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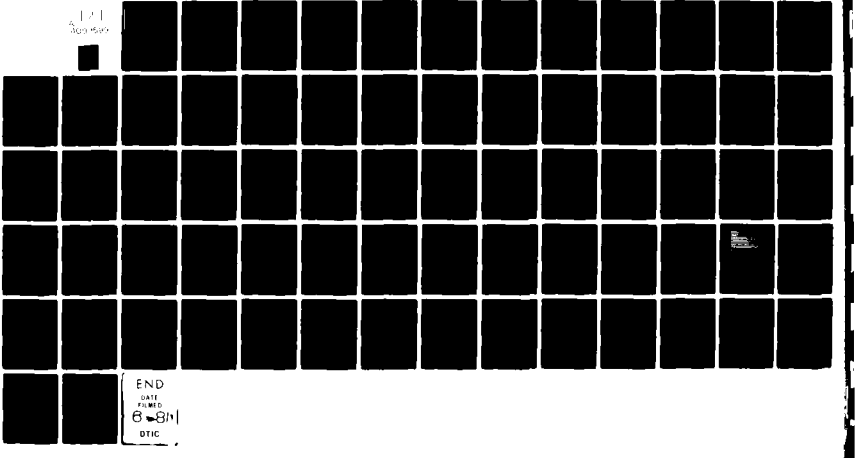

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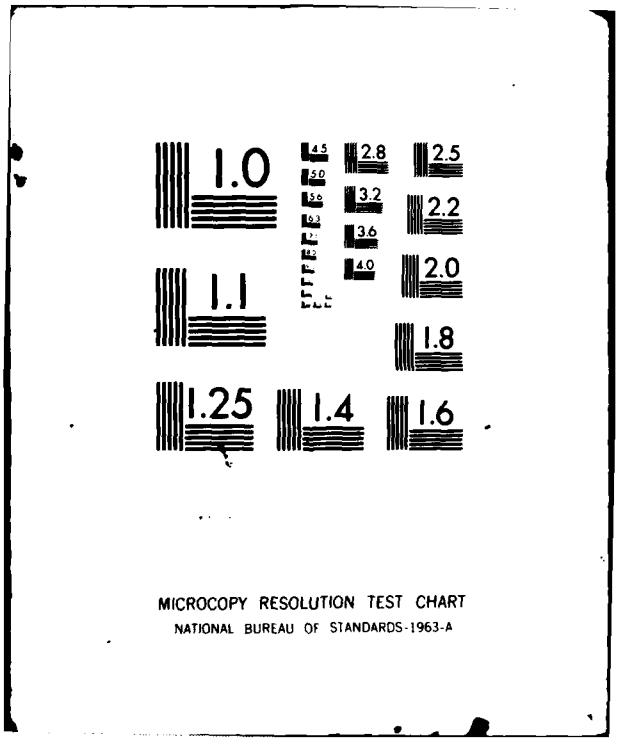
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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: Technical Report No. 4	2. GOVT ACCESSION NO.: AD-A099 599
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle): Histocompatibility Typing	5. TYPE OF REPORT & PERIOD COVERED: Interim (annual)
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): David D. Eckels, Ph.D.	8. CONTRACT OR GRANT NUMBER(s): N00014-77-C-0747
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Immunologic Oncology Division, Dept. of Pediatrics, Georgetown University School of Medicine, 3900 Reservoir Rd., NW, Washington, D.C. 20007	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: Task No. NR 207-101
11. CONTROLLING OFFICE NAME AND ADDRESS: Office of Naval Research, Biophysics (Code 44), 800 N. Quincy Street, Arlington, VA 22217	12. REPORT DATE: Apr[illegible] 81
	13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office): TR-4	15. SECURITY CLASS. (of this report)
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

See enclosed list

DISTRIBUTION STATEMENT A
Approved for public release;
Distribution Unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Histocompatibility typing, LD typing, bone marrow transplantation, homozygous cells, HLA-D, human histocompatibility, mixed lymphocyte culture, primed lymphocyte test, T-lymphocyte clones

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

During the last contract year, our laboratory has made significant progress in understanding the genetics of the human major histocompatibility complex (HLA). Ten new homozygous typing cells (HTCs) were identified specific for known HLA-D region antigens. For each cell stored in our liquid nitrogen freezers, complete personal and genetic information is now maintained in a computer data bank. This facilitates easy access and inventory control. In collaboration with the Naval Medical Research Institute, a system using

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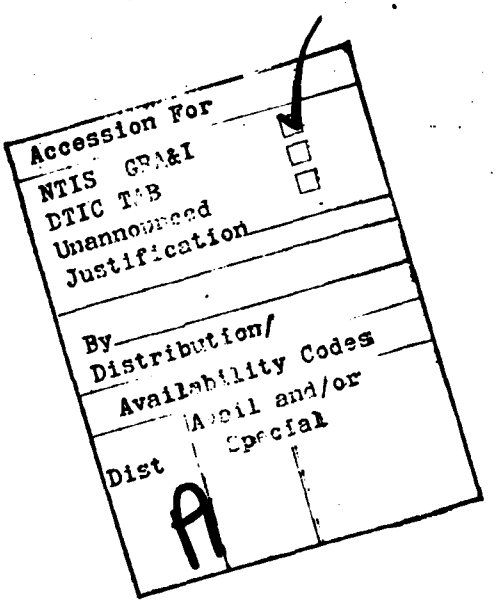
PHA-stimulated homozygous typing (PLT) cells has been developed for rapid transplant matching. The technology for T-lymphocyte cloning has been extended and utilized to begin analysis of D-region genetics in detail. This important technique has already begun to yield results which suggest the presence of D-region complexities derived from previously undefined specificities and new gene products. We have provisionally designated one such new locus "TLC-A" (T-lymphocyte clone-A) as the first of what may prove to be a long series of different loci linked to HLA genes. Many of these data were presented formally and informally during the Seventh Annual meeting of the American Association for Clinical Histocompatibility Testing.



OFFICE OF NAVAL RESEARCH

Contract N000-14-77-C-0747

Task No. NR 207-101

TECHNICAL REPORT NO. 4

HISTOCOMPATIBILITY TYPING

By

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27 April 1981

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A. ABSTRACT

During the last contract year, our laboratory has made significant progress in understanding the genetics of the human major histocompatibility complex (HLA). Ten new homozygous typing cells (HTCs) were identified, specific for known HLA-D region antigens. For each cell stored in our liquid nitrogen freezers, complete personal and genetic information is now maintained in a computer data bank. This facilitates easy access and inventory control. In collaboration with the Naval Medical Research Institute, a system using PHA-stimulated homozygous typing cells has been developed for rapid transplant matching. The technology for T-lymphocyte cloning has been extended and utilized to begin analysis of D-region genetics in detail. This important technique has already *begun* to yield results which suggest the presence of D-region complexities derived from previously undefined specificities and new gene products. We have provisionally designated one such new locus "TLC-A" (T-lymphocyte clone-A) as the first of what may prove to be a long series of different loci linked to HLA genes. Many of these data were presented formally and informally during the Seventh Annual meeting of the American Association for Clinical Histocompatibility Testing.

B. INTRODUCTION

The following technical report summarizes the fourth year's progress on ONR contract N000-14-77-C-0747 "Histocompatibility Typing". This contract was initiated on September 15, 1977 for the purpose of developing HLA-D histocompatibility testing to permit the potential availability of non family member bone marrow transplant donors, and to develop rapid and accurate methods for HLA-D region typing.

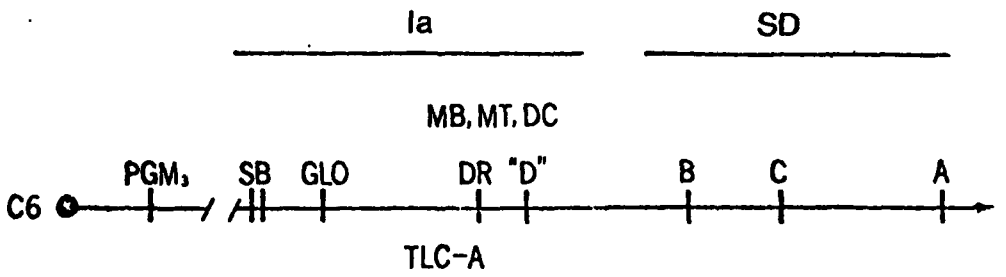
C. BACKGROUND

Military personnel working in hazardous areas are at far greater risk of contact with bone marrow toxic agents than the civilian population. The use of various toxic fuels, chemicals and radiation sources are hazards which can induce aplastic anemia and require the availability of bone marrow transplantation for treatment. Treatment of aplastic anemia by bone marrow transplantation is currently funded in civilian institutions by health insurance plans. Although transplantation is an accepted form of treatment, the only long term survivors receiving this therapy have occurred when HLA identical siblings have been used as the marrrow donor. Because only one third of the potential transplant recipients have matched sibling donors, it is necessary to identify methods of tissue matching which will allow the use of unrelated donors. Improved methods of tissue typing would then allow a much broader use of bone marrow transplantation. Furthermore, the identification of those specificities which must be closely matched in donor and recipient versus those specificities with little clinical significance will broaden the number of potential transplant donors.

It is clear that the success of transplants is greatest with the most compatible grafts, and is much less successful with poorly matched tissues. We have therefore initiated a program of tissue typing in collaboration with Navy researchers and other contract facilities to define the essential components of graft rejection and to seek ways to alter the body's natural rejection mechanisms.

At the present time, tissue typing is essential for all current transplant programs. However, it is clear that typing of histocompatibility antigens must be extended and further refined if it is to fulfill its potential as a major clinical tool. To outline the complexity of the HLA tissue typing system, it now appears that there are at least ten allelic series which control histocompatibility antigens on the human cell surface which have been called HLA-A, -B, -C, -D, -DR, MB, MT, DC, SB, and TLC-A and code for approximately 100 unique antigens. While some of these histocompatibility genes are responsible for the rejection or acceptance of tissue grafts, they may also be linked to loci which modulate immune responses. Of these, the HLA-D region loci (Figure 1) are the least understood; however, they seem to play the

Figure 1:



minant role in afferent control of the immune response. Therefore, an understanding of HLA-D region genetics is essential to understanding the antigenic criteria for graft acceptance and graft versus most disease. The research that we have initiated is directed toward the problem of examining this HLA-D genetic region, and cataloging the gene loci and allelic antigens in such a manner that individuals could be easily typed for the most important determinants.

D. RESEARCH DESIGN AND PLANS:

The initial plans for this contract were to follow a sequence of events:

1. Establishment of a contract facility with a laboratory capacity to study the human histocompatibility system and do routine mixed lymphocyte culture testing.

2. Preparation of a panel of homozygous typing cells (HTCs) capable of identifying the common HLA-D specificities.

3. Preparation of panels of primed lymphocyte typing (PLT) cells for use in confirming the results of initial HTC typing, and for use when rapid knowledge concerning an indivual's HLA-D type is essential.

4. Cryopreserving and storing these reagents in such quantities that they would allow typing of a large numbers of individuals whenever necessary.

5. Identify and quantitate the relative role of each of the major histocompatibility complex genes on clinical tranplantation as it relates to graft and donor survival, graft versus host disease and reconstitution of normal immunologic function.

6. Collaborate with the Naval Medical Reseach Institute and other contract facilities in identifying the genes that control immunologic responses in humans.

E. RESULTS

1. Homozygous Typing Cell (HTC) Panels

a. New HTC specificities.

In a continued effort to collect homozygous typing cells which represent the core of internationally accepted reagents used for HLA-D region typing, thirty individuals, previously screened for homozygosity at HLA-A and -B, were tested in large matrix experiments. Preliminary data indicate that we have identified ten new HTCs specific for HLA-Dw 1,2,3,4,5,6,7(two), 9 and 10. With the acquisition of these reagents, we now maintain a complete panel of HTCs representing all currently accepted specificities as well as many in the "workshop" status.

b. Cell inventory.

A major effort has been directed at developing a complete computer inventory of the HTCs stored in liquid nitrogen at Georgetown. This has been accomplished using AIMS Automated Information Management Service), a data management program which runs on the Wang 2200 MVP minicomputer. As currently configured, the cell inventory can maintain information on up to several thousand cells. Data stored for each sample include donor identification, date of bleeding, number of vials, location, full HLA type, RBC and isoenzyme specificities as well as other genetically relevant information. The AIMS software enables the file to be sorted by any one of, or many, different parameters. For example, data on cells from a group of individuals expressing HLA-A1 could be easily specified, sorted and printed out. This capability greatly facilitates locating cells for large experiments as well as keeping track of when certain cells are being depleted. Sophisticated AIMS applications have been programmed by Elizabeth Phillips which enable rapid updating of the large cell inventory. This greatly reduces the amount of time required by various personnel for "bookkeeping" purposes.

2. Primed Lymphocyte Typing (PLT)

a. PLT cell lines

We have continued to develop PLT reagents and expand them utilizing cell growth factor (TCGF). We have previously demonstrated that such PLT

cell lines detect both HLA-D and HLA-DR antigenic determinants. Reagents for all of the known D and DR specificities have been generated and provided to the Naval Medical Research Institute and the Walter Reed Army Medical Center for transplantation studies. The results of these clinical trials are still pending.

b. Rapid tissue typing.

In collaboration with Dr. Robert Hartzman at the Tissue Bank of the Naval Medical Research Institute, we have participated in the development of PHA-PLTs. Such typing reagents are derived from PHA-stimulated HTCs grown up in continuous culture and enable very rapid typing for HLA-D region differences. Georgetown primarily provided cells, from an inventory of genetically characterized individuals, to be used in defining the specificity of the PHA-PLTs developed by Dr. Hartzman. Results from these experiments were presented at the Seventh Annual Meeting of the American Association for Clinical Histocompatibility Testing, held during March 1981 in Orlando, Florida. This technology offers the possibility of very rapid (12-24 hours) analysis of donor-recipient compatibility.

3. Cloning of Allospecific T-Lymphocytes

a. Background.

During the last year, a substantial amount of work has focused on the development and characterization of T-lymphocyte clones which are

capable of recognizing discrete HLA-D region specificities. This technology is now available here at Georgetown. In order to understand the significance of this capability, a brief background is necessary:

When this contract was first initiated, the human major histocompatibility complex (HLA region) was thought to contain relatively few discrete loci encoding cell surface glycoproteins (antigens) important to transplantation and immune responses. Even at this date, though the number of HLA loci has increased, detection of new loci, using conventional serologic and cellular techniques, has progressed slowly. This is probably due to the fact that conventional cellular reagents respond to alloantigens in a polyspecific or polyclonal fashion, detecting "immunodominant" molecules. The problem is that although such immunodominant components (e.g., HLA-D) are matched for, residual alloreactivity still remains. A clinical corollary may be found in the fact that even among unrelated HLA-D identical individuals, bone marrow recipients often undergo moderate to severe graft-versus-host (GVH) reactions. The clinical solution to this has been to transplant HLA identical siblings where the incidence of severe GVH disease is fairly low. However, even in this case, GVH reactions do occur.

In recognition of the problems with polyspecificity, an alternate approach has been to limit the range of antigenic determinants

recognized by the responding cell population. The ultimate extension of this, of course, is to clone cells recognizing individual antigenic determinants. This approach, though necessarily introducing greater numbers of different specificities, should enable precise matching of those HLA or other components required for enhanced graft survival. Furthermore, the identification of HLA-D subregion components will allow us to identify those which are most important in graft rejection and GVH disease and ultimately lead to more accurate and simpler typing.

Briefly, peripheral blood lymphocytes from one individual are primed against cells from another person. After four days, responsive cells are removed and cloned by limiting dilution. In order to insure the continued growth of responding cells, Interleukin-2 (IL2) and irradiated feeder cells are added. IL2 is a growth hormone which specifically supports the long-term growth of activated T-lymphocytes. By maintaining appropriate levels of IL2, feeders and cloned cells, cultures can be maintained for several months. Obviously, since optimal doubling times range from 24-48 hours, great numbers of cloned cells can be obtained by this method and used to determine the "fine specificity" of allogeneic cell-surface antigens.

b. Analysis of HLA-Dw1 using TLCs.

By selectively stimulating and cloning cells from certain individuals with cells expressing a particular HLA-D type, one should theoretically be able to obtain T-lymphocyte clones (TLCs) capable of recognizing discrete HLA-D region molecules. Using just such a protocol, we have investigated the response of TLCs generated against HLA-Dw1. When restimulated by a panel of cells expressing the known HLA-D specificities w1 through w10, TLCs could be grouped into several categories: a) TLCs which recognized cells expressing Dw1 plus "extra" cells which did not express Dw1; b) TLCs which reacted with only a subset of $Dw1^+$ cells; c) TLCs which did not react with any $Dw1^+$ cells, but nevertheless recognized a product encoded by a locus linked to HLA; d) TLCs restimulated by the original priming cells, but did not recognize antigens associated with HLA; and e) TLC which were not restimulated by the positive control, but which did react to other panel members. The most outstanding feature of these results was the fact that apparently no TLC, out of 58 tested, seemed to recognize the expected specificity exclusively (i.e., Dw1). That is, reactions were either too broad, too narrow or not correlated with HLA-D region genes. These observations have been verified in subsequent experiments and would suggest that HLA-D typing by conventional means (HTC, PLT or DR-serology) is not detecting a single antigenic entity but a composite of many different strong and weak antigenic determinants. These results were formally presented at the Seventh Annual Meeting of the American Association for Clinical Histocompatibility Testing during March 1980 in Orlando, Florida.

c. Identification of a new locus.

An important consequence of TLC typing will be the identification of new HLA-D region loci. Also presented at the Seventh Annual AACHT meeting was our preliminary evidence for a newlocus. From experiments described above, two TLCs were identified which reacted with the positive control (Dw1,3) but with no other unrelated panel member except a Dw5 HTC. Subsequent study in two families, each containing eight siblings, confirmed that these two clones, TLCs 14-14 and 14-86, recognized a previously unidentified antigenic determinant probably encoded by a new gene linked to HLA. Unfortunately, it has not been possible to map this new marker to a particular locus on chromosome #6 due to the lack of an informative recombinant family. Nevertheless, we have provisionally designated this locus as "TLC-A." Preliminary data from other experiments suggest the existence of many such new MHC-controlled determinants.

d. Kinetic analyses.

Kinetic studies have revealed interesting features of TLC function. It appears that each TLC, when restimulated by allogeneic cells, has a unique pattern of response: the time of peak response appears to be determined by the TLC; the level of response correlates with the antigen presenting cell. The earliest detectable proliferation appears at 12-24 hours, but some clones may peak as late as 120-140 hours. Once specificity questions are resolved, it should be possible to select for "rapid responding" TLCs for use in clinical transplant programs.

F. CONCLUSIONS FROM RESEARCH COMPLETED

Using many of the new techniques available to our laboratory we have been able to develop a sophisticated system for accurate histocompatibility typing and are pursuing research into further genetic parameters of transplant compatibility. Although the system is complex, it is likely that techniques can be developed for rapid large-scale typing which is essential for bone marrow transplantation.

G. PROPOSAL FOR THE CONTINUATION OF THIS CONTRACT

The initial success and yield of relevant typing data in the HLA field has encouraged us to continue this line of research.

1. Work Plan

a. Continued development of HTC and PLT typing system.

Under this contract we will continue to develop our capabilities to accurately identify and type random individuals. This will be accomplished by the screening of random donors in both the HTC and PLT systems, and the identification of new specificities. During the next contract year, particular emphasis will be given to typing minority groups in order to characterize "blank" specificities.

b. Cloning of PLT cells.

During the following year, we plan to expand our panel of cloned typing cells to include 20-40 TLCs each specific for HLA-Dw1, w2 or w3.

Using the ORTHO Fluorescence Activated Cell Sorter (FACS), the cloned PLT cells and their precursors will be evaluated by surface-antigenic phenotype, using a combination of monoclonal antibodies against human cell surface antigens and physical parameters. By selecting precursors on the FACS, it may be possible to develop useful, highly reactive clones in a much faster and less costly fashion.

c. Evaluation of TLC cytotoxic cells.

During the generation of T-lymphocyte clones, killer cells also arise in the mixture. It is possible that such clones may be useful for typing. Work will be done to characterize these cytotoxic TLCs.

d. Continued statistical approaches

Because clonal typing responses are qualitatively different from the MLC or PLT systems, continued effort is required to determine statistically appropriate analytical methods.

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5. Eckels D.D., Woody J.N., and Hartzman, R.J. Monoclonal and xenoantibodies specific for HLA-DR inhibit primary responses to HLA-D but fail to inhibit secondary proliferative (PLT) responses to allogeneic cells. Human Immunol. (submitted)

6. Eckels, D.D. and Hartzman, R.J. Does HLA-D exist? Human Immunology (submitted).

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I. ABSTRACTS

1. Eckels, D.D., Hartzman, R.J. Smoot, D., Robbins, F., hargrove, R. and Lionetti, T. Does HLA-D exist? Seventh Annual meeting of the American Association for Clinical Histocompatibility Testing, Orlando, Florida, 1981. (oral presentation).

2. Eckels, D.D., Hartzman, R.J., Smoot, O., Robbins, F., Hargrove, R. and Lionetti, T. Description of a new HLA-linked locus using cloned PLT cells. Seventh Annual meeting of the American Association for Clinical Histocompatibility Testing, Orlando, Florida, 1981. (oral presentation).

3. Robbins, F. and Hartzman, R.J. Phytohemmagglutinin stimulated human lymphocytes for rapid transplant matching. Seventh Annual meeting of the American Association for Clinical Histocompatibility Testing, Orlando, Florida, 1981.(oral presentation).

MONOCLONAL AND XENOANTIBODIES SPECIFIC FOR HLA-DR INHIBIT PRIMARY RESPONSES TO HLA-D BUT FAIL TO INHIBIT SECONDARY PROLIFERATIVE (PLT) RESPONSES TO ALLOGENEIC CELLS

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These results were presented in part during the Second International Conference on the Primed Lymphocyte, April 13-17, 1980 in Bethesda, Maryland.

ABSTRACT

Monoclonal antibodies (DA-2 and CA-2) and xenoantisera (rabbit anti-human, p23,30) specific for HLA-DR framework determinants were added to primary and secondary mixed lymphocyte cultures. Although such antisera were shown to inhibit primary MLC, primed lymphocytes were much less sensitive to the blocking effects of these antibodies. In the studies shown here, the concentration of antibody required to inhibit primary MLC reactions was 0.1-1.5μg/ml whereas that required to block the PLT reaction was approximately 50-fold greater (50-100μg/ml).

INTRODUCTION

When lymphocytes from unrelated individuals are mixed together *in vitro* a strong proliferative reaction generally occurs. This reactivity is controlled by lymphocyte cell surface glycoproteins, encoded by genes within the human major histocompatibility complex (HLA region) on chromosome #6, and in particular by products of the HLA-D sub-region. Current concepts regarding HLA-D envisage at least two detectable components: HLA-D, detected in mixed lymphocyte reactions (MLR); HLA-DR, detected serologically using alloantisera from multiparae. Some controversy exists regarding whether or not the HLA-D and HLA-DR "determinants" are on the same molecule and also how much either or both contribute to *primary* lymphoproliferative responses.

Primed lymphocytes can be generated by mixing cells from individuals different for the HLA-D region as in primary MLC and allowing the reaction to proceed past the peak of proliferation. The remaining memory cells, upon subsequent rechallenge with the original stimulating cells, will undergo a vigorous secondary proliferative response (1). Similarly, cells which share antigens with the original stimulator will also induce strong secondary proliferation. Most evidence suggests that such primed lymphocyte typing (PLT) responses detect *both* HLA-D and HLA-DR antigenic components (2-4). Additionally, other antigens which restimulate in PLT have been detected (5-9).

Descriptions of recombination between "genes" encoding D and DR have been reported (10). Recombination has generally been considered formal proof of discrete loci, however, the data are technically explainable in terms of intracistronic cross-overs. In contrast, blocking experiments using anti-B-cell alloantisera have demonstrated inhibition of primary and secondary MLC (11,12) and have been interpreted as evidence that D and DR reside on the same

molecule or physically associated molecules. Although a serum component which specifically binds HLA-D has not been reported, the presence of such a factor in alloantisera would render this interpretation uncertain.

We have investigated whether monoclonal and xenoantibodies specific for HLA-DR can inhibit in vitro primary or secondary lymphocyte reactions. We hypothesized that should the D and DR antigenic moieties reside on the same molecule, anti-DR antibodies ought to be able to inhibit lymphoproliferation by sterically blocking receptor recognition. Data are presented which demonstrate the failure of anti-DR antibodies to completely block secondary primed lymphocyte (PLT) responses even though they inhibit primary MLC reactions. One interpretation of the data would suggest that D and DR are not closely associated on the cell surface.

METHODS

Cells. Peripheral blood lymphocytes (PBL) were obtained from whole blood diluted 1:1 in RPMI 1640 (Grand Island Biologicals Company, Grand Island, NY) and centrifuged over Ficoll-(Sigma Chemical Company, St. Louis, MI) Hypaque (Winthrop Laboratories, New York, NY) at 400xg for 30 minutes. After washing, cells were diluted to approximately 10^7/ml in RPMI 1640 medium containing 10% AB plasma, 25mM HEPES, 2mM L-glutamine, 50 micg/ml gentamicin, 25 IU/ml Na-heparin (preservative free) and 7.5% v/v dimethylsulfoxide at 4°C. PBL were frozen using a programmed rate-controlled freezer (Cryoson, Associated Biomedic Systems, Buffalo, NY): -1°C/min for 20 minutes; -5°C/min down to

-80°C. Following this procedure, cells were transferred to the vapor phase of a liquid nitrogen freezer (MVE Cryogenics, New Prague, MN) and stored at -180°C until use.

One milliliter cell samples were thawed in a 37°C water bath until the last ice crystal disappeared and transferred to 15ml conical test tubes (Corning, Corning, NY). While constantly agitating, 5ml of medium (as above but without DMSO) was added dropwise over the course of 30-60 seconds in order to reduce hypo-osmotic shock. Suspensions were finally brought up to 15ml with medium and washed at 200xg for 10 minutes. Supernatants were decanted and medium containing 10% AB serum (MA Bioproducts, Bethesda, MD, lot #94896) was added to bring cells up to the appropriate volume. This serum lot was previously screened for inhibitory activity and was found to produce optimal cell growth in a number of different proliferative assays.

HLA typing. Cells were serotyped for HLA-A, B, w4/w6, C and DR in the 8th International Histocompatibility Workshop using multiple sera from the 6th, 7th and 8th Workshops for each specificity in microcytotoxicity assays.

HLA-D typing was performed using homozygous typing (HTC) cells from the 8th International Workshop. A minimun of two HTCs were used to define each specificity and each experiment was performed at least twice. Double normalized values (DNV) were computed: DNV<40 was considered a positive response, 40-60 was doubtful and DNV>60 was negative.

The HLA phenotypes of all cells used in these experiments are given in Table 1.

Antisera. RIa was kindly provided by Dr. M. Crumpton and is a rabbit anti-human Ia generated against p23,30 from a human lymphoblastoid cell line. DA-2 and CA-2 are mouse monoclonal antibodies which react with framework residues of the DR molecule; Genox 3.53 is a murine monoclonal antibody which

binds cells bearing HLA-DR1, DR2 or DRw6 and may be supertypic for DR antigens or it may bind another antigen such as MB1 (13). Control antibodies consisted of the following: monoclonal murine anti-Thy 1.2 provided by Dr. P. Lake; 3A-1, a murine monoclonal specific for human T-lymphocytes provided by Drs. B. Haynes and A. Fauci; a murine monoclonal, M8, specific for human β_2-microglobulin provided by Dr. A. Sanderson. Separate experiments in this laboratory have shown that RIa and DA-2 profoundly inhibit mitogen-induced and antigen-specific proliferative responses while M8, 3A-1 and normal rabbit serum have no effect (14).

Primary mixed lymphocyte culture. Fifty thousand responder cells were combined with $5x10^4$ γ-irradiated (2500 rads, ^{137}Cs, 313 r/min) stimulator cells in triplicate cultures in 96-well U-bottom sterile microtiter trays (Linbro, Hamden, CN). Antisera were added to wells in serial two-fold dilutions; the highest concentration of antibody was approximately 100μg/ml. At 108 hours, each well was pulsed with 1.0μCi ^3H-methylthymidine (New England Nuclear, Boston, Massachusetts). Cells were harvested at 120 hours and radiolabel incorporation was quantitated by liquid scintillation spectroscopy.

Secondary primed lymphocyte culture. Primed lymphocytes were prepared by incubating equal volumes of responders and irradiated stimulator cells at $1x10^6$ cells/ml in 10% AB plasma and RPMI 1640 medium containing 25 mM HEPES, 2mM L-glutamine, 50μg/ml gentamicin, 25 IU/ml preservative free sodium heparin. Cultures were allowed to progress for 11 days in 5% CO_2 at 37°C at which time they were resuspended to 10^5 cells/ml in medium containing 10% AB plasma and plated in 0.1 ml aliquots. To each well of U-bottom microtiter trays containing 10^4 primed responder cells was added $2x10^4$ γ-irradiated stimulator cells in 10% AB plasma and medium. Antibody was serially diluted as for primary cultures, added to each microculture and incubated 48 hours at

which time 1μCi ^{3}H-methylthymidine (New England Nuclear) was added. Cultures were incubated an additional 12 hours, harvested and the radiolabel incorporation was quantitated as for MLC.

RESULTS

Inhibition of primary MLC by anti-DR antibodies. The addition of antisera specific for human HLA-DR antigens to primary cultures of cells from AAHM (Dw?; DR2,3; MB1,2; MT2) responding against stimulator cells from $RHAR_x$ (Dw5,?; DR5,6; MB1,3; MT2) ablated proliferation as seen in Figure 1. Untreated cultures yielded 4531±301 cpm (mean ± SEM) while cultures in the presence of high concentrations of RIa, DA-2 or CA-2 were not significantly above the background level of 155±90 cpm. DA-2 and CA-2 were not significantly inhibitory below 1.6μg/ml and 6.25μg/ml, respectively, although RIa significantly inhibited proliferative responses above approximate concentrations of 0.1μg/ml. Control antisera, including monoclonal anti-Thy 1.2, M8 (anti-β_2-microglobulin) and 3A-1 (anti-human-T-cell), produced no biologically significant inhibition of primary MLC (Figure 2). However, 3A-1 appeared to enhance ^{3}H-methylthymidine uptake in some cultures (32824±4805 cpm) at a concentration of 100μg/ml. Similar results using the same antisera have been observed irregardless of the HLA phenotypes of responding and stimulating cells (data not shown).

Effects of anti-DR antisera on PLT cultures. Addition of the anti-HLA-DR framework antibody, DA-2, to primed lymphocyte cultures generally failed to block secondary responses completely (Figure 3) unlike MLC. Of seven separate experiments, prepared using seven different batches of primed cells, three

revealed no significant inhibition of PLT responses by AAHM's cells restimulated with RHAR's cells, two experiments demonstrated partial inhibition of responses at concentrations above 25μg/ml and two further experiments using the same responder and stimulator combination showed decreased PLT responses at concentrations above 3.1μg/ml. Similar experiments with CA-2, RIa and Genox 3.53 demonstrated no inhibition of secondary proliferation (Figure 4). In control experiments with 3A1,M8 and anti-Thy 1.2, no suppression of PLT responses were observed (Figure 5), although in one of three experiments, M8 produced approximately 40% inhibition at 25-100μg/ml. Additionally, 3A-1 enhanced proliferative responses at high concentration's in some cultures similar to that observed for MLC.

DISCUSSION

We have presented evidence that monoclonal anti-DR can inhibit MLC responses to D-region differences, and shown that primed lymphocyte typing responses are not completely inhibited by anti-DR antibodies or rather that they are less sensitive to inhibition by these reagents compared to the primary MLC. Several groups (11,12) have interpreted similar data using alloantisera as indicating a near identity for HLA-D and HLA-DR. In contrast, there are data from the primed lymphocyte typing (PLT) test that D and DR are, at least antigenically, quite distinct (2-4) and can be detected independently of each other. Our data could be interpreted to support this latter view.

At the cellular level in the primary MLC, interactions may be inhibited at a number of different places. It is conceivable that anti-DR antibodies simply bind to D/DR molecules on the stimulator cell and thus physically hinder recognition. Such inhibition could be effected by interfering with

transduction of the activation signal across the cell membrane. This possibility is suggested from reports documenting inhibition of responses to polyclonal activators by anti-Ia antisera (15,16). Alternatively, since the primary MLC is monocyte dependent (17), blocking could occur at the presentation level similar to that observed for murine responses to soluble antigens (18). This suggests that monocytes carry DR antigens which may be important in antigen presentation; the fact that responses to D and DR, were inhibited favors this view.

Another less attractive hypothesis would include the induction of suppressor cells in MLC by anti-Ia antisera as has been suggested by Broder et al. (19).

The insensitivity of PLT cultures to anti-DR antibodies raises several interesting considerations. The first derives from the work of Wollman et al. (17) demonstrating that in contrast to primary MLC, activation of PLT cells is not as monocyte dependent. Therefore, anti-DR antibody would be less likely to interfere with presentation of alloantigen during the monocyte-T interaction in secondary cultures as it might in primary cultures. The second is that other alloantigenic stimulating determinants may be detected in the absence of responses to HLA-D/DR, as has been demonstrated by Shaw and others (5-9). An argument against this possibility, however, is suggested by work in the mouse (20) showing that secondary responses to weakly stimulating MHC determinants are effectively masked by priming in the presence of immunodominant Ia antigens. Alternatively, it is possible that a binding equilibrium is established which depends on the relative affinities of antibody and cell-surface receptor for activating determinants; clones of sufficiently high affinity would be triggered while those of low affinity would not (Dr. P. Parham, personal communication). If this is true, then boosting antibody

concentrations beyond 100μg/ml into the milligram range should further suppress PLT proliferation. It is also possible that clones which recognize DR are blocked by antibody while those recognizing HLA-D are not and are therefore capable of being activated since the PLT priming combination recognizes both D and DR differences. This would also suggest that secondary responses to HLA antigenic differences are additive.

Specific antibodies produced different effects in different cultures. 3A-1 enhnaced proliferation in a minority of MLC and PLT assays probably similar to the reported enhancing effects of antilymphocyte serum (21). Similarly, two of seven identical PLT experiments have shown complete inhibition of proliferation with the highest concentration of antibody used. Presently, we have no explanation for these results but we believe they may correlate with cell viability, serum components present in the medium or other technical aspects of the procedure.

ACKNOWLEDGEMENTS

The authors thank Ms. Bonita Minor for her assistance in the preparation of this manuscript and Drs. Marc Feldmann, John Neefe and Phil Lake for many helpful discussions. The expert technical assistance of Ms. Peggy Lamb and Mr. Thomas Lionetti is also greatly appreciated. Special thanks are due Drs. Armead Johnson, Fran Ward and Bernard Amos for HLA serotyping.

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FIGURE LEGENDS

Figure 1. The influence of xenogeneic and murine monoclonal antibodies on primary mixed lymphocyte reactions. Fifty thousand AAHM responder cells were added to $5x10^4$ RHAR γ-irradiated stimulator cells per well in the presence of serially diluted (1:2) antibody. Each point represents the mean of triplicate 0.2ml cultures. Two other experiments gave identical results. (——), DA-2; (– – –), RIa; (·····), CA-2.

Figure 2. The influence of control antibodies on primary MLC. 3A-1 and M8 are murine monoclonal anti-human T-cell and anti-human β_2-microglobulin antibodies, respectively. Fifty thousand AAHM responder cells were added to $5x10^4$ RHAR γ-irradiated stimulator cells per well in the presence of serially diluted (1:2) antibody. Each point represents the mean of triplicate 0.2ml cultures. Occasionally, 3A-1 induced obviously enhanced uptake of radiolable in MLC. (——), 3A-1; (·····), M8; (– – –), Thy 1.2.

Figure 3. The effect of monoclonal DA-2 (anti-DR) antibody on secondary primed lymphocyte typing (PLT) reactions. Cells from AAHM were primed against γ-irradiated cells from RHAR (see Methods Section). For PLT, 10^4 AAHM PLT cells were added to $2x10^4$ γ-irradiated RHAR stimulator cells per well in the presence of serially diluted (1:2) antibody. Each point represents the mean of triplicate 0.2ml cultures. Of seven identical experiments using separate batches of primed cells, two (dotted line) demonstrated complete inhibition of PLT responses.

Figure 4. The effect of other anti-DR antibodies on PLT responses. Cells from AAHM were primed against γ-irradiated cells from RHAR (see Methods Section). For PLT, 10^4 AAHM PLT cells were added to $2x10^4$ -irradiated RHAR stimulator cells per well in the presence of serially diluted (1:2) antibody. Each point represents the mean of triplicate 0.2ml cultures. (——), RIa; (– – –), CA-2; (·····), Genox 3.53.

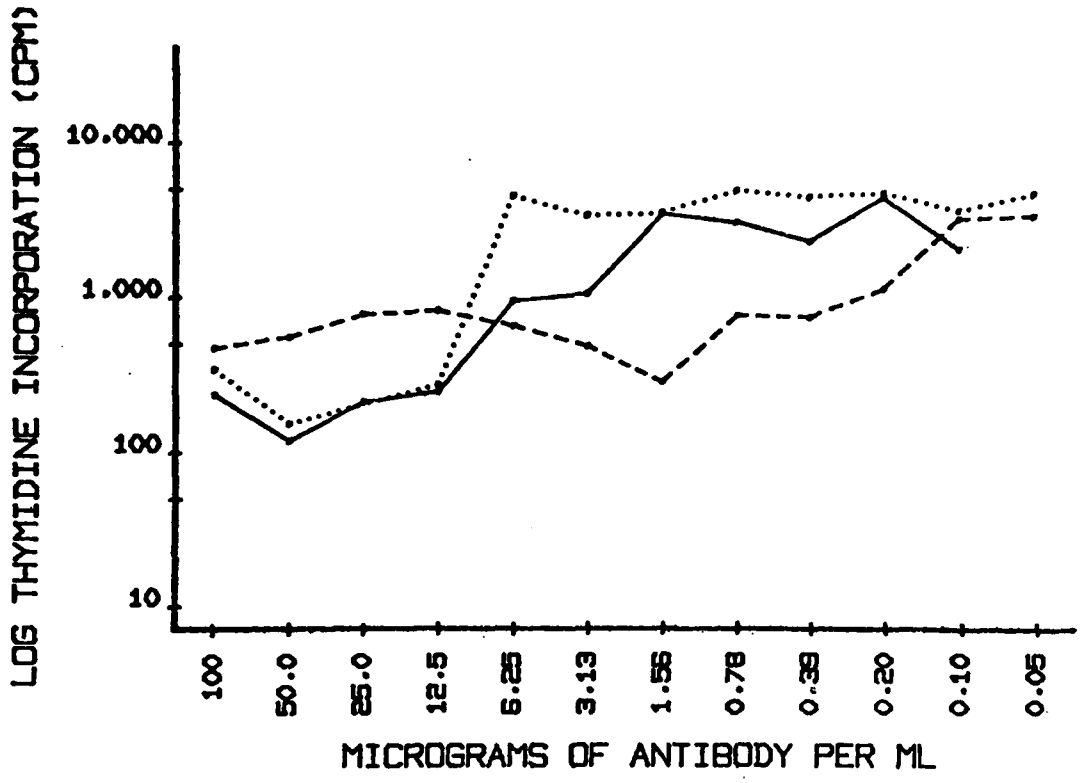
Figure 5. The effect of control antibodies on PLT responses. Cells from AAHM were primed against γ-irradiated cells from RHAR (see Methods Section). For PLT, 10^4 AAHM PLT cells were added to $2x10^4$ -irradiated RHAR stimulator cells per well in the presence of serially diluted (1:2) antibody. Each point represents the mean of triplicate 0.2ml cultures. (——), 3A-1; (·····), M8; (– – –) Thy 1.2.

Table 1

HLA PHENOTYPES OF CELLS USED IN ALL EXPERIMENTS

Cell	A	B	w4/w6	C	D	DR
AAHM	2,26	8,w53	w4,w6	?	?	2,3
RHAR	2,26	17,w41	w4,w6	w6,?	w5,?	5,?

Cells were serotyped for HLA-A, B, w4/w6, C and DR using several sera for each specificity defined in the 6th, 7th and 8th International Histocompatibility Workshops. HLA-D typing was performed using at least two HTCs for each specificity from the 8th Workshop; a DNV<40 in two experiments was required in order to assign an HLA-D specificity to a particular cell. Cells AAHM and RHAR, although possessing undefined D-region antigens, nevertheless give strong MLC and PLT responses when cultured together and are therefore non-identical in the HLA-D region.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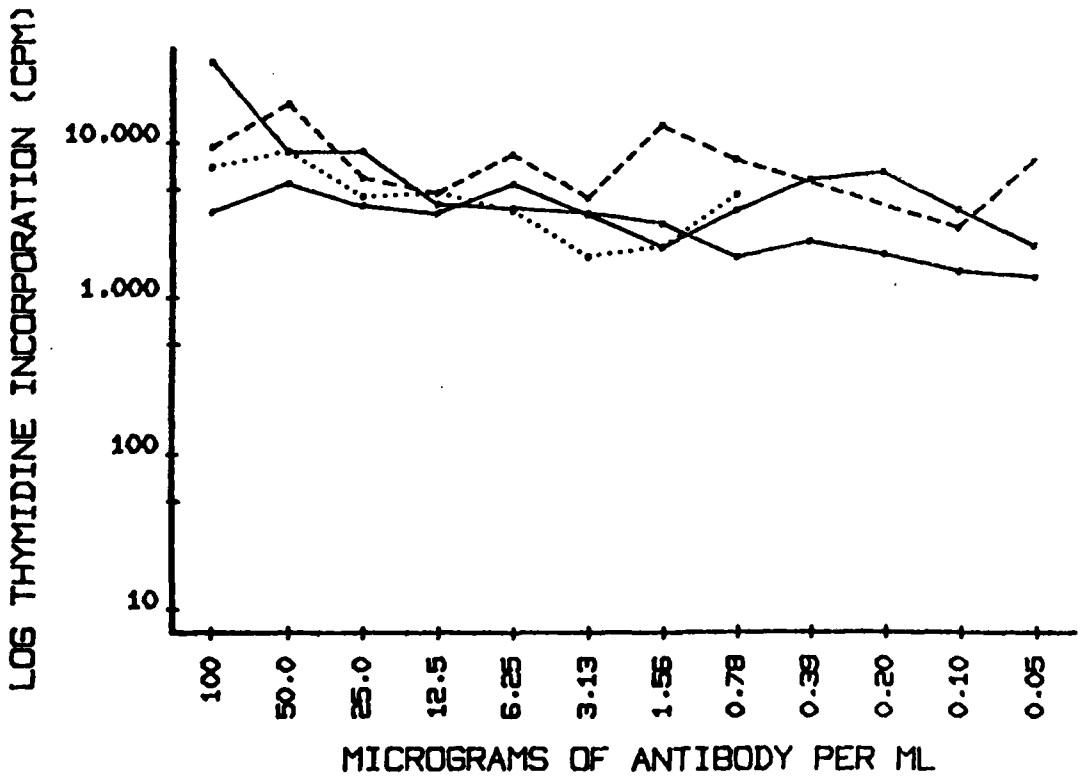
MICROGRAMS OF ANTIBODY PER ML

1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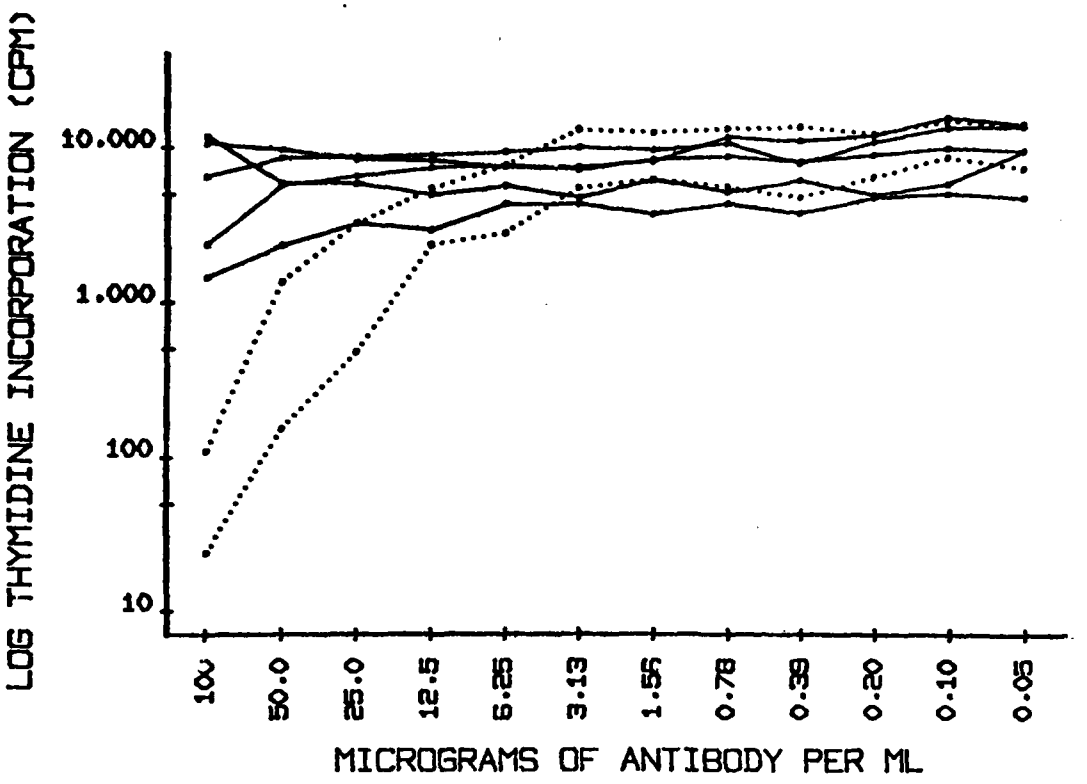
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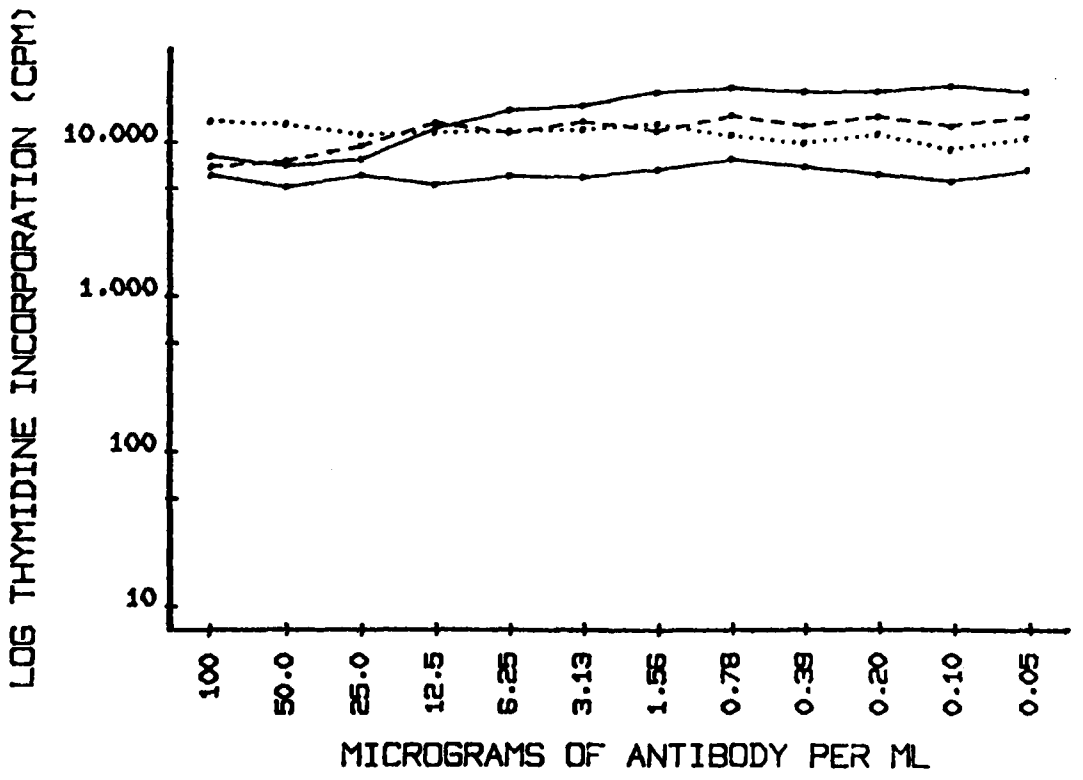
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MICROGRAMS OF ANTIBODY PER ML

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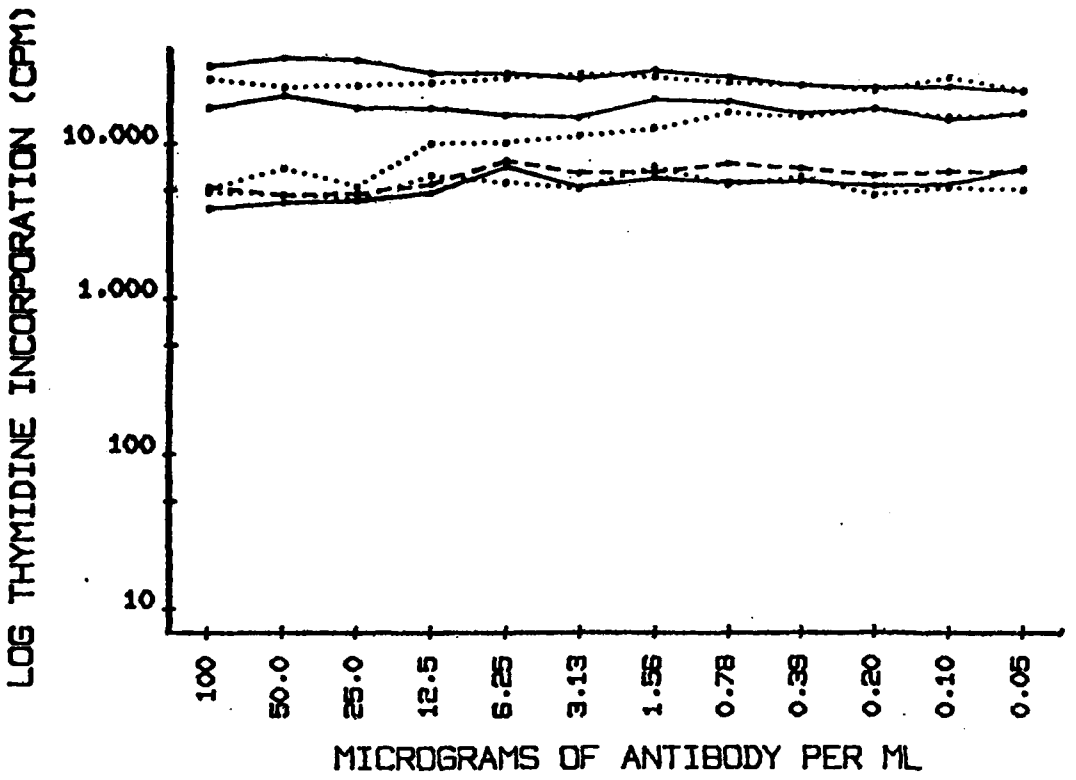
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MICROGRAMS OF ANTIBODY PER ML

5

EVIDENCE FOR A PRIMED LYMPHOCYTE (PLT) RESTIMULATING DETERMINANT ENCODED BY A NEW GENE LINKED TO HLA

ABSTRACT

Human peripheral blood T-lymphocytes were stimulated by allogeneic cells in primary MLC and subsequently cloned by limiting dilution in the presence of lymphocyte conditioned medium (LyCM). Following expansion, clones were tested for specific proliferation against a panel of 32 stimulator cells including cells from the family of the original stimulator (FLAM). Two clones, TLC 14-14 and TLC 14-86, responded to FLAM and a cell homozygous for Dw5 (JPSU), but not to other unrelated panel members; reactivity segregated with the haplotype containing Dw1 in FLAM's family. In separate experiments, TLCs 14-14 and 14-86 were restimulated by an antigen encoded by the maternal "c" haplotype in JPSU's family. This example, using cloned primed lymphocytes, of an MHC gene other than HLA-D which encodes a lymphocyte activating product is provisionally designated TLC-A.

Current conceptions of the human major histocompatibility complex (MHC) include at least four loci or regions: HLA-A,B,C and D/DR. Each region encodes distinctive cell surface glycoproteins which are presently detected using one of two methods. HLA-A,B,C and DR are detected in microlymphocytotoxicity assays using allospecific antibodies primarily from multiparae (1,2). HLA-D (3) has traditionally been defined by testing cells from different individuals and observing proliferation in mixed lymphocyte reactions (MLR).

HLA-D region antigens can also be detected in the primed lymphocyte typing (PLT) test. Lymphocytes are primed in primary mixed lymphocyte cultures and upon subsequent rechallenge with the original stimulating cell, or cells sharing certain antigenic components with the stimulator, vigorous proliferation ensues (4). Such primed lymphocytes detect both HLA-D and HLA-DR determinants (5). Although it has not been finally determined whether D or DR comprise distinct genes, there have been several reports of a recombination between D and DR (6-8). Finally, there is immunochemical, serologic and cellular evidence for multiple genes in tight linkage disequilibrium with the D-region (e.g., MB, DC, MT and SB, references 9-12 respectively).

A major obstacle preventing resolution of human HLA complexities is the fact that reagents used in serology, MLC and PLT are polyspecific. In this regard, newly available monoclonal antibodies against human MHC products should help to resolve questions of serologic polyspecificity. Recently however, with the work of Bach et al. and others (13-15), the use of monoclonal cellular reagents for HLA-D typing became possible. Similarly, we have used clones of primed lymphocytes to analyze stimulating determinants present on human lymphocyte cell surfaces and present evidence here for a new gene, linked to HLA-D, which is provisionally designated TLC-A.

Ten million human peripheral blood lymphocytes (PBL) from an individual homozygous for HLA-Dw3 (GHOV) were primed against 10^7 cells from a Dw1,3 heterozygous individual (FLAM) in 10% human AB serum (MA Bioproducts, Bethesda, Maryland, lot #94896) and RPMI 1640 (Grand Island Biologicals Company, Grand Island, New York) containing 25mM HEPES, 2mM L-glutamine, 50 micg/ml gentamicin and 25 IU/ml sodium heparin. Cells were incubated for 4 days at 37°C at which time they were centrifuged over 35% Percoll (Pharmacia, Uppsala, Sweden) to enrich for responding blast cells. Blasts at the interface were diluted in 10% ABS, 20% lymphocyte conditioned medium (LyCM, 16) and RPMI 1640 and plated at 0.3 cells/well in 20 micl (total volume) aliquots in Terasaki trays. Each well also received $5x10^3$ responders and $5x10^3$ stimulators as feeder cells (γ-irradiated, 2500 Rads). The presence of irradiated feeders and LyCM, which contains TCGF, were required for continued growth of T-lymphocyte clones. Trays were incubated for one week at which time growing clones were transferred to 24-well trays (Linbro Chemical Co., New Haven, Connecticut) containing 2 ml 20% LyCM per well with $5x10^5$ feeders/ml and thus maintained throughout the course of the experiments.

For specificity testing, clones were diluted to 10^5 cells/ml in 10% ABS and medium after washing and 0.1ml was plated into triplicate cultures of $5x10^4$ -irradiated stimulator cells in 96-well, U-bottom trays. Cultures were incubated for 48 hours at 37°C, pulsed for an additional 12 hours with 1.0 micCi ^{3}H-methylthymidine (6.7 Ci/mM, New England Nuclear, Boston, Massachusetts) and harvested. Radiolable incorporation was measured by liquid scintillation spectroscopy. Data are expressed as median cpm of triplicate cultures. The HLA specificities of 32 stimulator cells are shown in Table 1. This panel included cells expressing HLA-Dw1 through Dw8 and cells from the family of the original stimulating cell (FLAM).

In the first experiment, proliferation by ten of 58 clones segregated with HLA in FLAM's family. Two T-lymphocyte clones, TLC14-14 and TLC14-86, responded to JPSU (homozygous Dw5), FLAM (positive control) and only those individuals from FLAM's family who carried the Dw1 ("a") haplotype (Table 1); all other panel members failed to restimulate these two clones. Positive responses were typically 50-fold over a background of 200 cpm. To further demonstrate that TLC14-14 recognized a product encoded by genes linked to HLA, JPSU's family was typed in five separate experiments. The HLA specificities and reaction patterns of the family are shown in Figure 1. In this family, reactivity segregated with the maternal "c" haplotype.

This is the first formal demonstration, using cloned PLT cells, of a unique new D-region product. That PLT clones may detect new loci has been suggested previously (17). Other cell surface components, different from previously known HLA specificities, can restimulate primed lymphocytes as has been shown by a number of different groups using PLT cells (12, 13, 18-20). TLC14-14 and TLC14-86 do not detect MB, MT or SB because cells positive for such specificities do not restimulate these clones. These data suggest that there are several genes, in close priximity but not identical to those within the currently described HLA region, which can encode antigens capable of eliciting lymphocyte proliferative responses. It is clear that cloned T-cells detect such specificities without the concomitant oligoclonal problems observed using populations of PLT cells. Hopefully, PLT clones will enable precise definition of HLA-D region genes as well as other restimulating determinants.

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21. The authors thank D. Smoot, F. Robbins, R. Hargrove and T. Lionetti for expert technical assistance, Ms. B. Minor for assistance in preparing this manuscript and Drs. P. Lake, J. Neefe, A. Johnson and J. Woody for helpful criticisms of the manuscript. Special appreciation is due Drs. D.B. Amos, F.E. Ward and A.H. Johnson for HLA serotyping and Dr. S. Shaw for SB typing cells. This work was supported by funds received from the Office of Naval Research Contract N000-14-77-C-0747, Research Task No. NR-207-101 and Navy Medical Research and Development Command Task No. M0095-PN.001.0045. The opinions expressed herein are those of the authors and are not to be construed as those of the Naval Department or the Naval Service at large.

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Table 1

SPECIFICITY OF RESPONSES BY T-LYMPHOCYTE CLONES

	HLA PHENOTYPES									PROLIFERATION**	
Cell	A	B	w4/w6	Cw	Dw	DR	MB	MT	SB	TLC	
-Panel-										14-14	14-86
JAYR	1,11	8,w62	6,?	1,?	3,?	3,w9	2,?	2,3	nt	37	138
CBER	1,3	27,w51	4,?	2,?	3,5	3,5	2,3	2,?	nt	243	783
SJOH	1,3	27,w51	4,4	2,?	3,5	3,5	2,3	2,?	nt	96	500
CGRE	w30,?	w53,?	4,6	3,4	?	?	nt	nt	nt	185	175
OSCH	2,w33	w41,w48	4,6	3,?	3,6	3,w6	1,2	1*,2	1,4	43	53
BMEL	3,3	14,w41	6,?	8,?	1,4	1,4	1,?	1*,?	nt	83	329
HMEL	3,w24	14,w35	6,?	4,8	1,w12	1,2	1,?	1*,?	4,?	123	751
EPAP	2,3	w35,w35	6,6	4,?	1,5	1,5	1,3	1*,?	nt	146	230
FPAP	2,3	w35,w35	6,6	4,?	1,5	1,5	1,3	1*,2	nt	185	241
VSCH	1,w33	7,w58	4,6	3,?	1,6	1,w6	1,?	1*,2	nt	39	68
JSAU	1,26	8,w38	4,6	3	1,3	1,3	nt	nt	nt	64	104
RSCH	2,3	7,w41	6,6	?	1,3	1,3	1,2	1*,2	1,4	26	57
GHOV	1,?	8,?	6,?	7,?	3,3	3,?	nt	nt	nt	72	79
KCAR	2,?	7,?	6,?	?	2,2	2,?	nt	nt	4,5	72	455
RKEL	w32,?	8,?	6,?	7,?	1,3	3,?	nt	nt	nt	49	184
JKH	w24,26	w51,w60	4,6	3,?	8,?	w6,?	1,2	1*,?	nt	135	304
PBUR	1,2	14,?	6,?	?	7,7	7,?	nt	nt	nt	26	103
GYOD	2,2	w44,w44	4,4	5,5	4,4	4,4	3,?	?	4,?	54	52
JTED	2,29	27,w35	4,6	1,4	6,6	w6,?	1,?	1*,?	nt	22	35
RCUM	w30,?	7,w53	4,6	4,4	2,?	2,5,w6	1,3	1*,2	2,4	43	208
CCUM	1,3	7,w35	6,6	4,4	2,5	2,5,w6	1,3	1*,2	2,4	284	702
JPSU	2,28	27,w44	4,4	3,5	5,5	5,5	3,3	2,2	4,?	11,605	16,775

Table 1 (cont'd)

	HLA PHENOTYPES									PROLIFERATION**	
Cell	A	B	w4/w6	Cw	Dw	DR	MB	MT	SB	TLC	
-Panel-										14-14	14-86
FLAM†	2,w30	13,w41	4,6	6,?	1,3	1,3	1,2	1*,2	1,4	20,215	34,791
SLAM	2,29	w35,w60	6,6	3,8	8,9	4,w8	?	2,?	1,4	80	749
HLAM	2,29	w41,w60	6,6	3,?	nt	nt	nt	nt	nt	51	110
ELAM	2,2	w35,w41	6,6	8,?	3,8	3,w8	2,?	2,?	nt	460	331
FLAJr	2,w30	13,w35	4,6	6,8	1,8	1,w8	1,?	1*,2	nt	21,715	33,519
JLAM	2,w30	13,w35	4,6	6,8	1,8	1,w8	1,?	1*,2	nt	9,835	23,597
LLAM	2,w30	13,w35	4,6	6,8	1,8	1,w8	1,2	1*,2	nt	15,026	26,964
VLAM	2,w30	13,w35	4,6	6,8	1,8	1,w8	1,?	1*,2	nt	10,549	25,845
OLAM	2,w30	13,w35	4,6	6,8	1,8	1,w8	1,?	1*,2	nt	9,351	25,216
GLAM	29,w30	13,w60	4,6	3,6	1,9	1,4	1,?	1*,2	nt	8,298	18,623

* =MT1 is operationally defined by MB1, but is probably not part of the MB allelic series.

** =Median cpm of triplicate cultures.

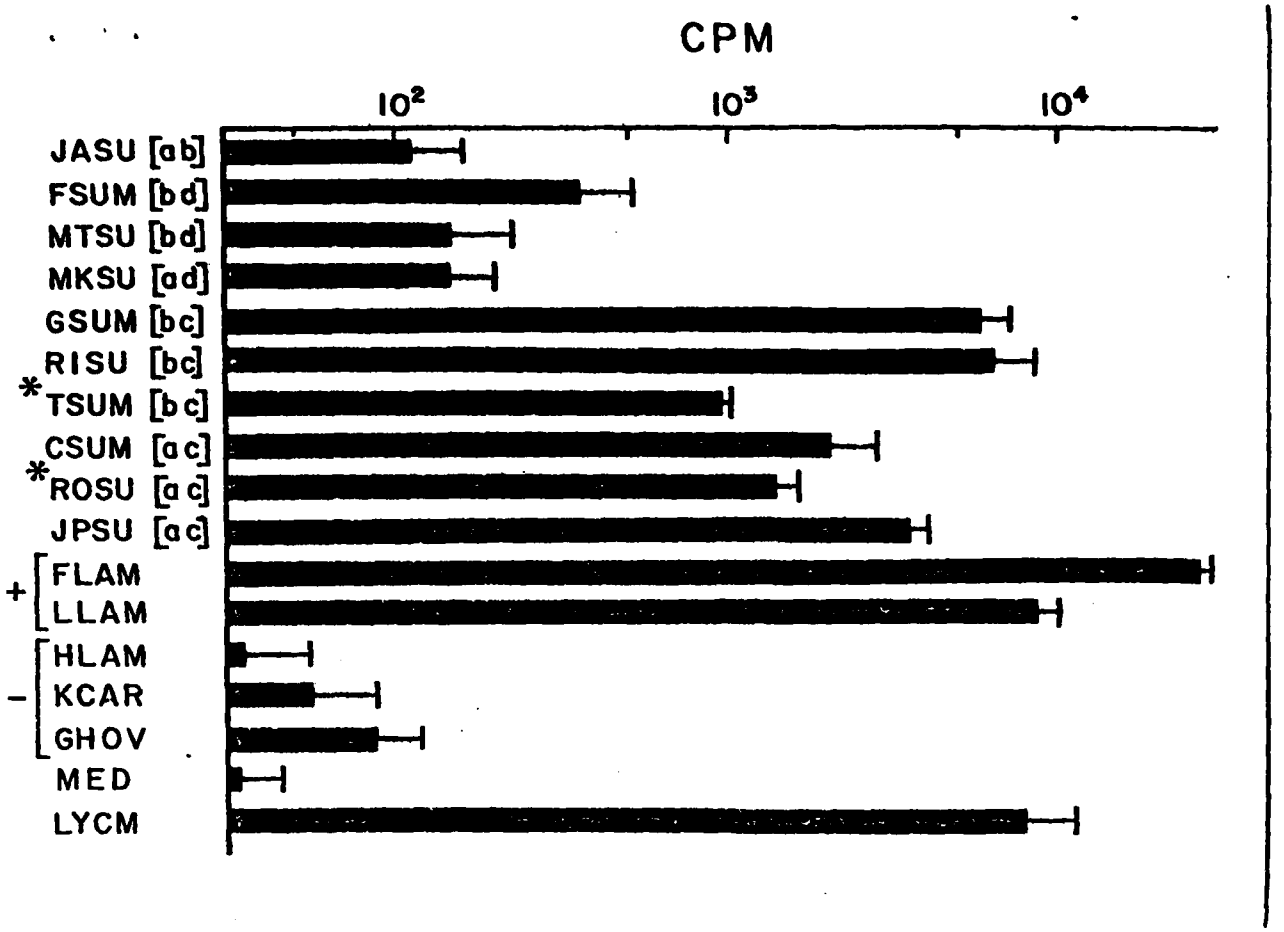
† =Original stimulating cell.

? = Specificity unknown.

nt = not tested.

FIGURE LEGEND

Figure 1: Response of TLC 14-14 to members of JPSU's family. Letters in parentheses indicate haplotypes. Father (JASU, haplotypes "a" and "b") was HLA-A2; B15,27; w4,w6; Cw3; Dw4,5; DR4,5. It was not possible to obtain a blood sample from the mother (MSUM, haplotypes "c" and "d") for this testing; she has been typed as HLA-A1,28; B37,w44; w4; Cw5,6; Dw2,5; DR2,5. Values were obtained from the means of five experiments ($\pm$ S.E.M.).


CPM
10^2
10^3
10^4
JASU [ab]
FSUM [bd]
MTSU [bd]
MKSU [ad]
GSUM [bc]
RISU [bc]
*TSUM [bc]
CSUM [ac]
*ROSU [ac]
JPSU [ac]
+
FLAM
LLAM
−
HLAM
KCAR
GHOV
MED
LYCM

DOES HLA-D EXIST?

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These studies were supported by funds received from the Ofice of Naval Research Contract N000-14-77-C-0747, Research Task No. NR207-101 and M0095-PN.001.0045. The opinions expressed herein are those of the authors' and are not to be construed as those of the Department of the Navy or the Department of Defense.

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These results were presented in part during the Seventh Annual Meeting of the American Association for Clinical Histocompatibility Testing, March 22-26, 1981 in Orlando, Florida

ABSTRACT

Cells from an individual homozygous for HLA-Dw3 were stimulated by cells from a Dw1,3 heterozygote (FLAM) in primary MLC for four days. Lymphoblasts were cloned by limiting dilution at 0.3 cells/well in the presence of T-cell growth factor (TCGF) and irradiated feeders. Following expansion in liquid culture for three weeks, T-lymphocyte clones (TLC) were tested for proliferation with a panel of 31 stimulators containing Dw1 through Dw9 as well as FLAM's family. Of 27 TLCs tested, 52% clearly responded to the positive control and 17% segregated with cells from siblings carrying the Dw1 specificity; 35% demonstrated complex segregation patterns in the family. TLCs were never restimulated exclusively by all Dw1/DR1+ cells and most were restimulated by subsets of Dw1/DR1+ cells. Generally, the difference between positive and negative responses was at least 20 fold. The simplest explanation of the data is that conventional MLC and PLT testing measure composite responses against many MHC and non-MHC antigens.

INTRODUCTION

The human major histocompatibility complex (MHC) is currently divided into four major regions or loci: HLA-A,B,C and D/DR. HLA-A,B,C and DR are detected serologically using the microlymphocytotoxicity assay (1,2) while D region antigens are defined in mixed lymphocyte reactions (MLR; 3).

Alternatively, HLA-D/DR and other antigens can be detected in the primed lymphocyte typing (PLT) test. Lymphocytes primed in primary mixed lymphocyte cultures (MLC) when rechallenged with the original stimulating cell, or cells sharing antigenic components with the stimulator, undergo vigorous and rapid proliferation (4). While PLT testing may be more precise than conventional MLC, the response is still oligoclonal. However, the recognition that supernatants from mitogen stimulated lymphocyte cultures (T cell growth factor, TCGF) can maintain the growth of T-lymphocytes in vitro has allowed the expansion and maintenance of allospecific T-lymphocyte clones (TLC) in long-term culture (5-10). Such TLCs presumably recognize much more restricted alloantigenic specificities and should, therefore, allow very precise analyses of cell surface determinants.

Using cloned alloreactive T-cells, we have investigated the response patterns of several different TLCs which responded to a panel of thirty one stimulating cells. The data suggest that in fact "HLA-D", as classically defined, consists of composite responses to many different antigens some of which are linked to HLA and others which are not.

METHODS

Cells. Mononuclear cells from peripheral blood (PBL) were obtained from screened donors diluted with an equal volume of RPMI 1640 (Grand Island Biologicals Company, Grand Island, NY) and centrifuged over Ficoll-Hypaque (Sigma Chemical Company, St. Louis, MO) at 400g for 30 minutes. After washing, the cells were resuspended at 10^7/ml in RPMI 1640 medium supplemented with 10% screened, pooled human AB plasma, 2mM L-glutamine, 25mM HEPES buffer, 50 micg/ml gentamycin, 25 IU/ml sodium heparin and 7.5% v/v dimethylsulfoxide at 4°C. The cells were frozen at -1°C/min for 20 minutes using a rate-controlled freezer (Cryoson, Associated Biomedic Systems, Buffalo, NY), then at -5°C/min down to -80°C. Cells were stored in the vapor phase of a liquid nitrogen freezer (MVE Cryogenics, New Prague, MN) at -180°C until required.

HLA phenotyping. Cells were typed in primary MLC using Seventh and Eighth International Histocompatibilty Workshop HTCs and local typing cells corresponding with workshop reference cells. Cells were assigned a specificity (Dw1-9)if they were typed with 4 defined HTCs with an average DNV of less than 40. HLA-A,B,C,DR serotyping was performed at Duke University by Drs. D.B. Amos, F.E. Ward and A.H. Johnson using Seventh and Eighth International Histocompatibility Workshop sera and local sera in a modification of the workshop method (2).

T-cell growth factor (TCGF) production. PBLs from screened donors were cultured at $1x10^6$/ml in RPMI 1640 supplemented with 0.1% purified phytohaemagglutinin-P (PHA-P; Difco Laboratories, Detroit, MI) and 1% autologous plasma (7). After 48 hours, supernatants were harvested, passed through 0.22 micm filters and assayed for their ability to support the growth

of a TCGF(Interleukin II)-dependent cell line as assayed by tritiated thymidine incorporation. Acceptable lots of TCGF were stored at 4°C or diluted as required to 20% TCGF in 10% AB plasma and medium.

Priming and cloning of allospecific T-lymphocytes. Ten million human peripheral blood lymphocytes (PBL) from an individual, homozygous for HLA-Dw3 (GHOV), were primed against 10^7 cells from a Dw1,3 heterozygous individual (FLAM) in 10% screened, pooled human AB serum (MA Bioproducts, Bethesda, MD, lot #94896) and RPMI 1640 (Grand Island Biologicals Company, Grand Island, NY) containing 25mM HEPES, 2mM L-glutamine, 50 micg/ml gentamicin and 25 IU/ml sodium heparin. Cells were incubated for 4 days at 37°C at which time they were centrifuged over 35% Percoll (Pharmacia, Uppsala, Sweden) to enrich for responding lymphoblasts. Blasts were removed from the interface, diluted in 10% AB serum, 20% TCGF and medium and plated at 0.3 cells/well in 20 micl (total volume) aliquots in Terasaki trays. Each well also received $5x10^3$ autologous responders and $5x10^3$ autologous stimulators as feeder cells (γ-irradiated, 2500 Rads, ^{137}Cs). The presence of irradiated feeders and TCGF, was required for continued growth of TLCs. Trays were incubated for one week at which time growing clones were transferred to 96-well trays (Linbro Chemical Co., New Haven, CN) containing 0.2 ml 20% TCGF per well with $5x10^5$ feeders/ml. Growing TLCs were subsequently transferred to 24-well trays (Linbro) in 2ml of medium containing TCGF and feeders in the same proportion as before and thus maintained throughout the course of the experiments.

Proliferation assays. Ten thousand TLC cells diluted in 10% AB plasma and medium were added to 96-well U-bottom trays in 0.1ml aliquots. Fifty thousand peripheral blood lymphocytes from 31 different donors, phenotyped for HLA-A,

B,C,D,DR,MB,MT,SB, were -irradiated (2500 rads), suspended in medium containing 10% AB plasma and added in equal volumes to wells containing TLC. Cultures were incubated from 12 to 192 hours, pulsed for 8-18 hours with 1 micCi of ^{3}H-thymidine (6.7 Ci/mM; New England Nuclear, Boston, MA) and harvested onto glass fiber filters; proliferation, as correlated with ^{3}HTdR incorporation, was measured by liquid scintillation spectroscopy. Data was expressed as the median cpm of triplicate cultures.

RESULTS

Clonal growth. After seven days culture in Terasaki plates approximately 30% of those wells originally seeded with alloreactive lymphoblasts contained growing cells. Upon subsequent transfer to 96-well trays, and later 24-well trays, 95% of the wells still contained growing cells after seven or more days in culture. Removal of irradiated feeder cells or conditioned medium (TCGF) resulted in cessation of clonal growth in approximately 2 to 4 days.

Reactions of TLCs with phenotyped panel. Although twenty-seven clones were characterized in panel studies, only the data from seven typical TLCs are included herein (Table 1). Characteristic background responses to the autologous control (GHOV) were 100-200 cpm. No clone was stimulated by Dw1/DR1+ ells exclusively. Furthermore, most cells which were restimulated by FLAM (positive control) reacted with only a subset of cells bearing Dw1/DR1. For example, TLC 14-65 was restimulated by cells expressing Dw1/DR1 but also proliferated in response to SLAM (Dw8,?;DR4,?; 12,276 cpm) and CCUM (Dw2,5;DR2,5,w6; 2,494 cpm). Like TLC 14-65, 14-71 was restimulated by all Dw1/DR1+ panel members and one individual (Dw3,6;DR3,w6; OSCH) whose cells

"ced low level stimulation (1,094 cpm). TLC 14-12 was strongly restimulated by a subset (6/8) of cells bearing Dw1/DR1 but was also stimulated at lower levels by five non-Dw1/DR1 panel members (range 1,008 to 1,695 cpm). Interestingly, TLCs 14-20 and 14-43 reacted with 4/6 of the Dw1/DR1+ cells restimulating 14-12, missed RSCH and gave no "extra" reactions with other panel members. In addition to being restimulated by FLAM the positive control, TLC 14-15 reacted also to SLAM (25,920 cpm), RKEL Dw3,?;DR3,?; 29,989 cpm), CGRE (Dw?;DR?; 7,497 cpm) and HMEL (Dw1,12;DR1,2; 1,500 cpm). Finally, TLC 14-2 was not highly stimulated by FLAM (675 cpm) although it did recognize EPAP (Dw1,5;DR1,5; 1,856 cpm), KCAR (Dw2,?;DR2,?; 6,312 cpm) and GYOD (Dw4,4;DR4,4; 7,868 cpm).

Segregation of TLC Responsiveness. Cells obtained from the family of the original stimulator (FLAM) were tested with T-lymphocyte clones to look for segregation of proliferative responses with known HLA markers (Table 2). Although TLC 14-65 responded to the mother (SLAM) who was negative for Dw1/DR1, only those siblings which expressed the Dw1/DR1 marker on the paternal "b" haplotype restimulated this particular clone. TLCs 14-71 and 14-12 reacted similarly although they were not induced to proliferate by SLAM. TLCs 14-43 and 14-20 gave complex patterns of segregation in the family. TLC 14-15 was restimulated by both the positive conrol (FLAM) and SLAM (25,171 and 25,920 cpm, respectively) although only one child (HLAM) was able to stimulate this particular TLC (1,436 cpm). Similarly, proliferation by TLC 14-2 did not segregate with HLA in this family although it was restimulated by some panel members.

Kinetics of Response. Clones 14-1, 14-12, 14-20 and 14-72 were assayed for their kinetic responses to cell LLAM from 12 to 192 hours (Figure 1). Peak responsiveness was observed at 72 hours for TLC 14-1 (16,482 cpm), 120 hours for TLC 14-12 (83,625 cpm), 120 hours for TLC 14-20 (13,441 cpm) and 96 hours for TLC 14-72 (15,718 cpm).

DISCUSSION

In this paper, we have presented data which suggest that the response to HLA-D, as detected in a classic MLC or PLT test, is in reality the result of composite reactions inherent in the clones of the responding cell population. Thus, no particular clone recognized HLA-D/DR exclusively. To the contrary, most clones appeared to react with subsets of the Dw1/DR1 stimulating cell population (e.g., TLC 14-20 and 14-43). Additionally, other TLCs such as 14-65, 14-71 and 14-12 clearly detect non-HLA-Dw1 related antigens. This includes subsets of clones which are restimulated by the positive control (FLAM) and segregate with the Dw1 haplotype in FLAM's family, but which are also restimulated by other cells not expressing Dw1/DR1. In fact, using previous cell typing methods (MLC and PLT), these other cells bear no detectable relationship to FLAM. A minority of TLCs (e.g., TLC 14-15), though positive with the positive control, did not segregate with HLA antigens in family studies even though they may detected determinants present on other panel cells. In contrast, a small percentage of TLCs similar to 14-2, were unstimulated by the positive control but were nonetheless induced to proliferate in response to cell surface antigens on other cell types. Furthermore, the complexity of this situation is compounded by the fact that

kinetic influences may alter the interpretation of positive and negative responses. That is, TLCs like 14-20, which had late kinetics but was nonetheless capable of recognizing a particular antigen, would be missed if assayed early. Furthermore, unexpected results might be caused by a clone which was cytotoxic for some antigens but which proliferated in response to other discrete or composite determinants.

One question remains: Are these clones? Several factors militate against this possibility. When lymphoblasts were originally seeded at 0.3 cells/well, each well was inspected microscopically to insure that cell clumps were not plated. No such clumping was observed. Assuming a uniform, single-cell suspension, by Poisson probabilities, more than 99% of the wells should have contained one or fewer cells. Additionally, only a subset (30%) of those cells originally seeded eventually produced viable lines. At least two clones from this experiment had absolutely identical response patterns (10) which one would not expect of oligoclonal populations. While each of these individual arguments may not prove the clonal nature of our TLCs, in aggregate, they strongly support the probability that most of the TLCs are, bona fide, clones. In the worst possible case, if all possible wells with more than one cell was called a clone, one proliferating well per plate (14%) might be derived from two or more cells. However, if we assume that rather than one functional unit per nine wells was plated, the probability that a given clone derives from two or more precursors in less than 10^{-4}. Until more sensitive techniques for assessing monoclonal features or methods for subcloning are developed, such questions cannot be finally answered.

Other cell surface antigens, different from currently defined HLA specificities, have been demonstrated by other groups using PLT, MLC and TLC techniques (10-16). However, no indication of whether such ancillary specificities correlate with the TLCs defined in these experiments is possible until TLC typing reagents can be exchanged among investigating laboratories. Meanwhile, the use of T-lymphocyte clones provides a powerful tool for the differentiation or definition of lymphocyte cell-surface restimulating structures. We hope that this may ultimately lead to a better understanding of the genetic factors influencing control of human immune responses.

ACKNOWLEDGEMENTS

The authors thank D. Smoot, F. Robbins, R. Hargrove and T. Lionetti for expert technical assistance and Ms. B. Minor for typing this manuscript. Special appreciation is due Drs. D.B. Amos, F.E. Ward and A.H. Johnson for HLA serotyping as well as continued encouragement in this work.

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FIGURE LEGEND

Figure 1. Kinetic responses of four clones, TLC 14-1 ( ), 14-12 ( ), 14-20 ( ) and 14-72 ( ), stimulated by peripheral blood lymphocytes from LLAM. Five thousand TLC cells were cultured with $5x10^4$ irradiated stimulators and assayed at the various time intervals indicated. Although peak response was relatively uniform for each TLC, the magnitude was dependent upon the nature of the stimulating cell present.

Table 1

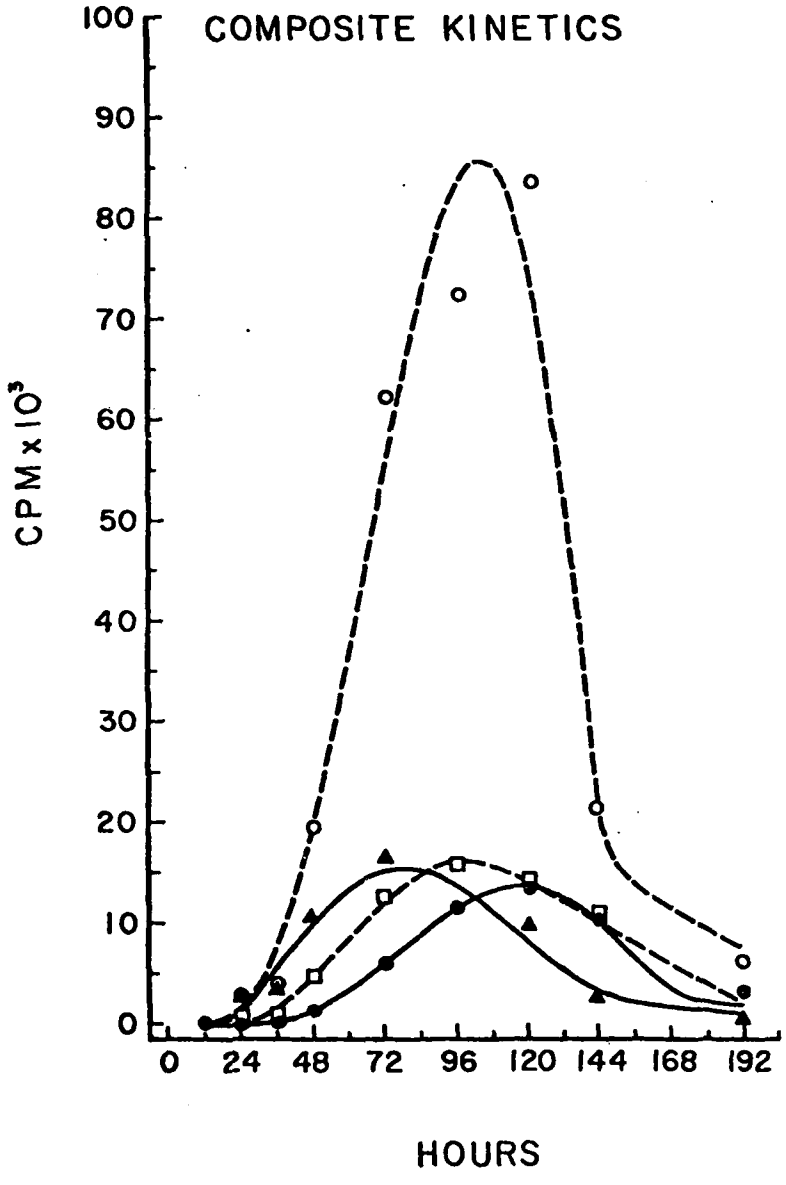
Medians (cpm) of TLC Responses to Cell Panel

			T-Lymphocyte Clones						
Panel	Dw1	DR1	14-65	14-71	14-12	14-20	14-43	14-15	14-2
FLAM	+	+	38,288	20,162	15,959	5,815	8,625	25,171	675
JSAU	+	+	44,807	25,231	15,407	9,767	14,968	304	539
EPAP	+	+	43,142	22,953	15,183	10,460	9,855	329	1,856
FPAP	+	+	36,536	21,542	15,193	4,280	8,330	342	474
BMEL	+	+	37,415	7,470	754	146	130	218	193
HMEL	+	+	33,878	2,665	541	168	92	1,500	102
VSCH	+	+	22,835	16,716	10,335	4,007	5,928	64	166
RSCH	+	+	10,900	11,366	8,556	746	206	110	121
OSCH			64	1,094	310	59	41	136	44
SLAM			12,276	227	735	214	126	25,920	223
CCUM			2,494	161	1,118	187	31	326	575
RCUM			254	256	791	115	142	161	482
CGRE			89	156	1,008	79	37	7,497	72
RKEL			156	186	1,232	186	27	29,989	149
KCAR			784	715	1,695	138	57	341	6,312
SJOH			62	148	710	76	29	322	56
JAYR			274	190	575	217	43	160	106
CBER			206	189	1,903	301	96	159	704
GYOD			399	85	119	53	27	136	7,868
PBUR			51	79	119	44	24	177	37
JKH			67	207	655	236	84	493	103
GHOV			197	155	249	282	54	367	149
JSUM			1,078	609	355	139	22	284	65

Table 2

Segregation of TLC Responsiveness (Medians)

				T-Lymphocyte Clones						
Panel		Dw1	DR1	14-65	14-71	14-12	14-20	14-43	14-15	14-2
FLAM	F	+	+	38,288	20,162	15,959	5,815	8,625	25,171	675
SLAM	M	-	-	12,276	227	735	214	126	25,920	223
FLAM		+	+	34,998	20,135	16,218	6,604	3,272	308	416
GLAM		+	+	14,381	13,475	10,175	1,794	193	511	216
JLAM		+	+	24,515	12,641	10,320	1,009	3,486	327	784
LLAM		+	+	26,424	15,260	11,674	2,799	13	nt	nt
VLAM		+	+	24,130	11,476	12,234	1,887	3,583	172	592
OLAM		+	+	26,159	11,863	12,242	428	775	381	761
HLAM		-	-	2,030	155	185	87	29	1,436	70
ELAM		-	-	193	110	299	116	23	167	82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