AD-A139 748 HISTOCOMPATIBILITY TYPING(U) GEORGETOWN UNIV WASHINGTON DC LOMBARDI CANCER RESEARCH CENTER D D ECKELS ET AL. 15 MAR 84 TR-1 N00014-83-K-0410 1/1

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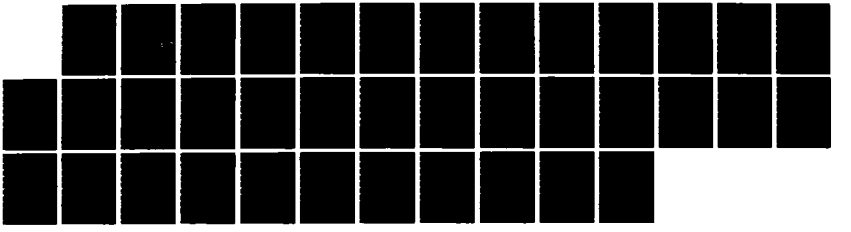

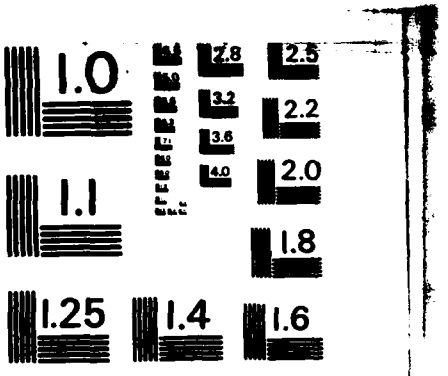

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OFFICE OF NAVAL RESEARCH
Contract N000[14]83-K-0410
TECHNICAL REPORT NO. 1

HISTOCOMPATIBILITY TYPING

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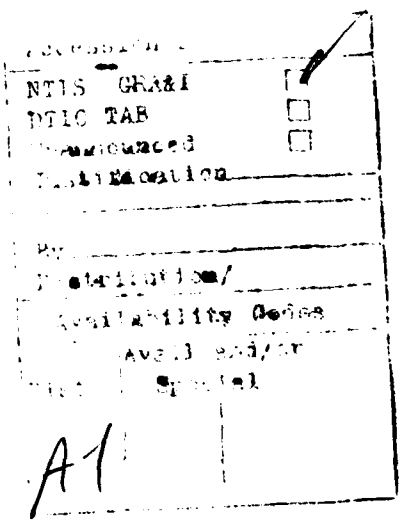


I. ABSTRACT

In order for bone marrow transplantation to be an effective and successful therapeutic measure in the treatment of irradiation casualties, the donor marrow cells and the recipient must be matched for HLA antigens. Matching for antigens encoded by HLA-D appears to be most critical, however, we are just beginning to comprehend the complexity and importance of the HLA-D region. This contract was established to systematically analyze those HLA-D antigens most important for bone marrow transplantation and to develop and implement methods for matching large numbers of unrelated individuals for clinical bone marrow transplantation. We describe herein our progress in using cellular methods for eludicidating the genetics of the HLA-D region and the initiation of biochemical approaches to clinical transplant matching. These methods include primed lymphocyte typing (PLT) generation of human T-lymphocyte clones (TLC's) that recognize histocompatibility antigens, homozygous typing cells (HTC) characterization and peptide map characterization. During the upcoming contract period, we will continue our efforts in cellular immunology, and will also continue to develop parallel programs in the immunochemistry of human alloantigens and serologic recognition of hLA antigens by human monoclonal antibodies.

II. INTRODUCTION AND BACKGROUND

The following technical report summarizes the first year's progress on ONR contract N000-14-83-K-0410 "Histocompatibility Typing". This contract was initiated on 1 May 1983 for the purpose of defining the antigens of the human major histocompatibility complex in order to facilitate bone marrow transplantation in unrelated donor/recipient combinations.

The purpose of these studies conducted here at Georgetown University is primarily directed at determining those factors that would facitate bone-marrow reconstitution by transplantation of military personnel lethally exposed to bone marrow toxic agents. The reason for this approach is that toxic materials such as aviation fuels, chemicals and electromagnetic radiation can induce aplastic anemia for which the only current viable therapeutic procedure is bone marrow transplantation. Unfortunately, long-term survival of bone marrow transplantation patients has only occurred within a certain percentage of sibling donor-recipient combinations indicating that there is a genetic component that determines the success of a bone marrow transplant. This conclusion is further supported by clinical experience which demonstrates good graft survival using genetically related individuals. However, in some bone marrow transplant cases, rather than the recipient rejecting the grafted marrow, immunologically competent cells (lymphocytes) in the donor's marrow can reject the recipient which is termed graft versus host disease or GvHD. Therefore, the aim of our program is to define those essential genetic components that contribute to graft rejection, immunologic reconstitution and GvHD. As additional genetic elements are ascertained, these will be matched up for appropriate matching for unrelated donors. These methods are used to define the level of matching for clinical transplants within families in concert with standard HLA typing procedures performed by in-house NMRI programs.

The major determinant of graft rejection and GvHD is a genetic region called the major histocompatibility complex (MHC) or HLA in humans located on the short arm of chromosome 6. The HLA system comprises a tight cluster of at least thirty different genes that:

1) When identical in different individuals, enhance the potential for acceptance of grafted tissue, and;
2) Code for proteins, some of which are found on all cells of the body and others which are found primarily on the cells of the immune system (lymphocytes and macrophages).

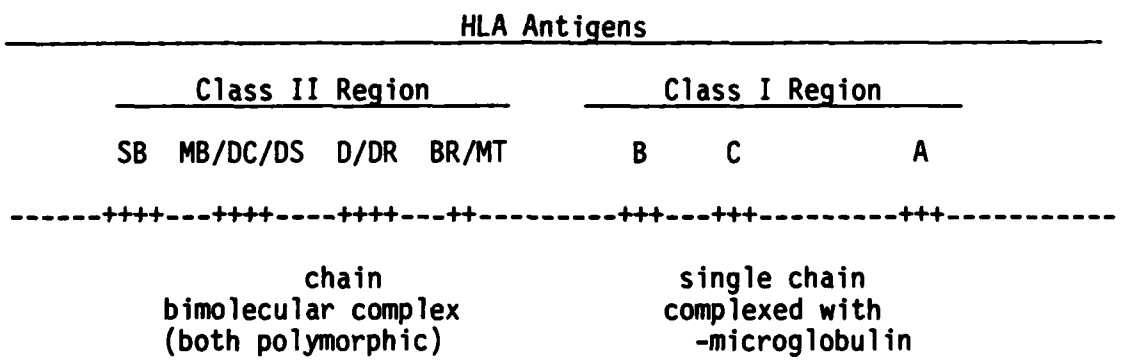
Therefore, methodologies for identifying HLA proteins provide important clues as to the genetic identity of different individuals and thus facilitate transplantation.

A molecular basis for the difficulty in obtaining successful transplants between unrelated donors has only been appreciated in recent years. The problem lies in the inherent diversity of the HLA system. Current models, based on protein chemistry and molecular genetic techniques, include not only multiple alleles but also many more gene products or loci than were previously thought to exist. The HLA system is currently divided into two categories:

1) Class I proteins which are detected by conventional serology, and;
2) Class II proteins which can be detected by a combination of serological and cellular techniques.

Orr et al. have shown by gene cloning techniques that multiple copies of human Class I genes are encoded within each haplotype. A similar situation exists for the HLA-D region which specifies the Class II proteins. Based on protein chemistry and gene cloning data, the D-region has been subdivided into at least four subregions (DR/D, DS/MB/DC, SB, BR/MT). Each subregion encodes unique molecules which are composed of a combination of two polymorphic proteins termed alpha and beta. There may be several alpha and beta chain genes encoded

within each subregion and even more complexity is postulated since a single alpha chain may combine with several different beta chains. Although alpha and beta combinations may not be completely random, they do add another layer of complexity to a genetic system already difficult to elucidate. The following schematic attempts to represent our current understanding of the human major histocompatibility complex:



In addition to controlling graft rejection, matching for Class II antigens also seems to be required for maintaining immunocompetency (i.e., Class II identity is required in order for the regulatory cells of the immune system to interact). Consequently, a lack of Class II matched responding and presenting cells may leave an individual at risk of severe infection as well as GvHD. However, now that the HLA-D region can be subdivided into at least four subregions (i.e., SB, MB/DC/DS, DR, BR/MT), an obvious question is whether or not different D-subregions control different immunological responses such as GvHD and normal immunocompetency. Our ability to identify and functionally test gene products which are needed for normal responsiveness is a potential method of determining which of the many gene products are critical for successful transplantation and long term clinical success.

In order to study HLA proteins two criteria obtain:

1) There must be a way to isolate and characterize the proteins chemically.

2) There must be a way to assign some relevant immunological function to them.

We are using two approaches to meet these criteria: cellular immunology and immunochemistry.

III. OBJECTIVES

Our concept of this research project is embodied in three basic questions:

1) How many D-region gene products are there and which of these products are critical in clinical transplantation?
2) How do D-region encoded molecules control immune responses in transplant recipients?
3) Are specific immune functions regulated by different HLA-D subregions?

A. Basis of Approach

HLA proteins and immunological functions can be studied using cellular methodologies. Fortunately for immunologists, it is the Class II HLA proteins that cause the lymphocytes from one individual, when combined with lymphocytes from another individual, to proliferate rapidly. It is possible to detect this proliferation in tissue culture systems and by controlling certain parameters, to glean information regarding the HLA-D region genetics of the two lymphocyte donors. Importantly, understanding the recognition of D-region proteins (called alloantigens) by cells of the immune system is a fundamental prerequisite to understanding immune regulation. Using this cellular model we can ascertain which D-region proteins are important for graft acceptance and rejection or which molecules serve as a stimulus for GvHD. Additionally, other questions can be asked such as: Do certain proteins cause attenuation of immune responses (suppression)? Are there others that might amplify (help) an ongoing response? We can begin to answer these questions by using monoclonal antibodies generated for immunochemical studies to selectively inhibit those cellular immune responses that may be controlled by a given D-region protein. Because cellular interactions involve recognition of sites on the molecule near or identical to the binding sites (epitopes) recognized by antibodies, these approaches will enable the creation of a functional map of Class II proteins.

Class II antigens can also be studied using biochemical approaches because D-region molecules can be isolated using appropriate antibodies. By analyzing the amino acid sequences of these isolated molecules, one can "count" the number of genes from which these proteins are derived. The protein sequence of each HLA-D molecule characterizes the molecule and gives us information concerning the function of the protein. Furthermore, the protein sequences of the numerous molecules can be compared among individuals to give us an idea of the amount and sites of variability in the HLA-D molecules. It is important to note that this approach is highly dependent on the effectiveness of the antibodies at recognizing amino acid variations in the HLA-D molecules. It is the amino acid sequence that primarily contributes to or dictates the 3-dimensional (tertiary) structure of the native protein and thus the binding sites (epitopes) recognized by antibodies. Obviously the most effective and ideal situation is one in which a single antibody (a monoclonal antibody) recognizes (binds to) a single protein epitope. Although each protein contains many different epitopes, it is the composite set of epitopes that distinguishes one protein from another. Once the molecule reactive with each monoclonal antibody is characterized, the antibody can be used to probe specific immune functions and to identify cells bearing those molecules. The cells and antibodies are then useful tools for studying immunological functions such as immune competance, graft rejection, and GvHD.

B. Specific Objectives of the Previous Contract Year

1. Stimulator cell bank

Maintain and update the large panel of HLA typed cells stored in liquid nitrogen at Georgetown.

2. PLT typing

Develop primed lymphocyte typing (PLT) reagents to replace homozygous typing cells.

3. TLC typing

Establish and characterize a panel of human alloreactive T-lymphocyte clones (TLC's) to detect epitopes on Class II antigens.

4. Functional T-cell clones

Further define those Class II antigens that function as restriction elements for TLC's that respond to influenza virus.

5. Inhibition studies with monoclonal antibodies

Characterize the ability of anti-Class II monoclonal antibodies to block clonal proliferation.

6. Retrospective bone marrow transplantation studies

Collaborate with transplant groups at the Naval Medical Research Instutute and Walter Reed Army Medical Center by providing consultative services and genetic characterization as needed.

7. Prospective transplant studies

Continue development of protocols for providing "state-of-the-art" tissue typing facilities for Naval transplant programs.

8. Protein chemistry

Begin a program aimed at the immunochemical characterization of human Class II antigens.

9. Molecular genetics

Begin to develop approaches for studying the structure and function of Class II molecules using molecular genetic techniques.

IV. PROGRESS REPORT

The following documents the progress made on the contract research in view of the above stated objectives.

A. Stimulator Cell Bank

The Georgetown laboratory now maintains in a liquid nitrogen storage facility one of the largest, most completely characterized panels of peripheral blood lymphocytes in the world. This panel is maintained and updated continuously, and materials are replenished as required. Maintenance is facilitated by a computer based software system for inventory control and retrieval of genetic information. Both cells and software have been made available to NMRI investigators and other collaborating research groups.

B. PLT Typing

In an effort to decrease the time requirement for cellular typing results and to improve the resolution of cellular specificities, we have begun the process of converting from HTC typing to PLT methodologies (for the justification see Proposal for Continuation, N000-14-77-C-0747, 6 December 1982). This is a laborious process involving the generation of a reagent and then the confirmation of its specificity by panel typing. Over the last contract period we have generated 16 PLT reagents as follows: 3 PLT's recognize DR/D associated antigens (one for Dw1, one for Dw2 and one for DB3, a DR4 associated split); 5 PLT's recognize SB related determinants (one for SB6 and 4 which recognize new antigens associated with the SB allelic series); 8 PLT's recognize apparently new D-region associated antigens. Our effort continues in further characterizing these reagents as well as developing and generating new typing cells.

This laboratory was a central contributor to the Ninth International Histocompatibility Workshop. Ms. Fu-Meei Robbins organized the expansion of 14 PLT cell lines recognizing the 6 SB alloantigens plus two PHA control cell lines that recognize most D-region specificities. Along with the expanded PLT cell lines for the International Workshop, we also provided 4 reference PBL's expressing SB 1-6. In addition to aiding the workshop participants, Ms. Robbins efforts have enabled 9 other labs worldwide to develop SB typing protocols.

Our laboratory also participated in the Workshop by PLT typing 83 sets of PBL's with 49 PLT reagents including those specific for D/DR-associated, SB-associated and unknown or new D-region antigens. The results of these studies will be presented in Vienna, Austria at the International Workshop during May 1984.

In collaboration with Dr. A.H. Johnson of Georgetown University, we have been involved with developing serological methods for detecting the SB series antigens. Since SB antigens currently are detected by cellular means, the confirmation of correlations between serospecificities and cellular specificities is an important aspect of this work. Therefore, we have been attempting to block SB recognition by SB-specific PLT reagents using reputed SB-specific alloantisera. These studies are ongoing, they will continue and should provide an improved method for detecting SB antigens.

C. TLC Typing

Major progress has been made in the direction of developing T-lymphocyte clone (TLC) typing using allospecific TLC's that recognize D-region antigens. Ms. Sandra Rosen Bronson, under the direction of Dr. Eckels, has generated over 200 allospecific TLC's and screened them on small panels of cells expressing

Dw1-Dw8. This panel of exquisitely specific cellular typing reagents will be used to type 200-300 individuals from the Georgetown cell bank. From the data obtained, Ms. Bronson intends to construct a genetic model of the HLA-D region. It should be emphasized at this point that this accomplishment is an important milestone in the course of the contract: such a wealth of TLC reagents will enable significant progress in other areas of the contracted research (e.g., biochemical and functional studies, below).

From our previous work we knew that PLT cells detected multiple specificities due to their oligoclonal nature. This has been born out by subcloning studies. We have subcloned one of the SB1-specific PLT cell lines mentioned above. In analyses of extended panels of SB1+ cells, heterogeneity within the SB series has been found analogous to that which we originally described for the D/DR subregion. Furthermore, there is preliminary evidence for the differential expression of these SB1-associated antigens on the cell surface. Studies are ongoing to determine the functional and structural ramifications of our observations.

Additionally, two new TLC's have been generated against the DR4-associated LD40 specificity and several others are specific for DR4-associated but undefined antigens. We are currently attempting to characterize these clones further.

D. Functional T-cell Clones

We continue to provide well-characterized panel cells for studies on the genetic restriction of anti-influenza responses. We also continue to discover new restriction specificities such as SB and other D-region associated determinants, some of which are closely related to known alloantigenic specificities, others are not. Such efforts augment our other genetic studies

and provide an important tie-in with questions related to the immunocompetency of bone marrow transplant recipients.

E. Inhibition Studies with Monoclonal Antibodies

Murine monoclonal antibodies (MoAb's) raised against human class II molecules tend to recognize framework determinants. Framework determinants are invariant among different individuals, but to some extent can define differences among antigenic subgroups (e.g., SB, DR, DS, BR). It was our hope that we could use MoAb's as probes of TLC specificity by using the antibody to block clonal recognition of alloantigens. While some progress has been made, there are a number of technical considerations that complicate the results. We can no longer assume that if a MoAb blocks a given TLC that the two reagents necessarily recognize the same determinant or epitope. Failure to block is also subject to a number of technical constraints (e.g., affinity, avidity, binding at 37^{o}C). Therefore, Dr. Eckels has been appointed by the American Society of Histocompatibility and Immunogenetics to chair a subcommittee on the structural and functional relationships of human class II MHC molecules. One of the tasks of this subcommittee will be to organize a workshop wherein some of the technical concerns regarding MoAb blocking studies can be addressed. The subcommittee was formed in January of 1984 and its work will be ongoing.

F. Transplantion Studies

We continue to collaborate with clinical groups at the Naval Hospital, Bethesda and the Walter Reed Army Medical Center who are performing renal tranplants and are referring patients from other facilities for clinical bone marrow transplantation. As these programs develop, we will be able to provide support at the level of research tissue typing with cellular (PLT and TLC)

reagents. We will also be able to provide reagents and protocol development for prospective studies.

G. Protein Chemistry

Dr. Carolyn Hurley has joined the group as of 15 December 1983 and has been developing a protein chemistry laboratory to study D-region, class II antigens. Spaces have been reconfigured for this purpose and equipment emplaced. Two high performance liquid chromatography units and a peptide sequencer have been purchased. One HPLC is currently operational as are standard chromatography and electrphoresis equipment and a facility to grow cell lines. The remaining HPLC and peptide sequencer will be in place by approximately June of this year.

H. Molecular Genetics

Collaborations have been established with Dr. M. Robinson and Dr. E.O. Long of the Laboratory of Immunogenetics, NIAID, Bethesda for the purpose of molecular genetic characterizations of the Georgetown cell bank as well as commencing gene transfer (transfection) studies. By transfecting cell lines with specific human class II genes, we hope to augment our efforts at identifying functionally important D-region molecules and the portions of these molecules which may be recognized during cellular interactions.

V. PROPOSAL FOR CONTINUATION

The primary goal of this contract is to facilitate cellular typing of substantial numbers of unrelated individuals from any ethnic background who might require bone marrow transplantation. HLA typing is essential in these instances in order to avoid graft versus host disease (GvHD), a situation in which the recipient is rejected by the immunocompetent cells in the grafted bone marrow. When the D-region was thought to be simply comprised of one or two determinants or loci (HLA-D and HLA-DR), straightforward tissue matching of individuals seemed appropriate. However, as this simple genetic model of HLA has proven incorrect, the original strategies for matching donors and recipients are probably inadequate as well. This problem may be somewhat alleviated by the fact that there may be clusters of genes (e.g., D/DR and DS/MB/DC) which contain several components in linkage disequilibrium. That is, although many genes may encode HLA-D region products, certain sets of these genes usually will be found together within any one population of common ancestral origin (e.g., Northern European Caucasians). However, these gene clusters probably vary to great extent between different anthropologic groups. In addition, it is likely that each of the many HLA-D region gene products is not of equal importance in the generation of GvHD. Detailed studies of clinical transplants between unrelated individuals is necessary to evaluate the risks of each mismatch. Therefore, in order to maintain the high level of inquiry targeted by this contract, some new approaches are being developed around a much more complex theoretical framework.

A. Cellular Approaches

1. Stimulator cell panels. A major strength of the Georgetown facility is the availability of a large panel of HLA phenotyped stimulator cells.

Genetic information is available here on more than 1000 individuals including 16 highly selected large families which include 12 HLA recombinant individuals. Many of these cells have been characterized in the International Workshops. Significant effort is involved in maintaining and updating information in such a cell bank and individuals, including family members, must be bled continually. As some of our families are not located in the Washington metropolitan area, arrangements must be made to bleed donors in their local communities. Organizational efforts are made easier by a computerized inventory of all cells stored in our liquid nitrogen freezers and also a data base containing all current genetic information on each donor can be accessed by the computer. We will continue to improve our cell panel, replenishing cells that have been depleted by experimentation as well as testing and characterizing cells from new donors. As new software innovations become available we will upgrade the computer inventory system. Frozen lymphocytes from minority groups will continue to be collected and characterized for HLA-D region antigens. A well defined reference panel is especially important for cross-indexing the results from the PLT, TLC, MoAb inhibition and immunochemical studies in progress here at Georgetown.

2. PLT typing. "HLA-D" usually was defined in a classical mixed leukocyte culture (MLC). It seems likely from data obtained in many different laboratories, including our own, that the response to "HLA-D" is really a composite response against several different D-region epitopes. That is, MLC typing with homozygous typing cells (HTC's) detects haplotypes or multiple epitopes on several gene products. Furthermore, the approach using HTC's is fraught with technical difficulties especially when attempting to separate positive and negative responses on unrelated panels. The MLC also requires 7-8

days before an answer can be obtained. Therefore, we will continue our efforts at converting from HTC to PLT typing for D-region antigens. The reasons for this conversion, in addition to those above, are manifold: 1) PLT reagents generally allow more reliable discrimination of positive and negative responses even with panels of unrelated stimulator cells; 2) the time factor is reduced by half, and; 3) finer resolution of specificity is obtained because, PLT cells can be selected to recognize subtle D/DR/DS/SB differences. The approach to be used is similar to that described in previous Technical Reports. Initially, cells will be primed to HTC's recognized by the World Health Organization as expressing each of the core of internationally accepted D/DR specificities. Second, reagents directed against separate HLA-D,DR, DS and SB specificities will be generated in cell combinations where the responder and stimulator differ only by a particular subspecificity. For example a Dw4,4;DR4,4;SB4,4 cell would be primed against irradiated cells expressing Dw4,10;DR4,4; SB4,4; since Dw10 is the only recognized antigenic disparity, the resulting PLT reagent should recognize the Dw10 cluster of specificities.

3. TLC typing. Clones of alloreactive T-cells are currently the most discriminating cellular reagents available. However, because TLC's recognize antigenic determinants that may be shared among distinct species of Class II molecules and also because a single Class II alpha/beta pair of molecules may have multiple determinants, the use of TLC's in routine tissue typing needs to be carefully considered. The reason for this is that the D-region probably encodes scores (perhaps hundreds) of distinct epitopes; an individual's tissue type then would correspond to a cluster of molecules each characterized by a series of unique epitopes. A particular set of epitopes, each recognized by a distinct group of TLC's could define the DR1 specificity, for example. The

approach necesary to establish tissue typing with TLC's is essentially the same that we have used all along. However, the results obtained will be interpreted differently. Approximately 200 alloreactive TLC's that have been generated against cells expressing Dw1-Dw8 will be screened and tested on the cell bank panel, but the analysis will be conducted differently. Instead of grouping TLC responses with known D-region phenotypes, TLC responses will be clustered among themselves. We expect that some TLC clusters will correspond with or split known specificities. Others may appear broad or supertypic. Once a cluster was defined in the population, attempts at dissecting out the different epitopes could be made using monoclonal antibodies to inhibit TLC responses (as described below). This constitutes an enormous effort. In this regard, Ms. Sandra L. Rosen, a pre-doctoral graduate student under the direction of Dr. Eckels, has already generated the necessary clones and will formulate a D-region genetic model for her thesis work. This work will provide an essential foundation for further investigation into the structural and functional significance of human Class II molecules. Furthermore, as uncloned PLT reagents probably define some alloantigenic clusters of epitopes, to a limited degree it will be important to subclone the PLT reagents generated under other segments of this contract and to analyze these TLC's for their spectrum of alloreactivity.

This year we will also initiate studies combining cellular and immunochemical techniques for differentiating DR from D. Using serologic typing reagents to measure polymorphism, 10 alleles of HLA-DR have been identified (DR(w)1-10). Another means of assessing HLA-D region complexity has been through the use of cellular reagents such as homozygous typing cells. These cellular reagents detect determinants called HLA-D(w) via mixed leukocyte reactions (MLR) . While, in general, there is a good correlation between DR

and D typing (i.e., most DR2 individuals are Dw2), these cellular reagents have detected complexity additional to that observed by serotyping. For example, five HLA-D specificities have now been associated with DR4 and at least three HLA-D specificities have been associated with DR2. There are two alternatives for this D-DR variability: 1) DR and D specificities reside on different molecules or; 2) D and DR specificities reside on the same molecule as different epitopes. The latter means that among different individuals there may be several different DR molecules, all carrying the same DR specificity but different Dw specificities. Biochemical data on the DR4 disparity by Bach and Watson suggest that there are different DR molecules associated with each D type; however, this study did not identify the specific differences nor did it analyze the other HLA-D molecules like SB and DS for variability as well. We will examine whether the DR specificity resides on the same molecule as that expressing HLA-D or whether discrete molecules are involved. Distinct TLC's that recognize DR2, Dw2, Dw12 and TB24 will be generated and used to characterize antigen expression on homozygous B-lymphoblastoid cell lines. Parallel immunochemical studies by Dr. Hurley will define sequence differences in the D region molecules expressed by these LCL's.

4. Functional T-cell clones. We are currently, and will continue to be, involved in studying the genetic restrictions of T-cell clones reactive to nominal antigens. It will be particularly important to correlate genetic restriction specificities with the specificities detected by alloreactive TLC's. Furthermore, in antigen specific systems it may be possible to learn more about the immunological functions of various D-region molecules and therefore we will provide genetically well-characterized antigen-presenting cells for restriction studies.

Since one goal of bone marrow transplantation is immune reconstitution, analyses of antigen-specific clones are viewed as fundamental prerequisites for determining which Class II molecules are absolutely essential for normal immunological function and which molecular structures induce undesirable effects in transplant recipients (e.g., GvHD).

5. Inhibition studies with monoclonal antibodies. We will continue to use MoAb's to probe the epitope specificity of TLC's. We have already demonstrated the potential of this approach and will continue to obtain and utilize well-characterized reagents to block TLC proliferation.

B. Biochemical Approaches

Since one of the major goals of the Navy bone marrow transplant program is the use of donor marrow from unrelated individuals, it is critical to precisely and rapidly identify those antigenic determinants which stimulate allogeneic responses. These allogeneic responses are responsible in large measure for transplantation failure due to the recognition of foreign transplant antigens Under this contract, increasingly sophisticated methods have been developed to detect these transplant antigen differences using cellular techniques (e.g., mixed leukocyte culture utilizing homozygous typing cells, primed lymphocyte typing and T-cell cloning). Such cellular techniques have been able to detect small alloantigenic differences but additional information is required to: 1) determine the molecular basis of both cellular and serologic observations and; 2) to initiate the development of more rapid methods of detecting significant allogeneic differences. Biochemical techniques must be used in conjunction with cellular and serologic identification methods as well as studies of immune function to determine which molecular differences are relevant to clinical

transplantation. We would like to be able to identify a relatively small number of critical molecular differences among the general population which will permit us to select marrow donors and eventually to select stem cells with certain antigenic characteristics (being developed as part of the in-house Navy program).

This proposed study will focus on the biochemical dissection of the DR-D difference observed in DR2+ cells and will interface with cellular studies using the same materials (see above). Studies of the D-DR polymorphism will aid in analyzing this additional polymorphism and will help identify those molecules important in HLA-D typing and will allow more accurate typing for clinical bone marrow transplantation.

DR2 homozygous cell lines will be obtained that express different Dw specificities. These lines are already in existence and can be readily obtained. These cells will be characterized for all known D-region specificities, DR, D, MB, MT and SB by TLC, PLT and MLR typing using the Georgetown facilities. This will alert us to any possible differences at other loci which could complicate interpretation of the data. The cell lines will be grown in the presence of radiolabeled amino acids to biosynthetically label all of the HLA-D region molecules. Following detergent lysis and isolation of a glycoprotein pool, preparations will be passed over a variety of monoclonal antibody affinity columns to isolate DR, DS (MB, MT) and possibly SB molecules. Monoclonal antibodies with specificity for these D region molecules are already on hand in the investigator's laboratory. Examples of monoclonal antibodies that will be used are 1) L203 (anti-DR), 2) Genox (anti-MB1/MT1), 3) Tu22 (anti-DS), 4) IVA12 (anti-DR,DS,SB), and 5) I-LR1 (anti-SB2,3). Initially, molecules will be compared using high performance liquid chromatography (HPLC) peptide mapping. In this way, DR molecules from a Dw2

line will be compared to DR molecules from a Dw12 line; DS(Dw2) to DS(Dw12) and so forth. This will determine if DR, DS (or SB) molecules from the two cell lines differ. At least two different radiolabeled amino acids and two different enzymes will be used to analyze variability in this way. Once variability is observed, the variable molecules will be isolated and the position of variability determined. To do this, variant peptides will be isolated using the HPLC and analyzed by amino acid sequencing to identify the region within the molecule that is variable. This should aid in defining the HLA-D determinants. Several Dw2, Dw12 and TB24 cell lines will be analyzed in this way. If other DR2-associated D specificities are found, cell lines with those specificities will also be analyzed.

C. Serological Approaches

Under this contract cellular methods have been used to define HLA-D region (class II) specificities. Results to date have shown that many more specificities have been found than were first expected. In addition, newly developed T cell clones have been utilized to uncover multiple specificities and reactivities. By employing these cell-mediated immune assays, we can now recognize and define many different HLA-D region molecules important in transplantation.

Currently, we are advancing along two avenues of investigation: 1) to determine and classify new class II specificities and; 2) to examine the protein structure of these molecules. An area which we are proposing to investigate is that of developing human-human monoclonal antibodies with specificity for class II molecules. Currently, monoclonal antibodies are made by immunizing a mouse or rat with human B-cells expressing the HLA-D molecules of interest. Following immunization, the rodent splenic B-cells which are

secreting antibodies directed against HLA molecules are isolated and fused with a mouse myeloma cell line. The vast majority of these rat or mouse-derived monoclonal antibodies to human class I & II antigens are directed primarily against framework specificities. One can, therefore, use these antibodies to distinguish DR from DS but not, for example, DR4 from DR5. It is likely that subtle HLA-D allelic differences are generally not recognized in rodent immunizations because of stronger and more immunodominant antigenic disparities between the species. One clear solution to the development of monoclonal antibodies with allospecificities for human class II molecules lies in the advancement of human-human monoclonal antibody technology. Such allospecific antibodies would be useful in identifying class II alloantigens and could also be used in isolating HLA-D molecules for bichemical characterization.

There have been two major drawbacks in regards to human-human fusions: 1) no source of stable, drug-sensitive, human myeloma partners capable of secreting milligram levels of antibody and; 2) the low numbers of antigen specific (in this case, HLA-D specific) antibody producing cells. Recently, investigators have developed human myeloma lines which fuse with high frequency and produce relatively high quantities of immunoglobulin. One such line, HFB-1, has been developed in a cooperative effort between the Naval Medical Research Institute and Georgetown.

The intended goal of this research effort will be to utilize these new myeloma partners in producing human-human monoclonal antibodies with specificity for human class II molecules. Antigen-specific, antibody-producing cells will be derived from hyperimmunized human spleen cells. These cells are obtained from a patient who underwent multiple renal transplants and rejection due to the production of very high levels of anti-HLA antibodies (including a high level of anti-DR2). We plan to preselect enriched subpopulations of

antigen-reactive cells by in vitro allogeneic restimulation and/or alloantigen specific B cell "panning" prior to fusion. Screening for specific hybridomas will be performed by first incubating Ig-secreting cells in the presence of tritium labeled amino acids and then looking for radiolabeling of test cells which express appropriate HLA specificities. Confirmation of specificity will be performed using the fluorescence activated cell sorter, ELISA or radioimmunoassay using activated T cells which express class II molecules but not surface immunoglobulin and by cytotoxicity against B-cell panels.

The advantages and uses of such antibodies are many and varied. They can be used to identify new class II specificities. Alloantigenic determinants could be localized and biochemical and molecular genetic appreaches facilitated. Monoclonal antibodies could replace typing sera thus eliminating many of the artifacts such as autoantibodies and the cumbersome complement mediated cytotoxicity typing method. These monoclonal antibodies should also provide a more reliable and standardized source of antibody than typing sera. In addition, human monoclonal antibodies would greatly augment or possibly replace cellular typing assays for class II antigens. Finally, because these monoclonal antibodies would be from humans, they could be administered as passive treatment for or prevention of GvHD in HLA mismatched transplants thus creating a potential therapeutic product for transplant recipients.

D. Clinical Approaches

As new D-region epitopes are defined their clinical relevancy must be established. One avenue of approach will be to collaborate with clinical groups performing bone marrow and kidney transplantation. We are currently collaborating with transplant groups at the Naval Medical Research Institute and Walter Reed Army Medical Center. Particularly interesting will be the

typing for HLA-D subregion differences in donor-recipient combinations where HLA-D matching nevertheless failed to insure graft retention or prevent severe GvHD.

E. Collaborations

1. We continue to provide HLA phenotyped cells to ONR scientists and contract facilities and make available cellular reagents of interest to various laboratories. We will also continue to provide tissue typing expertise to investigators at the Naval Hospital, Bethesda and Walter Reed Army Medical Center as they seek to establish stronger bone marrow and organ transplantation programs. As the Navy bone marrow transplantation program is developed, the Georgetown laboratory can provide typing reagents to define HLA-D region gene products. Thus, research quality typing is available to Naval clinical investigators and information can be accumulated on the effect of various HLA mismatches on the success of transplantation and immune reconstitution.

2. It is now possible to isolate the genes that encode human Class II molecules. In the near future direct genotyping of D-region genes at the DNA level will become possible. Furthermore, as genes can be isolated, packaged into viral vectors, transmitted and "turned on" in non-expressing host cells it will be possible to study the function of isolated gene products for the first time. A collaborative link-up has been initiated between our current T-cell cloning group and the recombinant DNA laboratory of Dr. Eric Long in the Immunogenetics Laboratory, NIAID.

3. Dr. Eckels has been appointed as chairman of the ASHI subcommittee on structural and functional relationships of human Class II MHC molecules. There are three tasks for the committee to accomplish:

1) Develop a core panel of 30 HTC LCL's expressing HLA-Dw1 through Dw10.
2) Define a set of monoclonal antibodies that recognize all the major subclasses of D-region antigens (e.g., D/DR, BR/MT, DS/DC/MB, SB).
3) Organize a workshop in which the monoclonal antibodies are used to block TLC function as well as immunochemically characterize class II molecules immunoprecipitated from the LCL panel.

This effort will hopefully lead to a better understanding of the function and specificity of D-region antigens as well as serving to clarify and help coordinate discussion and understanding of HLA-D region genetics among different laboratories.

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