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Point mutations define positions in HLA-DR3 molecules that affect antigen presentation

(major histocompatibility complex/class II molecule/mutant mapping)

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ABSTRACT **Allelic differences in major histocompatibility complex (MHC)-encoded class II molecules affect both the binding of immunogenic peptides to class II molecules and the recognition of MHC molecule–peptide complexes by T cells. As yet, there has been no extensive mapping of these functions to the fine structure of human class II molecules. To determine sites on the HLA-DR3 molecule involved in antigen presentation to T cells, we used monoclonal antibodies specific for HLA-DR3 to immunoselect mutants of a B-lymphoblastoid line. We located the sites of single amino acid substitutions in the HLA-DR3 molecule and correlated these structural changes with patterns of recognition by HLA-DR3-restricted, antigen-specific T cells, allospecific T cells, and allospecific anti-DR3 monoclonal antibodies. We analyzed seven mutations. One mutation, at position 74 in domain 1 of the DR β chain, affected recognition by all T cells tested, whereas others, at positions 9, 45, 73, 151, and 204 of the DR β chain and position 115 of the DR α chain, altered recognition by some T cells, but not others. Each of the substitutions resulted in a unique pattern of T-cell stimulation. In addition, each T-cell clone recognized a different subset of the mutants. These results indicate that different residues of the DR3 molecule are involved in presentation of antigen to different DR3-restricted T cells. These studies further show that substitutions which most likely affect peptide binding alter recognition of DR3 molecules by an alloreactive T-cell clone and some allospecific antibodies.**

Major histocompatibility complex (MHC) molecules are highly polymorphic cell surface glycoproteins whose most evident and best understood function is to present immunogenic peptide antigens to T lymphocytes (1, 2). In addition, allelic variation in MHC class II molecules is associated with susceptibility or resistance to autoimmune diseases (3). The essential relationship between the polymorphism of MHC molecules and their function is well documented (4) and suggests that the locations of the hypervariable regions of MHC class II molecules are likely to identify functional domains (5, 6). However, only a few studies have examined the particular contribution of individual class II residues to antigen presentation, in murine (7–9) or human (10) systems.

Brown *et al.* (11) have proposed a structural model of the class II binding domain for antigen, based on the crystal structure of an MHC class I molecule. This model identifies amino acid residues that are involved in peptide binding or in T-cell interactions based on their locations and the orientation of their side chains. One experimental approach for determining the function of individual amino acid residues, and thus testing the model's predictions, is to generate somatic cell mutants with single amino acid substitutions in class II molecules, to map their mutations, and to characterize their functional defects. Using a B-lymphoblastoid cell line (B-LCL) as progenitor, we have immunoselected mutants with single amino acid substitutions in the DR3 molecule. Here, we report seven mutant B-LCL clones in which DR3 mutations are associated with altered antigen-presenting function. We have mapped the mutations and found that changes outside as well as within the putative peptide-binding domain perturb antigen presentation by DR3 molecules. The results also suggest that the reactivities of some allospecific antibodies and an allospecific T-cell clone are sensitive to alterations in peptide binding by class II molecules.

MATERIALS AND METHODS

Antigen-Presenting Cell (APC) Lines. With the exception of mutant clones 7.25.6, 10.22.6, 7.31.6, 10.3.6, 10.77.6, and 8.39.7, the B-LCLs have been reported. Clone 8.1.6, derived from the T5-1 progenitor line (12), is deleted for all *DR* and *DQ A* and *B* genes on one haplotype. Clone 8.1.6 retains expression of all HLA genes of the other (DR3) haplotype, including *DRB1*0301*, which encodes the β chain of the DR3 molecule, and *DRB3*0301*, which encodes the β chain of the DRw52 molecule (13). Clone 9.22.3 is a homozygous *DRA* deletion mutant derived from 8.1.6; it lacks expression of both the DR3 molecule and the DRw52 molecule (14). Clone 9.4.3 is an 8.1.6-derived mutant that lacks *DRB1* mRNA but expresses the DRw52 molecule at normal (8.1.6) levels (15). The DR3 mutant clones 7.25.6, 10.22.6, 7.31.6, 10.3.6, and 10.77.6 were isolated from 8.1.6 by ethyl methanesulfonate mutagenesis, then immunoselection with anti-DR3 monoclonal antibody (mAb) 16.23, followed by complement-mediated lysis. Conditions for immunoselection with mAb 16.23 have been described (14). Mutant 8.39.7 was isolated by the same protocol, using a different anti-DR3 mAb, CD6.B1 (16). Clone 7.13.6, a previously described DR3 point mutant, was also isolated by 16.23 immunoselection (10).

Sequence Analysis of Mutant *DRA* and *DRB* Genes. Cytoplasmic RNAs were prepared from mutant cells by guanidine hydrochloride extraction, and poly(A)$^+$ mRNA was separated on an oligo(dT)-cellulose minicolumn (17). To prepare cDNA, 5–10 μg of poly(A)$^+$ RNA was incubated with 500 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in a first-strand reaction and then with 100 units of *Escherichia coli* DNA polymerase

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Abbreviations: MHC, major histocompatibility complex; APC, antigen-presenting cell; LCL, lymphoblastoid cell line; mAb, monoclonal antibody; TCR, T-cell antigen receptor; PPD, purified protein derivative of *Mycobacterium tuberculosis*; TT, tetanus toxoid; HBsAg, hepatitis B surface antigen; PCR, polymerase chain reaction; RR, relative response.

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(New England Biolabs) in a standard second-strand reaction (18). After phenol/chloroform extraction and ethanol precipitation, the cDNA was suspended in 50 μl of 10 mM Tris, pH 7.5/1 mM EDTA and 1/10th of the volume was used for amplification by the polymerase chain reaction (PCR). For amplifications of *DRB1*, primers B12 (plus strand, 5′ untranslated region, 5′-GTCGACCCTGGTCCTGTCCTGTTCTCC-3′) and B14 (minus strand, 3′ untranslated region, 5′-AAGAATAACAGCCAGGAGGGAAAGCTT-3′) were used. For amplification of *DRA*, primers were A10 (plus strand, 5′ untranslated region, 5′-GTCGACACTCCCAAAAGAGCGCCCAA-3′) and A11 (minus strand, 3′ untranslated region, 5′-AAGCTTTAAGAAACACCATCACCTCC-3′) were used. After 25 cycles of denaturing at 94°C, annealing at 55°C, and elongation at 72°C, the reaction mixture was loaded onto a 2 × 3-inch (1 inch = 2.54 cm), 11-ml minigel and electrophoresed for 15 min at 80 V. After staining with ethidium bromide, the prominent band of the predicted size was excised and the DNA was recovered by using a silica matrix system (Bio 101, La Jolla, CA). For a sequencing reaction, 1/10th of the amount recovered was subjected to dideoxy sequencing using modified T7 DNA polymerase (Sequenase, United States Biochemical). The PCR primers and additional oligonucleotide primers covering 21-base-pair segments of the coding regions or its inverse complement were used to generate 100- to 300-base segments of sequence information, resulting in the complete sequence of the coding regions. The complete coding sequences of the *DRA* and *DRB1*0301* genes from 8.1.6 are identical to sequences reported previously (10, 19, 20).

Radioimmune Binding Assays. For binding assays, a panel of mAbs that bind to the DR3 molecule was used. mAbs 16.23 (21), CD6B.1 (16), and 7.3.19 (22) recognize polymorphic determinants expressed on both DR3 and DRw52 molecules. mAb UK8.1 (23) recognizes a polymorphic determinant expressed on DR3 molecules; mAb VI.15 (24) recognizes a monomorphic DR determinant. The mAbs are likely to react with different epitopes on DR3 molecules by virtue of their different specificities and the inability to cross-inhibit one another (data not shown). For binding assays, saturating amounts of mAbs were used; bound antibody was detected with ^{125}I-labeled rat anti-mouse κ chain. The binding assay has been described (24).

T-Cell Proliferation Assays. Human T-cell lines specific for soluble protein antigen were generated as described (25). The antigens tetanus toxoid (TT), purified protein derivative of *Mycobacterium tuberculosis* (PPD), and hepatitis B surface antigen (HBsAg) were used as described (10, 25). Antigen-specific T-cell clones were obtained by standard methods of limiting-dilution cloning in the presence of antigen, exogenous interleukin 2, and irradiated peripheral blood mononuclear cells as APC. The gp350/DR3 clone is specific for a major envelope glycoprotein of Epstein–Barr virus (B.C., unpublished work). The allospecific, anti-DR3 clone was isolated as described (26). T-cell clones are designated by antigen/restriction element. The restriction element used by a T-cell clone was identified by comparing the stimulation observed with a panel of class II antigen-loss mutants derived from 8.1.6, as described (25). For example, PPD/DR3 is stimulated by antigen presented by 8.1.6, but not by the DR3-loss mutant 9.4.3, indicating restricted presentation by the DR3 molecule. Clones with the same apparent specificity were isolated independently. For the antigen-specific T cells, T-cell stimulation was measured by incorporation of [^{3}H]thymidine into DNA: cpm in the presence of soluble antigen − cpm in the absence of soluble antigen (25). For the allospecific T-cell clone, stimulation was measured as cpm in the presence of stimulator APCs − cpm in the presence of DR-negative, control APCs. Assays were performed in triplicate and relative response (RR) to antigen presentation by the mutants was calculated as (median T-cell stimulation by the mutant APC/median T-cell stimulation by progenitor 8.1.6) × 100. The RR of the alloreactive T-cell clone to 2× APCs was calculated as response to 2 × 10^5 mutant APCs/response to 10^5 8.1.6 cells.

RESULTS

Sequence Analysis of the Mutant *DRA* and *DRB1* Genes. We derived DR3 mutants from a *DR/DQ* hemizygous progenitor B-LCL, 8.1.6, which expresses DR3, DR52a, DQw2, and DPw4.1. The mutant clones were isolated by immunoselection with a DR3-specific mAb, either mAb 16.23 (mutants 7.13.6, 7.25.6, 7.31.6, 10.77.6, 10.22.6, and 10.3.6) or mAb CD6B.1 (mutant 8.39.7). We chose seven DR3 mutants for sequence analysis. The selected mutants expressed an altered DR polypeptide, as judged by two-dimensional gel electrophoresis of immunoprecipitated DR α and β chains (data not shown), and/or demonstrated altered antigen presentation to DR3-restricted T cells (described below). We used the PCR to amplify the entire *DRB1* coding region from all the DR3 mutants as well as the *DRA* coding region from 8.39.7 and 10.77.6. We found a single point mutation in each mutant [Table 1; data from immunoselected mutant 7.13.6, previously reported (10), are also shown]. In six mutants, the mutation is in the *DRB1* gene, and in one, in the *DRA* gene. Each mutant *DRB1* gene differs from wild type by a G → A transition; G → A transitions are common ethyl methanesulfonate-induced mutations (27). Mutant 10.77.6 has a mutation in the *DRA* gene that is also a single nucleotide transition (C → T). In each mutant, the nucleotide substitution results in an amino acid change that is consistent with the isoelectric point of the mutant protein (ref. 10 and unpublished results). The protocol used for generating the mutants makes it likely that each mutant harbors only a single mutation (28).

Localization of DR β- and α-Chain Substitutions on a Model of Class II Molecular Structure. To locate the positions of the seven mutations on a structure that approximates the folded DR3 molecule, we identified residues of HLA-A2 that correspond to the mutant DR3 positions and located them in the class I molecular structure (Fig. 1 *Upper*) (29). Mutations in the antigen-binding domain were also located on the Brown and Wiley model of class II molecular structure (Fig. 1 *Lower*) (11). Three of the DRβ substitutions (in mutants 8.39.7, 10.22.6, and 7.31.6) form a cluster in the DRβ1 domain, whereas three other substitutions (in mutants 7.13.6, 10.77.6, and 10.3.6) are located at a distance from this cluster. The mutation in 10.77.6 is in the DRα chain. Based on the orientation of amino acid side chains at the mutant positions as predicted from the crystal structure of the HLA-A2 molecule (29), the DRβ substitutions in 7.13.6 and 10.3.6 are likely to affect the conformation of both the DRβ and DRα chains (see *Discussion* and ref. 10). All seven mutations alter the binding of mAb 16.23 (Table 2 and ref. 10), suggesting that

Table 1. Sequence changes in the DR3 mutants

Mutant	Nucleotide substitution	Amino acid substitution	Position	Domain
7.13.6*	GAG → AAG	$Glu^- \rightarrow Lys^+$	9	β1
7.31.6	GGG → AGG	$Gly^0 \rightarrow Arg^+$	45	β1
10.22.6	GGC → AGC	$Gly^0 \rightarrow Ser^0$	73	β1
8.39.7	CGG → CAG	$Arg^+ \rightarrow Gln^0$	74	β1
10.3.6	GGA → AGA	$Gly^0 \rightarrow Arg^+$	151	β2
7.25.6	GGG → GAG	$Gly^0 \rightarrow Glu^-$	204	TM (β)
10.77.6	CCA → CTA	$Pro^0 \rightarrow Leu^0$	115	α2

TM, transmembrane.
*Previously reported (10).

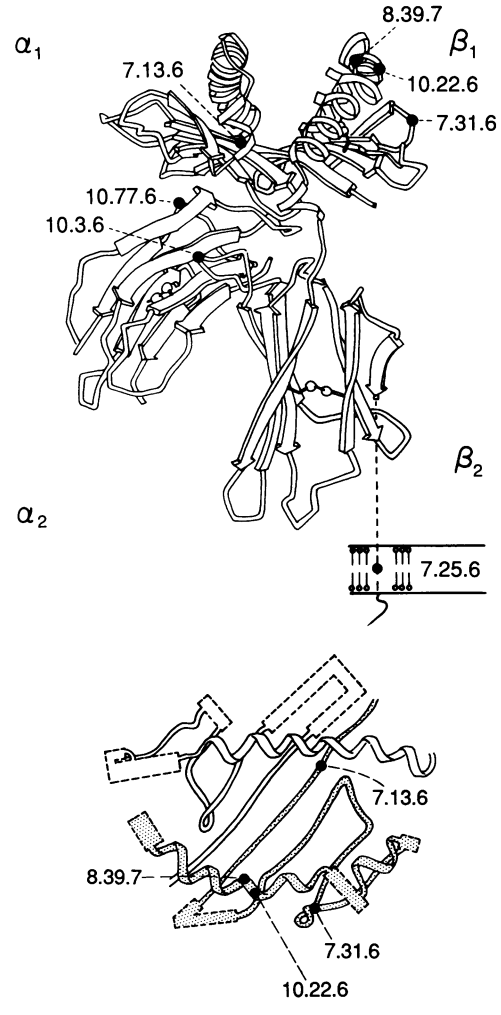

FIG. 1. Location of HLA-DR3 mutations on a schematic representation of the HLA-A2 structure (*Upper*) and on the model of the antigen-binding domain of the MHC class II molecule (*Lower*). Numbers designating the mutants are indicated. (*Upper*) The mutated amino acid residues shown on a schematic representation of the HLA-A2 molecule (heavy chain and β_2-microglobulin), which has shared structural features with class II molecules (11). The domains are labeled as the corresponding class II domains (α1, α2, β1, and β2). A portion of the β-chain transmembrane domain is shown schematically; the α-chain transmembrane domain is not shown. The residues that form the domains are as follows: α1, 1–85; α2, 86–182; α transmembrane–cytoplasmic, 183–233; β1, 1–95; β2, 96–189; β transmembrane–cytoplasmic, 190–238. The structural features of the HLA-A2 molecule have been described (29). (*Lower*) Schematic representation of the hypothetical class II molecular structure of Brown, Wiley, and coworkers (11). The α1 and β1 (*stippled*) domains are shown, as viewed from the top of the molecule. According to this model, the predicted orientations of amino acid side chains at the mutated sites are as follows: 7.13.6 and 8.39.7, toward the binding site; 10.22.6, up, toward the T-cell antigen receptor (TCR); 7.31.6, toward the β-strand (11).

both DRα and DRβ chains contribute to the determinant recognized by mAb 16.23.

Expression of Cell Surface HLA-DR3 Molecules in the DR3 Mutants. To determine the levels of cell surface DR3 molecules in the mutants, we measured cell surface binding of a panel of DR3-specific and DR-monomorphic monoclonal antibodies (Table 2). The near-normal binding of monomorphic anti-DR antibody VI.15 and some of the polymorphic antibodies to mutants 8.39.7, 7.31.6, and 10.22.6 indicates that these cells express approximately normal levels of mutant DR3 molecules on the cell surface; the mutant molecules, however, have lost expression of particular DR3 epitopes. The DR3 molecule in mutant 8.39.7, for example, has markedly reduced binding of polymorphic antibody CD6B1 but binds anti-DR monomorphic antibody VI.15 normally. In contrast, mutants 7.25.6, 10.3.6, and 10.77.6 demonstrate reduced binding of all antibodies tested, indicating that they express reduced levels of DR3 molecules on the cell surface. Three of the four DR3-specific antibodies react with both DR3 and a linked, minor DR molecule, DRw52, previously estimated to constitute 10–15% of the DR molecules expressed by progenitor 8.1.6 (15). Adjusting for the contribution of DRw52 molecules to antibody binding to the mutants, (see legend, Table 2), we estimate that mutants 7.25.6, 10.3.6, and 10.77.6 have reductions of cell surface DR3 molecules to approximately 55%, 30%, and 20% of 8.1.6 levels, respectively (Table 2); data from mutant 7.13.6, previously reported (10), are also shown.

Table 2. Cell surface radioimmune binding analysis of DR3 mutants and 8.1.6 with a panel of anti-DR antibodies

Cell line	Antibody binding ratio* × 100				
	16.23	CD6B1	7.3.19	UK8.1	VI.15
8.1.6	100	100	100	100	100
8.39.7	13 ± 6	25 ± 4	90 ± 2	73 ± 7	99 ± 10
7.31.6	17 ± 3	92 ± 9	35 ± 2	92 ± 4	88 ± 7
10.22.6	8 ± 4	103 ± 10	79 ± 6	98 ± 10	82 ± 6
7.25.6	35 ± 4	60 ± 9	40 ± 4	50 ± 8	45 ± 4
10.3.6	24 ± 3	24 ± 4	35 ± 5	37 ± 5	45 ± 3
10.77.6	21 ± 3	24 ± 7	28 ± 5	8 ± 1	35 ± 4
7.13.6	29 ± 4	45 ± 4	66 ± 2	25 ± 3	47 ± 6
9.4.3†	9 ± 3	4 ± 2	23 ± 2	0	13 ± 3

*Binding ratio was calculated as (cpm bound by mutant − cpm bound by negative control) ÷ (cpm bound by 8.1.6 − cpm bound by negative control). Data represent medians ± SEM from four or more experiments. Negative control was the DR-null mutant, 9.22.3.

†The contribution of DRw52 molecules to antibody binding was measured by binding to mutant 9.4.3, an 8.1.6-derived mutant that has lost expression of DR3 molecules but expresses DRw52 molecules normally (15). The level of DRw52 molecules expressed by the mutants is equivalent to that expressed by 9.4.3 as judged by the amount of DRw52 molecules in immunoprecipitates of DR molecules from 9.4.3 and from the DR3 mutants (data not shown).

Most of the Mutants Have Selective Defects in Antigen Presentation. To evaluate the capacity of the mutant DR3 molecules to act as restricting elements for antigen-specific T cells, we examined the ability of the DR3 mutants to stimulate a panel of DR3-restricted T cells specific for different soluble antigens (Table 3). Only one of the substitutions, at position 74 of DRβ (mutant 8.39.7), disrupts antigen presentation to all of the DR3-restricted T cells assayed. This profound effect results from the *DRB1* mutation in 8.39.7, and not from a generalized abnormality of antigen processing and presentation, as this mutant stimulates normal proliferation of T cells restricted by DRw52 and DP4. In contrast, the mutations in 7.13.6, 7.31.6, 10.22.6, 10.3.6, and 10.77.6 result in selective defects in DR3-restricted presentation. Each mutation is associated with a unique pattern of T-cell stimulation. The mutations in 7.13.6 and 7.31.6 ablate presentation to one antigen-specific, DR3-restricted clone (PPD/DR3), but allow normal presentation to several other T cells. Mutant 10.22.6 cannot stimulate one DR3-restricted, PPD-specific clone, stimulates the gp350-specific clone to a lower level than progenitor 8.1.6, and effectively stimulates three other DR3-restricted clones. Mutant 10.3.6 is affected for three of five antigen-specific clones tested (TT/DR3, PPD/DR3, and gp350/DR3). Mutant 10.77.6 is defective in the stimulation of four of five T-cell lines tested, but patterns of T-cell stimulation differ from that of the 10.3.6 mutant. The selective

Table 3. Response (RR) of HLA-restricted antigen-specific or allospecific T cells to stimulation by mutant APCs and by 8.1.6

	RR of T-cell clones (antigen/restriction element)									
							Allo/DR3*			
APCs	TT/DR3	TT/DR3	PPD/DR3	PPD/DR3	PPD/DR3	gp350/DR3	1×	2×	PPD/DRw52	HBsAg/DPw4
8.1.6	100	100	100	100	100	100	100	100	100	100
8.39.7	36 ± 5	0	0	0	0	0	3	6	110	104
10.22.6	75 ± 7	78	ND	0	85	42	104	ND	ND	99
7.31.6	100 ± 5	98	0	103	96	ND	102	ND	104	90
10.3.6	37 ± 6	78	ND	0	95	0	30	60	102	80
7.25.6	98 ± 8	93	94	100	98	ND	50	100	105	87
10.77.6	35 ± 5	12	ND	101	0	0	35	68	66	113
7.13.6†	ND	96	0	110	ND	69	6	8	ND	103

RR was calculated as described in *Materials and Methods*. Mean RR and standard error were calculated from at least three experiments, except for RR to gp350 and PPD/DRw52, which are the means of two experiments. The standard errors shown for RR of TT/DR3 are representative. Under the conditions of these assays, the observed defects in antigen-specific T-cell stimulation derive primarily from the qualitative, rather than quantitative, changes in the mutant DR3 molecules; defects in the presentation of soluble antigens observed with mutant APCs were not overcome by doubling the number of APCs used (data not shown). Moreover, each antigen-specific T-cell clone was stimulated effectively by at least one mutant APC line with decreased cell surface expression of DR3 molecules, indicating that these levels were sufficient for effective stimulation. ND, not determined.

*Two levels of APCs were used to stimulate the alloreactive T-cell clone: 1×, 50,000 B-LCL stimulators; 2×, 100,000 B-LCL stimulators.

†Similar data with TT- and PPD-specific T cells were previously reported (10).

nature of the presentation defects associated with mutations at positions 9, 45, 73, and 151 of DRβ and 115 of DRα indicate that different residues are involved in DR3-restricted presentation to different T cells.

Stimulation of an Alloreactive T-Cell Clone Is Affected by Quantitative and Qualitative Changes in DR3 Expression. To evaluate whether the residues altered in the mutants are involved in the stimulation of an alloreactive response, we tested the ability of the mutants to stimulate an anti-DR3 T-cell clone (Table 3). Mutants 10.3.6, 10.77.6, and 7.25.6, all of which express decreased levels of DR3 molecules, each stimulate the allospecific anti-DR3 clone approximately in proportion to the level of DR3 molecules on the cell surface. In each case, increasing the number of mutant APCs increases the T-cell stimulation (Table 3). Thus, it appears that the altered stimulation of the allospecific T-cell clone by mutants 10.3.6, 10.77.6, and 7.25.6 derives, in large part, from their altered levels of cell surface DR3 molecules. Mutants 8.39.7 and 7.13.6, on the other hand, are unable to stimulate the alloreactive clone, even if the number of APCs is increased (Table 3), and despite the fact that, in 8.39.7, the cell surface abundance of DR3 molecules is normal (Table 2). The inability of increased numbers of 8.39.7 and 7.13.6 cells to stimulate the alloreactive clone suggests that the residues altered in these mutants are critical for recognition by the alloreactive clone.

DISCUSSION

Analyses of structure–function relationships in MHC molecules are likely to reveal general rules governing MHC–peptide–T cell interactions as well as variations arising from species and allelic differences in MHC molecules. Identification of the unique features of particular MHC molecules may also be important for understanding the association of certain MHC alleles, such as HLA-DR3, with autoimmune disease. We have begun to dissect the relationship of fine structure to antigen presenting function in the DR3 molecule. In the present paper, we examine seven mutant cell lines with different point mutations in the DR3 molecule. Two salient points emerge from the analyses of the functional effects of these single amino acid substitutions. First, different residues of the DR3 molecule appear to be involved in presentation to different DR3-restricted T cells. Each mutation results in a unique pattern of stimulation of the T-cell clones, and each DR3-restricted T cell clone is stimulated by a different subset of the mutants. This complex relationship of fine structure to antigen-presenting function has been a consistent finding among the murine and human MHC class I and class II molecules examined to date (8, 30–37). Second, polymorphic position 74 of DRβ appears to play a critical role in DR3-restricted antigen presentation; responses of all DR3-restricted T cells tested are reduced or abolished by the mutation in 8.39.7, which alters the charge of the side chain at position 74. This disruption of function is unlikely to result from widespread conformational changes in the mutant DR3 molecule, given its normal binding of three antibodies that recognize distinct DR determinants (Table 2). A direct role in antigen presentation for position 74 is consistent with the predicted functional importance of polymorphic residues. Not all changes in polymorphic residues have the effect of the change at position 74, however. The substitution at polymorphic position 9, which also alters charge, has a more modest effect. Profound effects on T-cell recognition, like those observed with the mutation at position 74, have also been observed in I-Aβ mutants with substitutions at other polymorphic residues in this region of the β-chain α-helix (35, 36).

In contrast to the findings with mutant 8.39.7, most substitutions in the DR3 mutants are associated with limited alterations in antigen-presenting function. The positions of the mutations suggest that such selective effects may arise in several ways. The substitution (Gly → Ser) at residue 73 in 10.22.6 selectively abolishes reactivity with mAb 16.23 and one PPD-specific T-cell clone. In the Brown and Wiley model of class II structure, the side chain at residue 73, on the top surface of the β-chain α-helix, points upwards and projects into solution (11). Thus, mutating position 73 may disrupt its function as a contact residue for mAb 16.23 and a TCR. The selective changes in antigen presentation in 7.31.6, 10.3.6, and 10.77.6, on the other hand, are likely to result from conformational changes in the DR3 molecule at a distance from the substituted residues. Based on the crystal structure of HLA-A2, the bulky side chains introduced by the substituted arginines in mutants 7.31.6 and 10.3.6 should contact residues in the peptide-binding domain of the DR3 molecule (P. Bjorkman, personal communication). In mutant 10.77.6, a Pro → Leu substitution at a bend between two β-pleated sheets in the DRα2 domain most likely alters the conformation of both the DRα2 and DRα1 domains. The conformational changes in these mutant DR3 molecules may thus give rise to selective functional defects by altering interaction with some peptides, but not others. Recent crystallographic evidence suggests that the class I peptide-binding groove contains subsites that vary between alleles (38); thus, single

amino acid substitutions could alter some subsites but not others.

The sites on class II molecules that affect allorecognition provide insights into the nature of the target of alloreactive T cells. The proliferative response of the alloreactive, DR3-specific T-cell clone is abrogated by the mutations in 8.39.7 and 7.13.6, both of which alter the charge of side chains at predicted peptide interaction sites (11). These results suggest that this clone recognizes either an MHC–peptide complex or a conformation of DR molecules that is in part determined by peptide binding. A role for bound peptide in allorecognition has also been suggested by studies of the responses of murine alloreactive T cells to mutant class II molecules (30, 31, 37). These interpretations must be qualified, however, by uncertainty regarding both the overall structure of class II molecules (11) and the positions of amino acid side chains in particular class II alleles (38). As an alternative approach to defining the nature of the ligand of alloreactive TCRs, we have studied allostimulation by mutant APCs that are unable to generate class II–peptide complexes from soluble antigens (39). Three of four alloreactive T-cell clones fail to recognize these antigen-processing mutants, even though their class II molecules are of normal primary structure and abundance on the cell surface (T. Cotner, E.M. and D.P., unpublished work). These two and other lines of evidence (40) thus suggest an important role for bound peptide in class II allorecognition.

Mutant isolation by antibody-mediated selection might *a priori* be expected to select mutants altered in TCR interaction, since both antibodies and TCRs should interact with residues that are solvent-accessible. Indeed, in a set of antibody-selected class I mutants, 5 of 10 mutations in the antigen-binding domain mapped to putative TCR interaction sites, and only 1 of 10 mapped to a putative peptide interaction site (32). It is therefore of interest that five of seven mutants that have lost binding of mAb 16.23 appear altered in peptide interaction (8.39.7, 7.13.6, 7.31.6, 10.3.6, and 10.77.6). This finding suggests that the binding of mAb 16.23 is sensitive to the state of occupancy of the peptide-binding groove, a hypothesis that is further supported by the fact that immunoselections with this antibody have also resulted in isolation of mutants defective in antigen processing (39). Mutants immunoselected with antibodies whose binding is sensitive to occupancy of the class II binding groove should be particularly useful for dissecting the molecular basis of antigen processing and presentation.

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