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Immunology

Human T-cell clones used to define functional epitopes on HLA class II molecules

(HLA-D specificity/HLA-Dw14/HLA-disease associations)

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ABSTRACT **Polyclonal reagents have been used to define HLA class II molecules in conventional serologic and cellular typing. We generated human alloreactive T-cell clones to analyze the functional fine specificities of HLA class II molecules that might be important for the phenomenon of HLA and disease association. We chose to examine HLA-Dw14, an HLA-D specificity that has been associated with juvenile rheumatoid arthritis. In this paper we have presented data that suggest that conventional cellular typing does not reflect the distribution of T-cell epitopes on major histocompatibility complex class II molecules. We describe three alloreactive T-cell clones that have defined three separate Dw14-associated T-cell epitopes. Two of these epitopes were on a DR-region molecule; the third was located on a DQ-region product. In a panel of unrelated DR4-positive donors, these three Dw14-associated determinants were present in a high frequency but were not linked to each other. Within the tested panel of DR4-positive cells, all possible combinatorial arrangements of these three allodeterminants were seen. The concurrent expression of any two of the three allodeterminants was equivalent to a positive typing response for Dw14. Our finding that HLA-Dw14 is not characterized by a unique allodeterminant but by the combinatorial recognition of independently distributed T-cell interaction sites suggests that analysis of HLA and disease association may be more clearly demonstrated through the use of human T-cell clones.**

One of the most intriguing problems of modern immunology is the remarkable association of a large number of human diseases with the major histocompatibility complex (MHC) (1, 2). The majority of these diseases seem to have a strong association with the MHC class II molecules, the products of the HLA-D region, which may represent the human immune response genes (3, 4). HLA-D region cell surface products play an essential role in restricting the recognition of antigen by T lymphocytes. Speculations about mechanisms of HLA-D and disease association have included the concept that unique immune response genes represent the basis for the disease susceptibility by allowing pathogenic recognition of an environmental antigen.

Human MHC class II genes are characterized by an extensive polymorphism. This is partially reflected by the serological classification of the products of these genes into different HLA-DR haplotypes and further demonstrated by using the T-lymphocyte stimulating properties of these D-region products in mixed lymphocyte cultures to define HLA-D specificities (5). The genetic organization and biochemical nature of the HLA-D determinants, as well as their precise relationship to the serologically defined MHC-class II antigens, is unresolved.

HLA-D determinants represent target structures of alloreactive T lymphocytes and may play an essential role in the regulation of MHC class II restricted antigen-specific T lymphocytes. In HLA-associated autoimmune disease, altered immune responses may be involved in the induction and perpetuation of disease. Recent developments have made it possible to generate monospecific T-cell clones (6) that can be used as probes to analyze the functional fine specificity of class II polymorphism. The serotype HLA-DR4 is composed of at least five HLA-D specificities: Dw4, Dw10, Dw13, Dw14, and Dw15 (7, 8). We have generated alloreactive T-cell clones to dissect the HLA-D specificity Dw14, strongly associated with seropositive juvenile rheumatoid arthritis (9). We describe three alloreactive T-cell clones that recognized separate Dw14-associated determinants. While these clones recognized only Dw14-positive cells in a panel of homozygous typing cells, in testing a panel of cells obtained from unrelated donors with DR4 haplotypes, the T-cell epitopes recognized by these clones appear to distribute independently. These data demonstrate that HLA-Dw14 is not defined by a unique allodeterminant. In an unselected population, Dw14-negative individuals can express single Dw14-associated determinants. A positive typing response for HLA-Dw14 in conventional cellular typing requires the combinatorial association of two or more HLA-Dw14-associated allodeterminants.

MATERIALS AND METHODS

Monoclonal Antibodies. Purified monoclonal antibodies L368, L203, L227, and L243 were a generous gift from the laboratory of Ronald Levy (Stanford University Medical Center). Monoclonal antibody L368 is directed against β_2-microglobulin. Monoclonal antibodies L203 (IgG1), L227 (IgG1), and L243 (IgG2a) recognize monomorphic determinants of DR molecules (10, 11). P4.1 (IgG2), a monoclonal antibody from John Hansen (Fred Hutchinson Cancer Center, Seattle, WA), binds to framework determinants of DR molecules (12). In DR4 haplotypes, only one β chain can be immunoprecipitated by P4.1 (John Hansen and G. Nepom, personal communication). IVD12 (IgG1) that defines an HLA-DQ specificity (MB3) was kindly provided by Robert Giles (University of Texas Southwestern, Dallas, TX) (13). Leu10, a monoclonal antibody against nonpolymorphic HLA-DQ determinants (14) was purified by Becton Dickinson. P17.1, provided by John Hansen, reacts to HLA-DQw2 and -DQw3 determinants. B7/21 has been shown to recognize HLA-DP molecules (15, 16) and was obtained from I. Trowbridge (Salk Institute, La Jolla, CA).

Cell Lines. Homozygous typing cells were generously provided by John Hansen and Erik Mickelson (Seattle), Susan Hsu (Baltimore), and Nancy Reinsmoen (Minneapolis,

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Abbreviations: MHC, major histocompatibility complex; PLT, primed lymphocyte typing reagent(s); PBL, peripheral blood lymphocyte(s); EBV, Epstein–Barr virus.

MN). HLA-DR typing was performed by conventional cytotoxicity on nylon wool-purified, peripheral blood lymphocytes (PBL) or B-lymphoblastoid cell lines by using a panel of antisera recognizing HLA-DR1 through -DRw10 (17). HLA-D typing by primed lymphocyte typing reagents (PLT) was performed as described (18, 19). Briefly, PBL of selected responder cell lines were stimulated against Bin40 and LS40 cells. Primed lymphocytes (1×10^4) were cocultured with 1×10^5 irradiated PBL or $3–4 \times 10^4$ Epstein–Barr virus (EBV) blasts. Optimal discriminating results were obtained after 60 hr of culture.

Generation of T-Cell Clones. PBL of three different responders (C: DR4,4; T: DR2,−; G: DR4,w6) were separated by density gradient centrifugation on Ficoll/Hypaque. PBL (5×10^5 cells/ml) were stimulated by 5×10^5 cells/ml of irradiated lymphoblastoid cells (DR4,4; Dw4/Dw14) in 20 ml of RPMI 1640 supplemented with 10% (vol/vol) fetal calf serum and 2 mM L-glutamine. T cells were restimulated with 5×10^5 cells/ml of irradiated EBV blasts (10,000 rad; 1 rad = 0.01 Gy) at 2-week intervals. After 3–4 restimulations, T-cell blasts were cloned using the technique of limiting dilution at a cell density of 0.3 cells per well in the presence of 1×10^5 irradiated stimulator cells and 10 units of recombinant interleukin 2 per ml (Cetus, Emeryville, CA) in 96-well flat-bottom trays. Growing clones were transferred to 24-well Costar plates, restimulated with antigen and expanded in interleukin 2 containing medium.

T-Cell Proliferation Assay. Rested T cells ($1–2 \times 10^4$) were mixed with 1×10^5 irradiated PBL or 3×10^4 EBV blasts in triplicate cultures in 200 μl of complete medium in 96-well round-bottom plates. After 48 hr, cultures were incubated with 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) and harvested on glass-fiber filters 12 hr later. Thymidine incorporation was measured by liquid scintillation spectroscopy. Results are expressed as the specific thymidine uptake by subtracting the mean thymidine uptake without responder cells from the mean thymidine uptake from cultures with responder cells. Clones without antigen had <300 cpm, stimulators alone had 500–1500 cpm. Standard errors of the triplicate cultures were <20%. To compare results in different assays, Bin40, LS40, and an autologous control were included in all experiments. Proliferation induced by individual stimulators was divided by the maximal thymidine uptake in each assay (usually induced by Bin40) and expressed as relative response. A relative response of <20% was defined as negative.

For target inhibition studies, serial dilutions of monoclonal antibody (0.03–5.0 μg/well) were added in replicate cultures to 3×10^4 irradiated stimulator cells at the initiation of culture. Percent response was calculated by dividing the antigen induced thymidine uptake in the presence of antibody by the control proliferative response in the absence of antibody and multiplying by 100.

RESULTS

Generation of T-Cell Clones Specific for Dw14. T-cell lines of three different donors were established by repeated stimulation with a DR4-homozygous Dw4/Dw14-heterozygous lymphoblastoid line. T-cell blasts were cloned by limiting dilution at a density of 0.3 cells per well. Growing clones were expanded and tested for proliferative activity against the following: (*i*) the original stimulator cells, (*ii*) a Dw4-homozygous cell line, and (*iii*) a Dw14-homozygous cell line. Clones that proliferated in response to the original stimulator and to the Dw14$^+$ stimulator cells but not to the Dw4$^+$ cells were selected for further evaluation. The specificity of three selected clones was assayed on a panel of homozygous typing cell lines, and the results are shown in Table 1. These three clones exhibited clear specificity for determinants expressed by the Dw14$^+$ homozygous typing lines. None of the four Dw4$^+$-homozygous cell lines could induce proliferation in clones T4-31, C1-19, or GA1-5. To exclude reactivity to EBV-associated cell surface products, an autologous control (EBV-transformed cell line of donors), as well as fresh irradiated PBL from selected donors, were included within the test panel (data not presented).

Table 1. Reactivity of clone T4-31, clone C1-19, and clone GA1-5 on a panel of homozygous typing lines

Line	HLA-DR	HLA-Dw	Reactivity, Δcpm		
			T4-31	C1-19	GA1-5
BSM	4,4	4,4	421	<0	353
JAH	4,4	4,4	<0	115	18
BOD	4,4	4,4	438	481	<0
BAR	4,4	4,4	256	367	166
AL10	4,4	10,10	504	<0	<0
TS10	4,4	10,10	161	<0	205
KT2	4,4	13,13	22	<0	506
JHA	4,4	13,13	563	<0	86
Bin40	4,4	14,14	22,599	25,394	24,199
LS40	4,4	14,14	21,107	22,246	24,436
Has15	4,4	15,15	1,990	<0	<0
Autologous controls					
T	2,−		<0	37	234
C	4,4		78	528	2,286
G	4,w6		<0	19,854	290

Proliferation of $1–2 \times 10^4$ responder cells to 3×10^4 irradiated stimulator cells representing the HLA-D specificities of the DR4 haplotype in a 48 hr coculture assay. Results are expressed as the antigen-specific thymidine uptake Δcpm, which was calculated by subtracting the thymidine incorporation of the stimulator alone from the thymidine incorporation of the responder cells in stimulator coculture. Without antigen, cpm were <300. Stimulators alone had 500–1500 cpm.

Distribution of Dw14-Associated Determinants in Unrelated DR4 Haplotypes. To analyze the distribution of Dw14-associated specificities defined by clone T4-31 and clone C1-19, we tested these clones on a panel of 44 unrelated DR4$^+$ donors (Fig. 1). In addition to healthy controls this panel included patients with seropositive rheumatoid arthritis and patients with type I diabetes. Seven out of 40 of these individuals were Dw14$^+$ by the primed lymphocyte typing technique, which is in the range of reported frequencies (8, 9). Four additional Dw4/Dw14 heterozygous cell lines from patients with seropositive juvenile rheumatoid arthritis were provided by G. Nepom (University of Washington, Seattle) and are included in the panel. Surprisingly, these T-cell clones that were specific for Dw14-associated epitopes on the panel of homozygous typing cells (Table 1) were reactive against different subsets of the DR4 haplotypes and recognized non-Dw14$^+$ cells. The Dw14 determinant recognized by clone T4-31 was present at a high frequency, 20 out of 44 stimulators induced proliferation. Clone C1-19 was reactive against a Dw14-associated allodeterminant that was somewhat less frequent (15 out of 44) among the DR4 phenotypes but was clearly not included within the subset defined by clone T4-31. Concurrent expression of both epitopes was infrequent (6 out of 44 individuals) but the stimulator cells that were recognized by both clones typed Dw14$^+$.

The majority of stimulators seen by either clone T4-31 or by C1-19 were Dw14-negative by PLT indicating that the expression of only one Dw14-associated determinant did not elicit a Dw 14-typing response. The fact that cells from five persons, who were typed as Dw14-positive by PLT, induced proliferation in only one of the two clones suggested the possibility that there exist additional Dw14-associated determinants that were not defined by these two alloreactive T-cell clones. The functional importance of the determinants de-

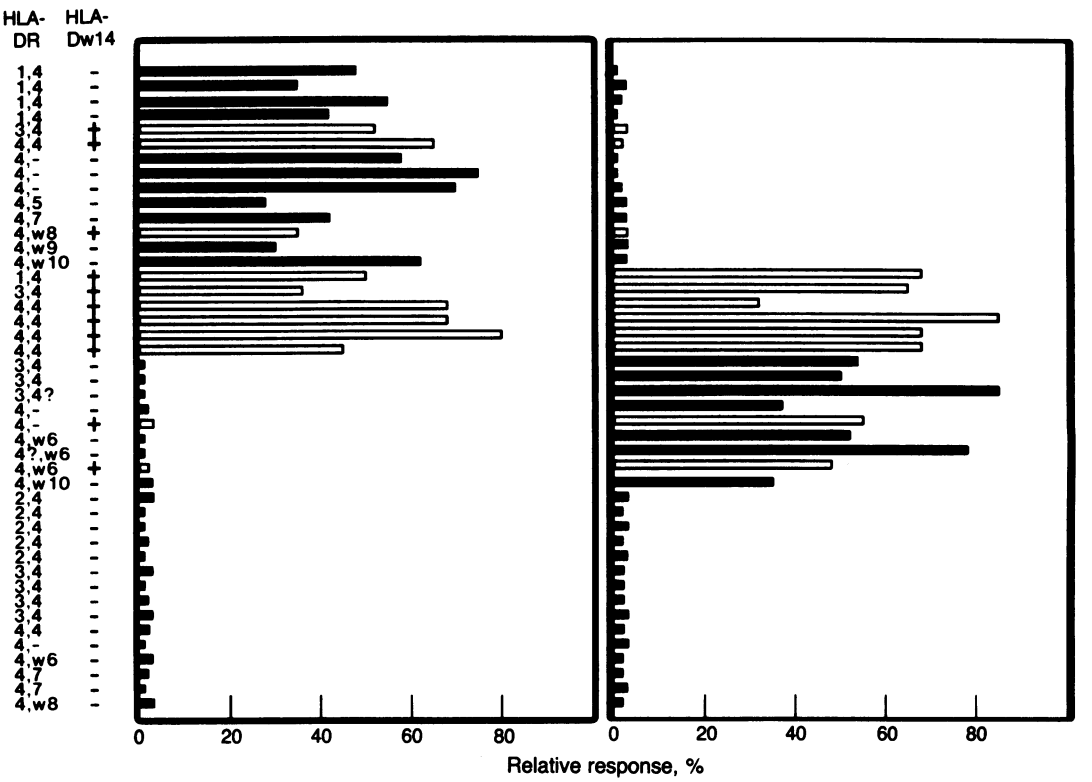

FIG. 1. Distribution of Dw14-associated determinants in 44 unrelated DR4$^+$ donors. Rested cloned cells (1×10^4 cells per well) were cocultured with 3×10^4 irradiated EBV blasts or 1×10^5 irradiated PBL. Individual proliferative responses are expressed as a percent of the maximum thymidine uptake induced by Bin40 cells. Maximal responses of clone T4-31 (*Left*) were in the range of 16,887 and 22,599 cpm, C1-19 (*Right*) incorporated between 17,542 and 25,394 cpm. Open bars indicate donors that typed Dw14-positive by cellular typing.

fined by these two clones was, however, emphasized by the finding that all Dw14-positive individuals were included within the subset defined by one or the other of these two clones.

Concurrent Expression of Two Out of Three Dw14-Associated Allodeterminant Defines the HLA-D Type Dw14. To prove the hypothesis that the expression of at least two Dw14-associated allodeterminants was necessary to give a positive typing response for Dw14, we generated additional T-cell clones from a DR4/DRw6 donor who typed Dw14-negative but whose cells were recognized by one of the Dw14-specific clones (C1-19). One such clone, GA1-5 was also defined as a clone with specificity for Dw14 on the homologous typing cell panel as shown in Table 1. Tested on the panel of cells from donors with unrelated DR4 haplotypes, clone GA1-5 reacted to a distinct subset when compared to the reactivity pattern of clone T4-31 or clone C1-19. Ten out of 44 individuals express the Dw14-associated allodeterminant defined by GA1-5 (data not shown), 6 of which belong to the Dw14$^+$ subgroup. The reactivity pattern of the three Dw14-specific alloreactive clones on the 11 Dw14$^+$ stimulators is presented in Fig. 2. All individuals who typed Dw14$^+$ by PLT but had been seen by only clone T4-31 or by clone C1-19 were recognized by clone GA1-5, support-

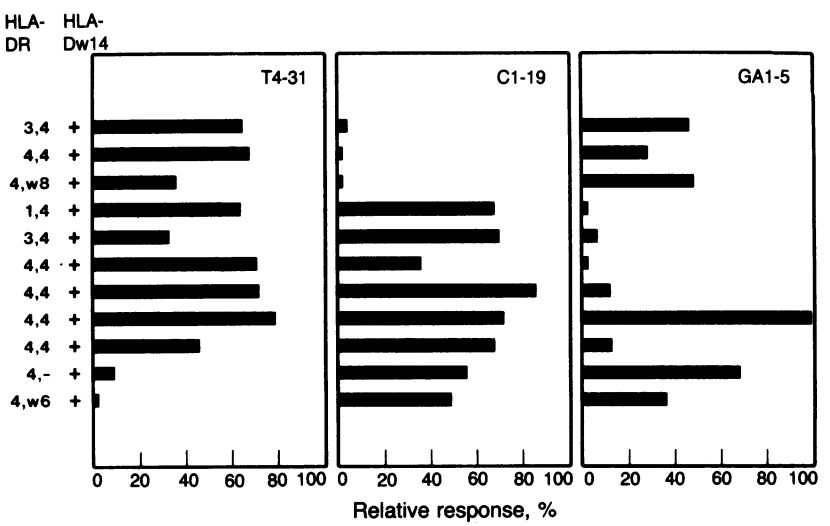

FIG. 2. Concurrent expression of at least two Dw14-associated T-cell recognition sites in Dw14$^+$ haplotypes. Proliferative reactions of clone T4-31, clone C1-19, and clone GA1-5 stimulated by cells from Dw14$^+$ individuals were assayed and evaluated as described in Fig. 1.

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Assignment of the Specificities Defined by T4-31 and C1-19 to Products of the HLA-DR and HLA-DQ Region. To assign the molecules recognized by the Dw14-specific clones to an HLA-D subregion, we tested the proliferative response of the clones in the presence or absence of a panel of monoclonal antibodies reactive against monomorphic determinants of the DR, DQ, and DP regions. In all target inhibition experiments, a monoclonal antibody against β_2-microglobulin (L368) was included as a control that should not affect proliferation of class II reactive T-cell clones. Blocking data presented in Fig. 3 provided strong evidence that T4-31 recognized a T-cell epitope on products of the HLA-DR region whereas C1-19 was directed against a DQ-encoded molecule. Proliferation of clone T4-31 was efficiently blocked by four different DR-specific monoclonal antibodies (Fig. 3*A*). In DR4 haplotypes, monoclonal antibody P4.1 immunoprecipitated only one DR β chain (J. Hansen and G. Nepom, personal communication). L243 and L203 bound to at least two species of DR antigens (10, 11). These data suggested that the epitope seen by T4-31 resided on an α–β dimer of the DR region. None of the three anti-DQ antibodies inhibited the proliferative response of clone T4-31 (Fig. 3*A*). Proliferation of clone C1-19 was not affected by any of the DR specific antibodies, but was significantly blocked by one of the DQ-specific antibodies, IVD12 (Fig. 3*B*). In the presence of monoclonal antibody IVD12, the proliferation response of clone C1-19 was reduced by 70%. Blocking of DP molecules by monoclonal antibody B7/21 did not influence the proliferation of either of these two clones (data not shown).

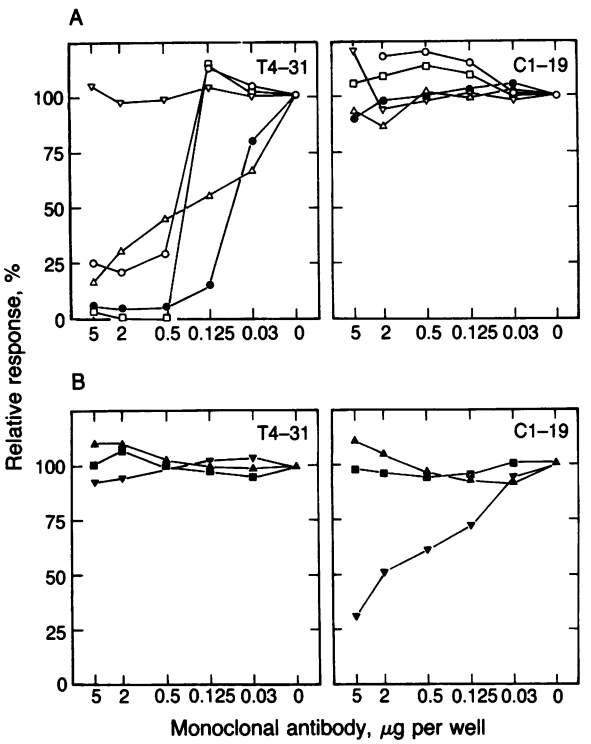

FIG. 3. Inhibition of proliferation induction of clone T4-31 and of clone C1-19 by monoclonal anti-DR or anti-DQ antibodies. Alloantigen-induced proliferation of clone T4-31 and clone C1-19 was measured as described in Fig. 1. At the initiation of culture, 0.03–5 μg of monoclonal antibody per well was added in replicate cultures to 3×10^4 irradiated Bin40 stimulator cells. Percent response was calculated by dividing the antigen-induced thymidine uptake in the presence of antibody by the control proliferative response and multiplying by 100. In the absence of monoclonal antibody, T4-31 incorporated 13,561 cpm, and C1-19 incorporated 19,195 cpm. The following antibodies were used: anti-DR [L203 (□), L227 (△), L243 (○), P4.1 (●)]; anti-DQ [IVD 12 (▼), Leu 10 (■), P17.1 (▲)]; anti-β_2-microglobulin [L368 (▽)].

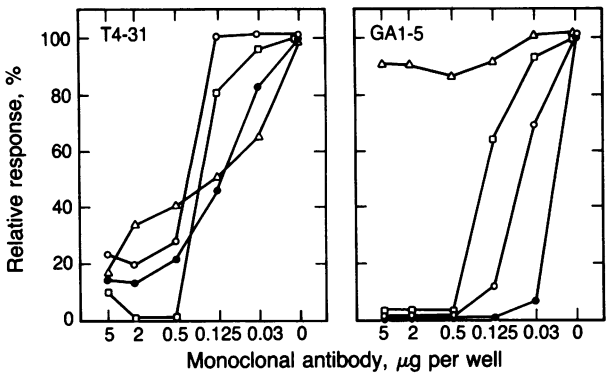

FIG. 4. Distinct inhibition patterns of two DR-reactive clones, T4-31 and GA1-5, by monoclonal anti-DR antibodies. Inhibition of proliferative responses of clone T4-31 and clone GA1-5 by an identical panel of monoclonal anti-DR antibodies as described in Fig. 3 showed that each clone recognized a distinct epitope.

T4-31 and GA 1-5 Are Directed Against Distinct DR Determinants. Target inhibition studies using an identical panel of monoclonal antibodies demonstrated that clone GA1-5 was directed against a DR-encoded molecule. Addition of anti-DQ or anti-DP antibodies did not affect the proliferative response of clone GA1-5. As shown in Fig. 4, monoclonal antibodies L203, L243, and P4.1 strongly inhibited the reaction of GA1-5; P4.1 was the most effective blocking antibody. In contrast to the DR-reactive clone T4-31, DR-reactive clone GA1-5 could not be blocked by monoclonal antibody L227. The differential blocking pattern induced by the four DR-specific antibodies indicated that T4-31 and GA1-5 recognized distinct T-cell epitopes.

DISCUSSION

Similar to the I region of the mouse, the human HLA-D region encodes a highly polymorphic family of cell surface glycoproteins. Gene products encoded within that region stimulate the proliferation of allogeneic lymphocytes, the basis for the definition of HLA-D specificities (5, 7). The technique of HLA-D typing by mixed-lymphocyte culture depends on the similarity of the donor cells (being typed) to a panel of commonly accepted homozygous typing lines. Interpretation of typing data has been complicated by the lack of a clear-cut differentiation between positive and negative typing responses (20). In the primed lymphocyte typing assay, as well as the typing utilizing homozygous typing cells, an arbitrary cutoff for positive and negative responses has been chosen (8, 19, 21) because complete or almost complete identity of HLA-D allostimulating antigens appeared to be the exception (21). More recent approaches to HLA-D typing included biochemical and genetic analysis of the HLA-D molecules and genes (22, 23). Nepom *et al.* (23) demonstrated by two-dimensional gel electrophoresis that five different DR β chains and three different DQ β chains could be distinguished among the different alleles of HLA-D within the DR4 family.

We generated human alloreactive T cell clones to analyze the HLA-D specificity Dw14 by yet another technique, the function of allostimulation. On a panel of homozygous typing lines, three different human alloreactive T-cell clones could be shown to distinguish between $Dw14^+$ and $Dw14^-$ stimulator cells. Target inhibition using monoclonal antibodies against DR, DQ, and DP molecules demonstrated that the three different clones recognized three different antigenic determinants: two Dw14-associated allodeterminants were

demonstrated to be on a DR α–β dimer, the third determinant was encoded by the DQ region. The two DR-reactive clones defined distinct epitopes. One of the clones, GA1-5, was not blocked by monoclonal antibody L227 (anti-DR) whereas the other DR-reactive clone, T4-31, was blocked by the entire panel of anti-DR antibodies used including L227 (Fig. 4). Monoclonal antibody P4.1 immunoprecipitated a single DR β chain in DR4 haplotypes and blocked the proliferation of both DR-reactive T-cell clones. These data suggested that these two T-cell epitopes were present on a single DR molecule.

In a population of unrelated DR4$^+$ individuals, the Dw14-associated determinants were present in both Dw14-positive and Dw14-negative donors. This suggested that Dw14 was not defined by a unique MHC class II determinant. The demonstration that stimulators that carried at least two out of the three Dw14-associated determinants seen by these three clones were Dw14$^+$ suggested that a combinatorial association of T-cell epitopes were required for HLA-Dw14 typing. The functional role of a similar gene–dose effect in allostimulation has been demonstrated in the murine system (24). In our group of Dw14$^+$ stimulators, we found all four possible combinatorial associations of the three different Dw14-associated determinants. The concurrent expression of the DQ-Dw14-associated T-cell epitope and one of the DR-Dw14-associated determinants as well as the combination of both DR-Dw14-associated determinants was sufficient to induce a Dw14$^+$ PLT response. The presence of all three Dw14-associated determinants was a characteristic finding for the Dw14 homozygous typing lines, but was seen on only one Dw14$^+$ donor from the tested panel. This suggested that the Dw14$^+$-homozygous typing lines might not be representative of Dw14$^+$ individuals in an unselected population. Such homozygous typing lines are selected by their unique stimulatory capabilities for polyclonal typing reagents and, according to our data, require the combinatorial expression of multiple Dw14-associated epitopes. Our data suggest that such Dw14 associated allodeterminants as defined by T-cell clones show varying patterns of linkage disequilibrium. Difficulties in evaluating PLT assays based on comparisons of their response to homozygous typing lines may reflect the heterogeneity of the HLA-D specificities in the tested population rather than the inefficiency of the method.

The distribution pattern of the Dw14-associated determinants defined by our three clones demonstrated that Dw14 was not characterized by a unique determinant but by a combination of different Dw14-associated determinants. Class II molecules act as restriction elements for antigen recognition by certain subsets of T cells. Determinants defined by alloreactive T-cell clones may reflect functional T-cell recognition sites. Our finding that Dw14-associated determinants were present in a high frequency and independently distributed in Dw14$^-$-DR4$^+$ donors demonstrated the limitations of HLA-D region typing for the functional analysis of class II antigens in transplantation and HLA-disease association. The use of T-cell clones may redefine HLA-disease associations in an interesting and more relevant manner.

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