STUDIES IN DEVELOPMENTAL IMMUNOGENETICS

Annual Progress Report

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ABSTRACT

This contract provides the research support for a 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 immune system. It extends into the area of cancer immunology and cell specificities as related to tumor systems, primarily from an immunogenetic viewpoint and with particular reference to leukemias in the mouse, and to disruptions of genetic control mechanisms in tumor development, especially as approached through the reappearance of fetal antigens associated with tumor development.

Functional Effects of Antibodies on Rat Peripheral Lymphocytes

The theta antigen of the mouse is carried by one or more molecules, possibly glycolipid in nature, which can be detected on the surface membranes of thymus-derived lymphocytes (T-cells) but not on antibody-producing cells or their precursors (B-cells). The two genetic variants of theta, Thy-1.1 and Thy-1.2, are the products of two autosomal codominant alleles, Thy-1^a and Thy-1^b respectively. Antibodies specific for one form of the antigen can be obtained by immunizing animals homozygous for one Thy-1 allele with T-cells from those homozygous for the other. In the presence of a series of serum proteins collectively referred to as the complement system, antibodies directed against the theta antigen present on a given cell can cause membrane damage and eventually cytolysis.

Peritoneal cells from inbred AKR/J mice (homozygous for Thy-1^a) previously immunized with EL4 mouse leukemia cells are capable of killing these tumor cells *in vitro* in the absence of complement. That this is dependent upon the action of T-cells is indicated by the observation that pretreatment of the peritoneal cells with anti-Thy-1.1 and complement completely abolishes their anti-tumor activity.

Despite the fact that rat thymocytes possess an antigen indistinguishable from Thy-1.1, rat peripheral T-cells were not killed by anti-Thy-1.1 antibodies and complement. But the possibility remained that such treatment might inhibit the anti-tumor activity of the peritoneal cells even though it did not cause cell death. Therefore peritoneal cells from rats immunized against mouse EL4 leukemia cells were pretreated with complement and either mouse normal serum, mouse anti-Thy-1.1 serum, or mouse antiserum against both Thy-1.1 and rat species antigens. Only the latter gave strong inhibition of peritoneal cell activity, indicating that antibodies against Thy-1.1 cannot be used for the selective inactivation of rat peripheral T-cells. The alternative explanation that tumor cell killing in this system might be mediated by cells other than T-cells is currently under investigation.

The Expression of Theta-like Antigen by Peripheral Lymphoid Cells of the Rat

In the mouse, less theta antigen is expressed by peripheral T-cells than by thymocytes. Although theta-like antigen can be detected on rat thymocytes by either antibody absorption or complement-dependent cytotoxic tests, neither of these methods gives positive results for rat peripheral T-cells. Others (Acton, 1974) have reached the same conclusion using radioactive antibodies, although theta antigen has apparently been detected using an indirect immunofluorescence assay (Micheel *et al.*, 1973).

Another way to approach the question of whether the theta-like antigen is present on rat peripheral T-cells is to raise antibodies against these cells. If part of the resulting antibodies is specific for theta, then this antigen must have been present on the cells in question.

Groups of AKR/Cum mice were injected weekly with 2.5×10^6 nucleated cells from rat thymus, spleen, lymph node, Peyer's patches, bone marrow, peritoneal exudate, or blood. During the six-week interval studied, all groups produced antibodies which were cytotoxic for thymocytes of the closely related strain AKR/J (Thy-1^a) but not for those of AKR/Cum (Thy-1^b). When tested against thymocytes from backcross mice, these sera were found to be cytotoxic for cells from Thy-1^a/Thy-1^b heterozygotes but not Thy-1^b homozygotes. It therefore appears that the theta-like antigen is present on rat peripheral T-cells. The possibility that transplanted rat cells might have developed the theta-like antigen after injection into mice is being examined.

Unexpectedly, animals in three of the groups described above were found to be producing low titers of antibody against AKR/J thymocytes before immunization. These groups made significantly stronger responses during the first week as would be expected in a secondary antibody response. Further experiments have indicated that approximately 18% of adult male AKR/Cum mice spontaneously make low titers of antibodies against Thy-1.1. The presence of "natural antibodies" against the theta antigen may be part of the reason that theta generally evokes stronger antibody responses than do most other mouse lymphocyte alloantigens.

References:

- Acton, R. T. (1974) Fed. Proc. 33: 778.
 Micheel, B., Pasternak, G. and Steuden, J. (1973) Nature New Biol. 241: 221-222.

In Search of New Genetic Polymorphisms in the Rat

Studies of the genetic polymorphisms for theta antigen, cytoplasmic malic enzyme, and transferrin (an iron-binding protein) of the mouse have been made previously in this laboratory. To date no corresponding polymorphisms have been reported for the common laboratory rat, Rattus norvegicus, although transferrin polymorphism has been observed in the black rat, Rattus rattus. The discovery of "mutant" forms of these genes, all three of which are linked in the mouse, would be very useful for genetic studies in the rat.

In complement-mediated cytotoxic tests thymocytes from rats of 13 inbred and two random-bred populations were all killed by antibodies specific for Thy-1.1 (θ-AKR) but not by those specific for Thy-1.2 (θ-C3H). Thus, no qualitative differences in the theta-like antigen of the rat were found. In order to look for possible quantitative differences in antigen expression, the number of thymocytes from a given strain required to absorb a standard amount of anti-Thy-1.1 antibody was determined. The amount of antigen present on the thymocytes of five different strains of rats (A/2, AUG, BN.B2, COP, WF/fz) was found to differ by less than 2-fold, indicating no large differences in antigen expression.

Starch block electrophoresis of serum samples from the 15 rat populations described above revealed no variation in the electrophoretic mobility of

transferrin. Electrophoretic analyses of kidney cytoplasmic malic enzyme samples from the same rats detected no variant forms. Enzymes from eight selected strains also ran similarly when a second buffer system was used.

Thus, no genetic variation in these traits has been found to date.

Wild Mouse Theta (Thy-1) Antigens

We have recently completed the first experiments designed to investigate the incidence of Thy-1 alleles in two feral populations of mice, and the possible representation on lymphoma cells of Thy-1 antigens. The study of the functional and morphological cellular origins of leukemias and lymphomas is currently of great interest to medical researchers. In the mouse, there are a number of antigens characteristic of certain cell types, and it was felt that the above approach was one good way of determining whether or not the lymphomas found in one wild mouse colony arose from a normal clone of Thy-1 positive, thymus-derived cells.

Animals for this project were obtained from wild mouse colonies maintained at the University of Southern California Special Cancer Virus Program facilities. Two colonies were studied: the Bouquet Canyon colony, which has a very low incidence of spontaneous lymphomas, and the Lake Casitas colony, which has a lymphoma incidence of 17% over a 12-month observation period. Serological techniques revealed that the Thy-1 antigen can be easily detected on brain and lymphoid tissue of normal young mice. Genotypes for 57 normal and lymphoma-bearing animals from the Lake Casitas colony were obtained. The observed frequency of the Thy-1^a allele is 0.43, and the frequency of the Thy-1^b allele is 0.57. The observed frequency of heterozygotes is 0.40, which is not significantly different from that expected in a population in Hardy-Weinberg equilibrium. The frequency of the Thy-1^b allele in the lymphomatous population (0.70, $n = 25$) was significantly different from that of the normal mouse group (0.47, $n = 32$); there were also fewer heterozygotes (29% vs. 48%). However, the presence of the Thy-1^a allele is restricted to the Lake Casitas colony: the Thy-1^b allele is apparently fixed in Bouquet Canyon mice. The implication of this result will be investigated in a proposed study of wild mice from other trapping areas, to see if the presence of the Thy-1^a allele is related to the presence of lymphomas. Our observation that the Mod-1^a allele and the Trf^b allele (two loci linked to Thy-1 on chromosome 9) are fixed in both Lake Casitas and Bouquet Canyon mice makes the segregation of Thy-1 in the Lake Casitas colony even more interesting.

Thy-1 antigen expression on brain tissue showed some variation with gene dosage (as detected by the α -Thy-1^b reagent) and with the day on which the experiment was conducted, but it did not vary with sex, age, or disease state. Thy-1 expression on normal lymphoid tissue did decrease somewhat with age, but not below detectable levels. 13 of 20 histologically-confirmed lymphomatous spleens (65%) were completely negative for Thy-1 antigen, 4 spleens were positive, and 3 gave ambiguous results. Thus, it does not appear that lymphomas from this colony are typically Thy-1 positive or thymus-derived. Other antigenic markers and functional characteristics of these lymphoma cells will have to be tested to determine their cellular origins.

Thymus and Bursa-Specific Antigens of the Pigeon

Lymphocytes of thymus-dependent and thymus-independent origins have separate roles in the immune response, but may cooperate in the antibody response to certain antigens. The lymphocytes look alike, but they can sometimes be distinguished from each other by serological means. The immune systems of birds are unique in that the functionally distinct populations of lymphocytes are anatomically separated in the central lymphoid organs--the thymus and the bursa of Fabricius. The avian immune system is used as a model for understanding mammalian immune systems. Cooperation between lymphocyte populations has been demonstrated in the avian antibody response although not with the two anatomically separate lymphocyte populations. Few serological markers are known for avian lymphocytes. Additional markers could more clearly identify distinct interacting cells and determine their roles in synergistic immune responses.

Antisera were prepared against pigeon thymus and bursa lymphocytes and against saline extracts of thymus and bursa. All antisera were prepared in rabbits--some as standard anti-lymphocyte reagents and some in rabbits injected neonatally with large doses of pigeon cells. The neonatal injections were made to reduce the rabbits' antibody response to species-specific antigens upon challenge with pigeon lymphoid tissue.

All unabsorbed sera were screened for differential reactivity against thymus and bursa-derived tissue by passive hemagglutination, immunodiffusion, leukoagglutination and complement-dependent lymphocytotoxicity. Sera identified in this manner were absorbed with thymus and bursa tissue to demonstrate specificity. One serum with specificity against thymus extracts was identified by immunodiffusion. The thymus specificity was absent from extracts of pigeon bursa, brain, liver and breast muscle, but present in spleen extracts. The thymus specificity was shared by extracts of thymus from two different breeds of pigeon but not by extracts of chicken lymphoid tissue. This thymus specificity does not appear analogous to lymphocyte specificities identified in other species.

Shared tissue specificities and a possible quantitative antigenic difference among the tissue extracts were also demonstrated by immunodiffusion and absorption analysis.

In lymphocytotoxicity tests, fresh rabbit normal serum is highly toxic for pigeon thymus and bursa cells. This toxicity resembles the natural antibody present in rabbit and guinea pig serum against heterologous thymus cells. The activity against pigeon lymphocytes can be absorbed by pigeon thymus and bursa cells and is heat labile. Unlike the natural antibody against thymus cells of other species, the toxicity against pigeon lymphocytes could not be absorbed by agarose.

A rabbit anti-pigeon gamma globulin serum was rendered specific for bursa cells by absorption with thymus cells. Some standard anti-lymphocyte sera were shown to contain an antibody fraction specific for thymus cells.

Some or all of these reagents may be useful for distinguishing cooperating cell populations in a variety of immune responses. Avian species, in addition to the chicken, may provide additional data on the phylogeny of functionally distinct lymphocyte populations.

Developmental Red Cell Antigen in the Pigeon

Several fetal red cell antigens are described in the literature for species other than the pigeon. In this study, a fetal red cell antigen was sought as a possible serological marker for white cells as well. There are several reasons for thinking that such an antigen might be present on white cells. First, these two cell types are derived from a common stem cell. Second, a number of other antigens are known to be shared by white cells and red cells. In addition, the thymus and the bursa of Fabricius, which are the primary lymphoid organs in birds, are transient sites of erythropoiesis. Both lymphoid organs could be classified as developmental structures. At least one fetal red cell antigen is known to exist on cells other than mature red cells and to persist in these sites even after the antigen is no longer detectable on red cells.

The studies reported here did in fact reveal an antigen on red cells from all pigeon squabs less than four days old. The antigen appears not to be secondarily adsorbed to the red cells from the fluids of the egg or the embryo. In vitro, masking of the antigen by components of adult serum does not occur under the conditions tested, nor can the antigen be made to reappear in the adult after frequent bleedings. Although the squab antigen behaves similarly to a known fetal red cell antigen in doves, it is probably qualitatively different from that antigen and from the known chick red cell antigen.

The squab antigen is not detectable on lymphocytes from the bursa or the thymus by either leucoagglutination or complement-dependent lymphocytotoxicity. The antigen does not appear to be a lymphocyte marker. It is, however, an additional developmental antigen. If it is like other developmental antigens, it could be an interesting marker in both normal development and some disease states.

The Relationship Between the Chicken Fetal Antigen and Avian Myeloblastosis Virus

Chickens possess a fetal red blood cell antigen which is present at the time of hatching but which disappears during the early development of the animal. The fetal antigen is present on certain adult tissues and particularly on cells in the bone marrow, presumably erythrocyte precursors. The fetal red blood cell antigen reappears on the red cells of adult chickens which have leukemia induced by avian myeloblastosis virus (AMV). It has been shown that leukemic chickens had the chicken fetal antigen at one day post-hatching, subsequently lost the antigen during development, and reacquired it upon contracting leukemia (Teplitz et al., 1974). Because of the association

of leukemia onset and reappearance of the chicken fetal antigen we are investigating the possibility that the chicken fetal antigen is the receptor for the AMV virus.

Hemocyanin has been glutaraldehyde-coupled to goat-anti-rabbit-IgG (Hcy-GARIGG) to serve as an electron microscopic visual label for the chicken fetal antigen. The AMV virus serves as the marker for the viral receptor. Examination of cell surface replicas by transmission electron microscopy with the Hcy-GARIGG reagent shows large quantities of the fetal antigen on young chick red cells and only background levels on the adult chicken red cells. We are currently carrying out competition and blocking experiments with the hemocyanin reagent and AMV virus to study the cell surface topological relationship of the AMV receptor and the chicken fetal antigen.

Reference:

Teplitz, R. L., Sanders, B. G., Brodetsky, A. M., Fung, H. and Wiley, K. L.
(1974) Cancer Res. 34: 1049-1053.

A Search for Mouse Alpha-Fetoprotein Variants

A number of proteins found in mammalian and avian fetal development appear again (in "retrogenic" expression) just prior to or during malignant tumor growth. The term carcinoembryonic antigen (CEA) is applied to such cell-bound or soluble antigens, and their detection in adults, especially human adults, may be correlated with or even diagnostic of tumor formation. One such molecule is alpha-fetoprotein (AFP), found in embryos of many mammalian species and in primary carcinomas of the liver. It is a protein of "alpha" mobility on agar or starch electrophoretic gels, and has a molecular weight of about 70,000 daltons. It closely resembles albumin, and it has been suggested that AFP is a fetal analog of adult albumin. AFPs from two different species may be cross-reactive in immunoprecipitation reactions with antiserum prepared in a third species.

Our purpose in this study is to investigate possible antigenic differences between AFPs from different inbred strains of mice. It would be useful to find molecular variants of AFP in order to prepare specific alloantiserum to AFP and eventually to locate the gene(s) responsible for its production.

Mouse AFP is prepared from homogenates of mouse 19-day embryos and partially purified by Sephadex gel chromatography. AFP-containing fractions from four different inbred mouse strains (A/J, CBA/J, C57B1/6J and AKR/Cum) have been prepared. There is no difference in mobility between them when the samples are subjected to electrophoretic separation on starch gels. Rabbit antiserum to AKR/Cum mouse fetal homogenate (containing AFP, albumin, transferrin, hemoglobin, and other minor proteins) was prepared and absorbed with adult mouse serum proteins which had been glutaraldehyde-cross-linked by the method of Avrameas (1969). The twice-absorbed reagent reacts with AFP, but not albumin, from column-purified fetal homogenates of all four

strains of mice. Experiments are in progress to test for strain-specific antigens detected by this reagent, by absorbing it with AFP preparations from each of the four strains of mice.

Reference:

Avrameas, S. and Ternynck, T. (1969) *Immunochemistry* 6: 53-66.

Effect of Immunosuppression on Primary Tumors

If immune surveillance suppresses spontaneous tumors which result from somatic mutation, environmental carcinogens, and oncogenic viruses, anti-thymocyte serum treatment should increase the tumor incidence and shorten the latent period. It might also allow the expression of tumors not normally seen in unsuppressed populations.

Virgin female mice of a single inbred strain, CBA/J, obtained after a variety of treatments, were observed for primary tumors until either tumor onset or death. Included were mice which were: (1) immunosuppressed as adults by injection of rabbit anti-mouse thymocyte serum (RAMTS), (2) injected with normal rabbit serum, (3) immunized with an irrelevant antigen (mouse serum β -lipoprotein) or (4) untreated. Data were collected on tumor histology, incidence and time of onset for all groups.

No tumors appeared during the period of ATS-immunosuppression or for several months following treatment. There was no evidence from this study or from the literature that anti-thymocyte serum has any carcinogenic effect in the absence of oncogenic viruses or carcinogens.

This strain carries mammary tumor virus as a result of neonatal infection via milk from infected mothers. The most frequently observed tumor, the mammary tumor, appeared at an average age of slightly less than two years. Although the first tumors appeared in ATS-treated mice, the mean age at tumor onset was not significantly affected by ATS-immunosuppression. No unusual tumors and no lymphomas were observed.

Tumor incidences among groups of mice purchased at different times were different, but unrelated to ATS-immunosuppression. Tumor incidence was not depressed by failure of mice to reach tumor age; the mean life span of non-tumor-bearing mice was as long as or longer than that of tumor-bearing mice. Cause of the fluctuation in tumor incidence is not known.

Although tumor immunity reportedly exists at a time when palpable mammary tumors are present, immune surveillance does not appear to be the determining factor in the long tumor latent period. This conclusion is supported by published data from other laboratories which indicate that cellular immunity to mammary tumors is often compromised by immunological tolerance and/or the presence of blocking factors in the serum. The failure of ATS-immunosuppression to affect mammary tumor growth is consistent with these observations.

A Possible Linkage Assignment for 6-Phosphogluconate Dehydrogenase in Mus Musculus

The availability of numerous inbred strains of mice, each differing from others at one or many genetic loci, has facilitated gene mapping and linkage studies in this organism. However, no variants for 6-phosphogluconate dehydrogenase (Pgd-1) have been reported in inbred mice, although electrophoretic variants have been detected in wild populations (Selander and Yang, 1969). It is probable that this lack of variation in inbred mice has delayed the assignment of the structural gene for this enzyme to a linkage group. While studying progeny of a cross between wild mice and the inbred strain C57Bl/10Sn we noticed a marked tendency for two alleles from the wild population, Pgd-1^a and Pgm-2^b (phosphoglucomutase-2), to segregate together. This linkage was detectable in only one family, the only family segregating for electrophoretic variants of Pgm-2. We have initiated crosses between F2 progeny, heterozygous for both of these markers, and the C57Bl/10Sn strain. The linkage between Pgd-1, Pgm-2 and other genes known to be on the same linkage group as Pgm-2 will be analyzed in more detail, using the progeny of this cross.

Reference:

Selander, R. K. and Yang, J. P. S. (1969) Genetics 62: 653-667.

Genetic Studies on Susceptibility to Lymphoma in Mus Musculus

Investigations of susceptibility to leukemia and lymphoma using inbred strains of mice have implicated a number of genetic loci (Lilly and Pincus, 1973). We have been collaborating with Dr. Murray Gardner, University of Southern California School of Medicine, on such a study using a colony of wild mice which has been shown to have a high frequency of spontaneous lymphoma (Gardner et al., 1973). The wild mice have been mated to members of an inbred strain of mice, C56Bl/10Sn, which is known to be highly resistant to leukemia and lymphoma. At six weeks of age, F1, F2 and backcross progeny are splenectomized and the tissue examined for the presence of murine C-type virus group specific (gs) antigen and for virus particles. The mice are then maintained in the laboratory to determine whether or not they develop lymphoma in later life. We have been testing blood samples obtained from these mice for a number of genetically determined protein polymorphisms, looking for linkage between any of these and gs antigen expression. Our preliminary data suggest a possible linkage between the structural gene for 6-phosphogluconate dehydrogenase (Pgd) and the locus controlling expression of gs antigen. We have data indicating that Pgd is located on chromosome 4, a chromosome which is also known to contain the locus Fv-1, which controls susceptibility to Friend leukemia virus (Rowe et al., 1973). If these preliminary data are confirmed by additional work now in progress, it would seem possible that the same genetic controls which regulate susceptibility to leukemia in inbred mice are operating in this feral population.

References:

- Gardner, M. B., Henderson, B. H., Estes, J. D., Menck, H., Parker, J. C. and Huebner, R. J. (1973) J. Natl. Cancer Inst. 50: 1571-1579.
 Lilly, F. and Pincus, T. (1973) Adv. Cancer Res. 17: 231-277.
 Rowe, W. P., Humphrey, J. B. and Lilly, F. (1973) J. Exptl. Med. 137: 850-853.

Do the Autoantibodies of Myasthenia Gravis Patients Inhibit Sarcoplasmic Reticulum Function?

Autoantibodies reactive with muscle as well as a variety of other tissues of human or animal origin are frequently found in the blood of patients suffering from myasthenia gravis (MG). Recently it was reported (Mendell et al., 1973) that these autoantibodies could be localized to the sarcoplasmic reticulum membranes of striated muscle. The ATP-dependent active transport of Ca^{++} ions by these membranes is necessary for muscular relaxation. Any interference with this process might contribute to the muscle dysfunction seen in this disease.

Partially purified sarcoplasmic reticulum membranes were prepared from rat striated muscle by differential centrifugation. These membranes were intact as judged by their ATPase activity and ability to accumulate Ca^{++} in vitro, in the presence of ATP. By electron microscopy this preparation could be seen to be composed of a mixture of thin- and thick-walled vesicles.

So far, the exposure of these membranes to sera from MG patients has not been found to affect their capacity to accumulate Ca^{++} in vitro. Further experiments along these same lines are currently in progress. The development of other methods for detecting the binding of autoantibodies to sarcoplasmic reticulum membranes is also planned.

Reference:

- Mendell, J. R., Whitaker, J. N. and Engel, W. K. (1973) J. Immunol. 111: 847-856.

Serological Investigation of Electrophoretically Determined Isozymes

Electrophoretic variants of enzymes have proven to be valuable markers in many types of genetic and developmental studies. Current methods used to detect the presence of enzymes employ indirect measurements of products and cofactors involved in the enzymatic process. Though colorimetric or spectrophotometric analysis coupled with electrophoresis is adequate for some investigations, a direct immunoassay would be more desirable, especially in histochemical studies requiring visualization or microquantitation of enzymes present in small amounts of tissue. We attempted to prepare specific antibodies against the electrophoretically defined genetic variants of two enzymes, glucose phosphate isomerase (GPI) (E.C.5.1.1.9) and malic enzyme (Mod-1) (E.C.1.1.1.40).

In order to detect possible antigenic differences between the electrophoretic variants both hetero- and alloantisera were prepared. The enzymes from AKR/Cum (Gpi^a and Mod-1^a) and CBA/J (Gpi^b and Mod-1^b) mice were partially purified by ammonium sulfate precipitation and Sephadex column chromatography. Preparation of alloantiserum involved cross-immunization of AKR/Cum and CBA/J mice with appropriate isozyme extracts. Rabbits were injected with the extracts in order to prepare heteroantiserum.

Analysis of the alloantiserum by immunodiffusion and immunoelectrophoresis failed to demonstrate the presence of allotypic antibodies. The heteroantisera were found to contain antibodies to all the variants as shown by immunodiffusion. However, they were highly cross-reactive and specificity for individual isozymes could not be shown even after extensive adsorptions. The lack of demonstrable allotypic antibodies may in part be due to an insufficient quantity of enzymes used in the immunizations. It is conceivable that given an adequate antigen dose allotypic antibodies could be elicited.

Cytotoxic and Immunological Properties of Ionene Polymers

The immune system affords a good model with which to investigate the relative contributions of specific as well as nonspecific cell surface characteristics involved in cell-to-cell interactions. The necessity for specific cell surface markers in immune recognition has been well documented. The role of nonspecific surface parameters, such as cell surface charge, has also been examined but to a lesser extent. Most investigators have employed enzymes (e.g., neuraminidase) to change the surface charge of cells. Another approach to modifying cell surfaces is the addition of polyanions or polycations which strongly adhere to cells and also change their net charge. In our investigations we have been using ionenes, a class of quaternary ammonium polymers, to alter the surface properties of lymphocytes and EL4 leukemia cells. We are attempting to assess the behavior of these modified cells in a variety of in vivo and in vitro immune mechanisms.

Previous work examining the immunological properties of these polymers showed that they were not immunogenic by themselves. However, they did show interesting cytotoxic and cell agglutinating behavior. We found that by varying the charge density and molecular weight of the polymer different biological activities could be conferred. Ionene 3,3 (I 3,3), the most densely charged, agglutinated murine erythrocytes and peripheral white blood cells to a greater extent than I 6,10, which is less charged. However, the toxicity of the polymers with respect to the cells is inversely correlated. The differential surface activity of the polymers suggested examining the ability of various ionenes to modify either afferent or efferent immune cell interactions.

Examinations of in vitro cell mediated immunity against ionene-coated EL4 cells showed no significant difference from controls (i.e., not coated EL4 cells). We are currently looking at the ability of ionenes to alter afferent immune mechanisms by means of in vitro mixed lymphocyte reactions and also by means of in vivo response to ionene-coated EL4 cells.

Quantitative Cell Surface Expression of Genetically Defined Bovine Red Cell Antigens

We have used the electron microscope to investigate the quantity and distribution of blood group antigens on the surface of bovine red cells. The antigens were visualized for electron microscopy (EM) by an indirect labeling method using hemocyanin from the snail Busycon canaliculatum, glutaraldehyde-coupled to rabbit-anti-bovine immunoglobulin. Gene dosage effects, reflected serologically in time of complement-mediated lysis for cells negative, heterozygous, and homozygous for the Z allele, were examined for the quantity of Z antigen per unit area of cell surface, on genetically defined cell populations. Homozygous cells were found to have approximately twice as much Z antigen as heterozygous cells. Cells negative for the Z antigen showed only background levels of label.

The J antigen is a soluble serum glycoprotein which secondarily adsorbs to the red cell surface. Some bovine red cells display a very low J titer and require large amounts of anti-J antibody to lyse them, while other cells have high J titers and require only minute amounts of antibody for cellular lysis. We have studied the cell surface quantitative expression of the J antigen using our EM labeling reagent, and have found that the J titer of a cell corresponds to the quantity of J antigen present on that cell's membrane. Anti-J antiserum was fractionated into the IgG and IgM components and these fractions were assayed for binding activity by EM. During the course of this study, we have identified an additional antigen on a population of J negative cells which does not appear to elicit lytic or direct hemagglutinating antibody, but which can be serologically detected by an indirect Coombs reaction with anti-immunoglobulin.

The expression of antigens on the cell surface in the presence of bound antibody directed against another antigen was studied by a sequential double labeling technique. Two specificities in the same blood group complex (C complex) as well as antigens in different complexes (C and Z complexes) were examined. In two experiments it was found that bound antibody directed against one specificity did not inhibit binding of antibody against another specificity. In another experiment with different red cell antigens it was found that bound antibody directed against one specificity enhanced the adsorption of antibody directed against a second specificity.

The Effects of Enzyme Treatment on the Expression of Cell Surface Antigens and Concanavalin A Binding Sites on Bovine Red Cells: Implications for Membrane Structure

Using an indirect labeling technique and hemocyanin as the visual marker for transmission electron microscopy, bovine red cells have been examined for their Concanavalin A (Con A) binding. A report by Hines *et al.* (1972) suggested that for bovine red cells the Con A receptor and the A antigen may be molecularly the same structure. Electron microscopic, hemolytic and

agglutination experiments in this laboratory using Con A and anti-A antiserum demonstrate that the two binding activities are involved with separate red cell surface structures. Red cells were either sensitized with Con A or anti-A antiserum and then tested for additional uptake of the other reagent in the above-mentioned assay systems. Untrypsinized bovine red cells were not agglutinated by Con A, but trypsinized A positive cells were agglutinated, as reported by Hines *et al.* (1972). Both A positive and A negative untrypsinized cells bound Con A, although the untrypsinized A positive cells bound more lectin than the untrypsinized A negative cells. Following trypsinization the Con A uptake of both cell types was increased, but the trypsinized A positive cells still bound more lectin than the trypsinized A negative cells. The uptake of immunoglobulin by both types of trypsinized cells was found to be largely nonspecific and due to an increased "stickiness" of the trypsinized cells.

Additional studies using the freeze-fracture technique for examining the internal structure of membranes have shown that for the bovine red cell A antigen the A antigenic determinants associate physically with the intramembranous particles seen on freeze-fracture replicas. This observation supports the theory of membrane structure proposed by others.

Reference:

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Bovine Leukocyte Antigens

Bovine red blood cell surfaces display over 60 serological specificities grouped into 11 genetic systems. These specificities and the phenogroups that they form have been well characterized serologically. Believing that a study of bovine leukocytes for the presence of bovine red cell antigens is in order, we have surveyed 28 bovine red cell typing reagents for their leukocytotoxicity in a microcytotoxicity system. Previously we have used the Amos one-step microcytotoxicity for our tests, but we found that additional positive leukocyte reactions were present using the two-step assay. We have now tested leukocytes from a panel of 13 animals from herds at University of California, Davis, and California Polytechnic Institute at Pomona. Using the Amos two-step assay we have tested representative reagents from each of the bovine red cell blood groups including 8 B complex antisera, 3 C complex antisera, F antiserum, Z antiserum, 7 S complex antisera, 2 A complex antisera, L_x, J antiserum, M₂, and R' and S' antisera. In all cases where the test panel consisted of cells from donors whose red cells displayed plus and minus reactivity patterns with the test reagents (28 of the 29 reagents), the pattern of leukocytotoxicity did not match the pattern of red cell hemolysis. Also, reagents that were not cytotoxic for leukocytes were nonreactive with leukocytes from donors whose red cells possessed the antigen, as well as with leukocytes whose donors lacked the antigen. These data indicate that bovine leukocyte antigens, as detected by the two-step microcytotoxicity assay, are distinct from red cell antigens. Because there

is no apparent association of leukocyte reactivity with bovine red cell antigens, we are probably detecting different antigenic systems on leukocytes and red cells.

H-2 and Ia Antigen Topology on the Cell Surface of Murine Lymphocytes

Within the past year a new system of murine lymphocyte cell surface alloantigens has been described by David *et al.* (1973) and Sachs and Cone (1973). The genetic region which controls the production of the Ia gene products is closely linked to the Ir region of the H-2 major histocompatibility locus in the mouse for additional information on the Ia antigen). The Ia antigens have been detected both serologically (David *et al.*, 1973; Sachs and Cone, 1973) and chemically (Cullen *et al.*, 1974) and shown to be distinct from the H-2 gene products.

Because of the close genetic linkage of the Ir region genes and the H-2 genetic region we are studying a possible cell surface association of the H-2 and Ir gene products. An electron microscopic labeling reagent has been prepared in which rabbit-anti-mouse immunoglobulin is coupled to hemocyanin (RAMIgG-Hcy). This reagent is being used to tag indirectly the cell surface H-2 and Ia antigens. Specific H-2K and H-2D end antisera from the National Institutes of Health and an anti-Ia antiserum from Dr. D. Shreffler are available. Cells are treated with the appropriate antiserum, reacted with the RAMIgG-Hcy, and prepared for electron microscopy by making carbon replicas of the cell surfaces. In some experiments the cells are treated twice with antisera, each time with a different antiserum followed by the hemocyanin reagent, to assess the blocking capacity of a particular antibody. Capping experiments using the hemocyanin reagent are also being undertaken to see if the Ia and H-2 products cap (e.g., are mobile on the cell surface) and if they co-cap. If the Ia and H-2 antigens are located on the same molecule they may cap together. If they are close to each other on the cell surface, but not on the same molecule they may block binding of antibody to one another but not co-cap. If they are sterically independent on the cell surface they may show neither blocking nor co-capping.

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Enzyme Studies in Caenorhabditis Elegans

We have examined seven strains of *Caenorhabditis elegans* for genetic polymorphisms in seven enzymes using starch gel electrophoresis. No substantiated

polymorphisms were found. Crosses for several apparent polymorphisms produced no evidence of Mendelian segregation.

In the process of screening for genetic polymorphisms, we observed two apparently exclusive forms of the enzyme acid phosphatase in populations of animals separated into large vs. small by filtration through a nylon mesh. This developmental polymorphism is being examined further.

In order to examine the development of the separable forms, we have devised a technique to grow large populations of nematodes synchronously in liquid culture. This is accomplished by taking heterogeneous cultures, separating gravid adults by settling through a 2 to 4% sucrose gradient and resuspending them in fresh culture media. After a suitable time for egg laying (2 to 6 hours), the adults are removed by settling. Synchronous progeny are maintained in liquid cultures and can be harvested at any desired developmental stage. In one such experiment six stages in development were collected and analyzed for enzyme.

Initial experiments have used α -naphthyl acid phosphate to stain for acid phosphatase. As an alternative, we are experimenting with 5-bromo-4-chloro-3-indolyl-phosphate as a possible higher resolution substrate.