species and the degree of sequence homology with mammalian κ or λ chains. In collaboration with Dr. Gerrie Leslie, Tulane University School of Medicine, New Orleans, Louisiana, we have isolated the heavy and light polypeptide chains from the 7S immunoglobulins of pheasant, pigeon, duck and goose. Quantitative studies with an automatic sequencer have been undertaken and the amino terminal 24 residues of pheasant light chains as well as the first 10 residues of duck light chains have been completed. The pheasant (order Galliformes) light chain sequence is virtually identical to the sequence reported for the chicken and is remarkably homogeneous. Although the amino terminal 10 residues of the duck were relatively similar to those of the chicken and pheasant, there was considerable heterogeneity which limited the analysis. Both pheasant and duck light chain sequence data suggest that the predominant light chain type is λ . It will be of interest to determine if goose light chain also has N-terminal sequence heterogeneity comparable with the duck, because both of these species belong to the order Anseriformes. Also, sequence analysis of pigeon (order Columbiformes) light chain should help to further establish whether or not λ chains are the predominant light chain type expressed in Aves.

Studies on the Biological and Physicochemical Properties of Pigeon Immunoglobulins

Investigations by others have revealed that the chicken synthesizes a 7S immunoglobulin that is distinctly different from any previously described class of mammalian immunoglobulin, and that approximately three-quarters of the total antibody produced in the duck is of a 5.7S variety, another unique type of immunoglobulin. These observations led us to investigate the properties of immunoglobulins produced in our colony of pigeons. There is little information regarding the immune capability of the pigeon or the classes of immunoglobulins it produces.

We have shown that pigeons are capable of responding to several antigens: Salmonella typhosa H antigen (STH), bovine gamma globulin and 2,4-dinitrophenol. Gel filtration of pooled sera from birds immunized with STH revealed elution profiles that corresponded to the void volume and the position where 7S and 5S standard molecular weight markers elute. Antibody activity could be demonstrated in the void volume and the 7S region. The protein fraction eluting in the 7S region represented approximately 40% of the total serum protein. The immunoglobulins in this region were further purified and we have isolated a 7S molecule that yields equal molar amounts of heavy and light polypeptide chains following extensive reduction and alkylation. The isolated heavy and light chains have molecular weights of approximately 65,000 and 22,500 respectively.

Presently we are observing individual birds hyperimmunized to 2,4-dinitrophenol conjugated to bovine gamma globulin to ascertain more clearly the size distribution of serum antibodies. Further studies will be directed toward a detailed characterization of these immunoglobulins.

Investigation of a Possibly Autoimmune Disease in a Strain of Rats

We have observed in our inbred rat colony a skin disease characterized by severe skin lesions, purulence, loss of hair, and localized dermal necrosis. The disease is not transmitted horizontally to other strains of rats and has not yet been successfully treated by injecting the rats with vitamins or by applying antiseptic solutions. Other treatments, such as steroids and antibiotics, are being tried. The disease is found most often in older females, but older males may become sick as well. It usually leads to general weakness and death; there have been three cases of apparent healing.

We suspect that the skin disease may be autoimmune in nature; that is, the sick animal's immune system may be in effect reacting to self-antigens. We are testing this hypothesis by examining the histological development of the thymus for possible pathology, testing for antibodies to self-components (i.e., red blood cells by the Coombs test), and characterizing the amounts and kinds of serum proteins found in the diseased animals as compared to normal rats. We are also trying to induce myelomas in the rats by injecting mineral oil into their peritoneal cavities. Pilot work on these experiments has provided some provocative results.

Structural Studies of the Gar Tetrameric Immune Macroglobulin

We wanted to compare the immunoglobulin chains of 2 animals which had diverged early in vertebrate evolution. The animal selected for these studies was a primitive bony fish, the gar. Previously we demonstrated that this animal can only synthesize a single class of immunoglobulin when immunized with a variety of antigens. Physical and chemical characterization of these immunoglobulins revealed a type of immunoglobulin molecule that differs from any previously described. Rather than the pentameric molecular form which has been demonstrated for the mammalian IgM class of macroglobulin, ultrastructural studies of the gar immune macroglobulin revealed a tetrameric configuration. Besides being tetrameric, the immune macroglobulin of the gar is distinct from any mammalian class of immunoglobulin on the basis of carbohydrate composition and heavy chain molecular weight.

Although the gar obviously has a unique immunoglobulin structure, detailed structural data are needed before the genetic and evolutionary implications of the molecule are clear. Presently we are attempting to sequence portions of heterogeneous pooled light chains. Although V region heterogeneity may preclude a complete analysis of these chains the C region should be amenable to sequence analysis.

An attempt is in progress to raise homogeneous antibodies against streptococcal group-specific carbohydrate. Antibodies comparable in homogeneity to myeloma paraproteins have been raised by others in rabbits and sharks. The light chains obtained from streptococcal immunization should be homogeneous enough to allow a complete amino acid sequence analysis. This study hopefully will permit us to compare V regions from animals which diverged more than 300 million years ago (e.g., shark and mouse).

Surface Antigens of Neural Cells

The theta (θ) alloantigenic system in mice shows promise as a serological approach to the study of development in the nervous system. This antigen, which is localized in the thymus and in the nervous system, is specified by a single gene with two codominant alleles (θ -AKR and θ -C3H). Virtually absent in neonatal brain, θ expression in the nervous system rises to the adult level during weeks 1-7 of postnatal life.

Appropriately tagged antibodies against θ may be localized by fluorescence or electron microscopy. In addition, quantitative absorption of specific antibody can be used as an indicator of the degree of θ expression.

Recent work by others suggests the presence in rat thymus of an antigen which is cross-reactive with the θ -AKR but not the θ -C3H antigen. We have recently found that this antigen also occurs in rat brain, and that it develops there during the first few weeks after birth.

The fluorescence and absorption methods are currently being employed in the localization of θ -antigen in normal neural tissue, and nervous system tumors are also being screened for the antigen.

Behavioral Biology of Simple Metazoans

As reported, in last year's annual report, we have been interested in studying behavioral biology in an organism which has a relatively simple nervous system and which is also amenable to genetic analysis. Originally, rotifers were chosen as having many desirable properties. However, it was found that culturing them was laborious and unpredictable. In addition, no clear-cut behavioral responses subject to experimental control were found. As a result, we have switched our attention to a nematode, which offers a more favorable genetic situation than do the rotifers, is much easier to culture, and has several clear-cut behavioral responses which can be controlled and analyzed. The work on the nematode is being done in collaboration with Professor Richard Russell.

In order to study how genes control patterns of synapse formation, it is necessary to have mutants with altered synaptic patterns. Such mutants should be identifiable because of altered behavior. A behavior of special interest in this respect is the attraction of the soil nematode Caenorhabditis elegans to the areas of an agar surface which contain its food, the bacterium Escherichia coli. In such areas, these worms reach a density about 100-fold higher than in bacteria-free areas.

On the assumption that this was a chemical attraction, about a hundred biochemicals (including most amino acids and cyclic AMP) were tested to see if they might also be attractive. A few of these appeared to be moderately attractive (giving about a 5-fold increase in worm density) but none were as effective as bacteria. In addition, one important observation was made: acids repelled the worms. pH gradients, formed in the agar, demonstrated that the worms avoided conditions more acid than pH 3, although they survived exposure to such low pH conditions fairly well.

Based on these observations, procedures were developed for selecting mutants. Agar plates were made relatively attractive (bacteria or neutral pH) on one half and repellent (no bacteria or acidic) on the other half. Mutagenized worms were placed on the attractive side, and after several hours any worms which crossed to the repellent side were removed. Control experiments indicate that this subpopulation of worms should be enriched for nonresponding mutants by a factor of about a hundred. Thus if worms are passed through several cycles of alternated selection and propagation, a mutant strain can be isolated from a population with a very small initial fraction of mutants.

Recently the first test of this procedure, using 10 plates of mutagenized worms, produced 2 strains with alterations in behavior. One is defective in its response to acid but not to bacteria, and the other produces numerous animals, of a certain pre-adult stage, which do not respond to bacteria.

A similar class of animals appears much less frequently in wild-type cultures and may represent a resting or dispersal stage. These methods should provide a useful way of selecting both behavioral mutants and mutants with developmental abnormalities.

Maintenance of Transferrin Polymorphism in Pigeons:
Differential Inhibition of Yeast Growth by Eggwhites
from Females Differing at the Transferrin Locus

Transferrin is an iron-binding protein found in all vertebrates. One of its functions is transport of iron in the blood. In birds it has long been thought to serve as a bacteriostatic agent in eggs. In pigeons the locus affecting transferrin has only two reported alleles (Tf^A and Tf^B). In our flock the alleles occur in nearly equal frequency and are in Hardy-Weinberg equilibrium. Females heterozygous at this locus hatch significantly more of their eggs than do homozygotes.

When yeast is grown in medium [1% yeast nitrogen base (Difco), 1% casein hydrolysate, 2% dextrose, tryptophan 0.2 mg/ml, biotin .01 mg/ml, streptomycin 100 µg/ml] containing 10% eggwhite, the following growth rates are observed: Type A, $.49 \pm .06$ doublings/hour; Type B, $.40 \pm .03$ doublings/hour; Type A + Type B, $.43 \pm .06$ doublings/hour; Type AB, $.16 \pm .07$ doublings/hour.

These differences are abolished by the addition of iron to the medium. The data strongly suggest that transferrin polymorphism is maintained by selective advantage of progeny developing in eggs laid by heterozygous females, due to their greater resistance to infection during embryonic and early posthatching development.

In collaboration with Dr. James Crow of the University of Wisconsin, we are developing an algebraic and computer-simulation model of balanced polymorphism maintained by selection against offspring of homozygous females. A population starting with the genotypes in Hardy-Weinberg equilibrium, and with the allele-frequencies at an equilibrium set by the selection coefficients, remains at equilibrium. Computer simulation of populations not starting at equilibrium indicates that after one generation the population comes effectively to Hardy-Weinberg equilibrium and remains at equilibrium while approaching the allele-frequency equilibrium set by the selection coefficients. This is a form of balanced polymorphism that does not display a significant excess of heterozygotes in the population even under intense selection.

The Possible Relationship of β -Lipoproteins to
Histocompatibility Antigens

We investigated the possibility, based on a published report of human studies, that β -lipoprotein of mouse serum might be related to histocompatibility antigens. An attempt was made to identify polymorphism for β -lipoprotein in inbred mice, in hopes of correlating this with histocompatibility type.

We isolated β -lipoprotein from A/J and BALB/cJ mouse plasma by flotation on salt ($\rho = 1.723$). A rabbit antiserum specific for β -lipoprotein was prepared. In vitro absorptions of this serum were performed using both whole serum and purified BALB/c lipoprotein in an attempt to obtain a reagent specific for A/J lipoprotein. This attempt was unsuccessful. Furthermore, in vivo absorption did not produce a specific reagent.

We attempted to sensitize CBA/J mice to A/J skin grafts by injection of purified β -lipoprotein from A/J, in Freund's complete adjuvant. No significant differences in rejection time were observed between the immunized and control animals. These studies have therefore failed to support the reported association between a major histocompatibility antigen and serum β -lipoprotein.

Effect of Antithymocyte Serum on Spontaneous Tumor Onset

The normal response to transplanted histoincompatible tissue is quick immune rejection by the host. With improved tissue matching and immunosuppressive therapy, kidney transplant recipients can obtain prolonged graft survival. Recent observations indicate that these patients have an incidence of tumors many times that of the normal population. The rationale for this finding has been that tumor rejection is analogous to foreign graft rejection and that suppression of cell-mediated immunity permits spontaneous tumors to survive in the incompatible host. This is the currently dominant dogma of "immune surveillance"—that the immune response has evolved as a scavenger for spontaneous neoplasms.

Using immunosuppressed (antithymocyte serum treated) mice to study skin graft rejection, it was noted that after 8 months some treated mice developed spontaneous tumors. Continued observation indicates that 13 out of 15 of the mice including all the control mice developed the same histological type of tumor. Although the mean time of tumor onset for the most immunosuppressed group is lower than for the control groups, the means are not significantly different. Time of tumor onset does not correlate with prior immunosuppression as determined by skin graft survival.

It remains provocative that the earliest tumors to appear were in some of the immunosuppressed animals. But tumors developed in other immunosuppressed animals later than in some of the controls injected only with normal rabbit serum.

In spite of the fact that the cellular immune system seems to function in the rejection of previously established tumors or large tumor inoculations, its role in the rejection of spontaneous neoplasms is not established. Further data are being collected.

Studies of Genetically Controlled Cell Membrane Specificities

A molecular and biochemical definition of cell surface specificities would help in understanding cell-cell interactions and cell specificity. The antigens of red blood cells are excellent surface specificities to study because they are readily defined serologically and can be obtained in assorted genetic combinations. Through the cooperation of Professor Clyde Stormont, of the University of California at Davis, red cells are available from 2 cows whose serological specificities are identical in 9 of 10 known genetic systems. Reagents were prepared by cross-immunizing the cattle and by cross-absorbing immune rabbit sera. The reagents are specific for the distinguishing blood group. A lytic inhibition assay is used to determine antigenic activity and specificity. Preliminary work has been concerned with obtaining the blood group antigens differing between the 2 cows in purified form, so that biochemical characterization can be carried out. Lyophilized membranes of lysed cells yield nonspecific antigenic material, as does mild trypsin treatment of the whole cells. A reported method for solubilizing histocompatibility antigens of lymphocytes with high salt concentration has been modified for red cells, and fractions of high-salt extracts of cattle red cells are currently being tested for inhibitory activity.