A SINGLE AMINO ACID SUBSTITUTION IN THE HUMAN HISTOCOMPATIBILITY LEUKOCYTE ANTIGEN DR3 β CHAIN SELECTIVELY ALTERS ANTIGEN PRESENTATION

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Activation of antigen-specific helper/inducer T cells requires that antigen be presented by APC in association with self MHC class II molecules (1). Recent evidence indicates that APC accomplish this by degrading exogenous soluble proteins to peptide fragments, which become bound to class II molecules (2, 3). The amino acid residues of human class II molecules that interact with immunogenic peptides, with the TCR or with accessory molecules such as CD4, are for the most part unknown. As an approach to mapping such residues, we are studying structure-function relationships in immunoselected mutant B lymphoblastoid cell lines (B-LCLs)[1] that express altered HLA-DR3 molecules. B-LCLs present soluble antigens to antigen-specific, HLA class II–restricted T cells (4), making it possible to assay the functional effects of mutational alterations in class II molecules. Mutational analysis has proved useful for mapping serologic epitopes and T cell restriction sites on human class I molecules (5) and murine class I and II molecules (5, 6). However, single amino acid substitutions that affect antigen presentation in human cells have not previously been reported.

To generate class II mutants in a human APC line, we mutagenized an HLA-DR3-expressing B-LCL with ethyl methane sulfonate. To simplify the analysis, we used as the progenitor line 8.1.6, a deletion mutant that lacks all *DR* and *DQ* genes of one haplotype, but expresses the class II loci of the other haplotype, DR3, normally (referred to in reference 6a). Immunoselection of mutagenized 8.1.6 with an anti-DR3-specific mAb, 16.23, yielded several types of mutants (7). Here, we describe one immunoselected mutant, 7.13.6, that expresses a structurally altered DR3 molecule and has a selective loss in DR3-restricted antigen presentation compared with its progenitor, 8.1.6. The mutation 7.13.6 maps to the first hypervariable region of the DR3 β chain.

Materials and Methods

APC Lines. With the exception of mutant clones 7.13.6 and 7.25.6, the B-LCLs have been reported. 8.1.6 is derived from T5-1 progenitor line (7); 9.22.3 is a homozygous DRα dele-

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[1] *Abbreviations used in this paper:* BLCL, B lymphoblastoid cell lines; 2-D, two dimensional; PCR, polymerase chain reaction; TT, tetanus toxoid.

tion mutant derived from 8.1.6 (6a); 9.4.3 is an 8.1.6-derived mutant that lacks DRβ_1 mRNA (7); 6.1.6 is a class II-negative regulatory mutant derived from T5-1 (7). 7.13.6 and 7.25.6 are DR3 mutants isolated by a described protocol (7). Mutant 7.25.6 expresses a DRβ_1 chain with a more acidic isoelectric point (pI) than that of 8.1.6 (not shown). 7.25.6 binds 45 ± 4% of 8.1.6 levels of anti-DR mAb VI.15, 35 ± 4% of anti-DR3 mAb 16.23, and 48 ± 6% of anti-DR3 mAb CD6B1.

Two-dimensional Gel Analysis. ^{35}S-labeled DR molecules were immunoprecipitated with anti-DR mAb VI.15 (7). Immunoprecipitates, with or without prior digestion with Endo F, were subjected to electrophoresis on two-dimensional (2-D) gels, as described (Cotner, T., submitted for publication).

Radioimmune Binding Assays. For binding assays, mAbs 16.23, anti-DR3 specific (8); CD6B1, anti-DR3 specific (9); 7.3.19, anti-DRw52 specific (10); VI.15, anti-DR monomorphic (7) were used. Cells were assayed with saturating amounts of mAb and ^{125}I protein A or ^{125}I-rat anti-mouse κ chain as a second reagent, as described (7).

T Cell Proliferation Assays. Human T cell lines specific for soluble protein antigen were generated as described (4). Antigens tetanus toxoid (TT), PPD, and *Candida albicans* extract were used as described (4); recombinant hepatitis B surface antigen (HBsAg), the generous gift of Merck Sharp & Dohme (West Point, PA) was used at 10 μg/ml. T cell clones (ND-PPD$_1$, ND-PPD$_2$, CT-TT, BJ-HBsAg) are designated by donor-antigen and were obtained by limiting dilution cloning in the presence of antigen, exogenous IL-2, and irradiated PBMC as APC. ND-PPD$_1$ and ND-PPD$_2$ are PPD-specific clones with different reactivity patterns; they are likely to recognize different peptides derived from PPD, which is a mixture of proteins. 1.5×10^4 T cell blasts were cocultured with 10^5 mitomycin C-treated B-LCLs as APC in the presence or absence of soluble antigen; stimulation was measured by the incorporation of [^{3}H]thymidine into DNA, as described (4).

Polymerase Chain Reaction (PCR) Amplification and Sequence Analysis. cDNAs prepared from poly(A)$^+$ RNA from 8.1.6 and 7.13.6 were size selected (>0.7 kb) on an agarose gel and subjected to PCR amplification using Taq I polymerase (11). *DR*α primers were selected based on published sequences (12). *DR*β primers were selected based on the sequence of a DR3β_1 cDNA clone from 8.1.6 (B. Arp, unpublished results). All primer sequences are available on request. Size-selected double-stranded PCR DNA was subjected to dideoxy sequencing using modified T7 DNA polymerase (Sequenase, U.S. Biochemical Corp., Cleveland, OH). The PCR primers and additional oligonucleotide primers covering 21 bp segments of the coding regions were used to generate overlapping 100–250 base segments of sequence information.

Results and Discussion

We initially analyzed DR molecules from mutant 7.13.6 and its progenitor 8.1.6, by 2-D SDS-PAGE electrophoresis (Fig. 1). 8.1.6, like other cells bearing the DR3 haplotype, expresses a single DRα chain and two DRβ chains, β_I and β_{III}, each of which forms a heterodimer with DRα. Comparison of the 7.13.6 and 8.1.6 2-D gel patterns reveals that mutant 7.13.6 expresses an altered DRβ_1 chain, whose pI is two charges more basic than that of 8.1.6 (see Fig. 1, legend).

To evaluate the level of cell surface expression of the mutant DR3 molecule expressed by 7.13.6, we compared cell surface binding with 7.13.6 and 8.1.6 by a panel of four mAbs that bind to DR3 molecules (Table I). On the basis of their different fine specificities (see Materials and Methods) and inability to crossinhibit each other (not shown), these antibodies are likely to react with distinct epitopes on DR3 molecules. At antibody concentrations that are saturating for 8.1.6, the binding of each antibody to 7.13.6 is moderately reduced (40–70%) from 8.1.6 levels. For both the DR3-specific mAb 16.23 and the monomorphic anti-DR mAb VI.15, binding to 7.13.6 saturates at 50% of the concentration required to saturate 8.1.6 (not shown). These data suggest that the mutant molecules in 7.13.6 are expressed in decreased

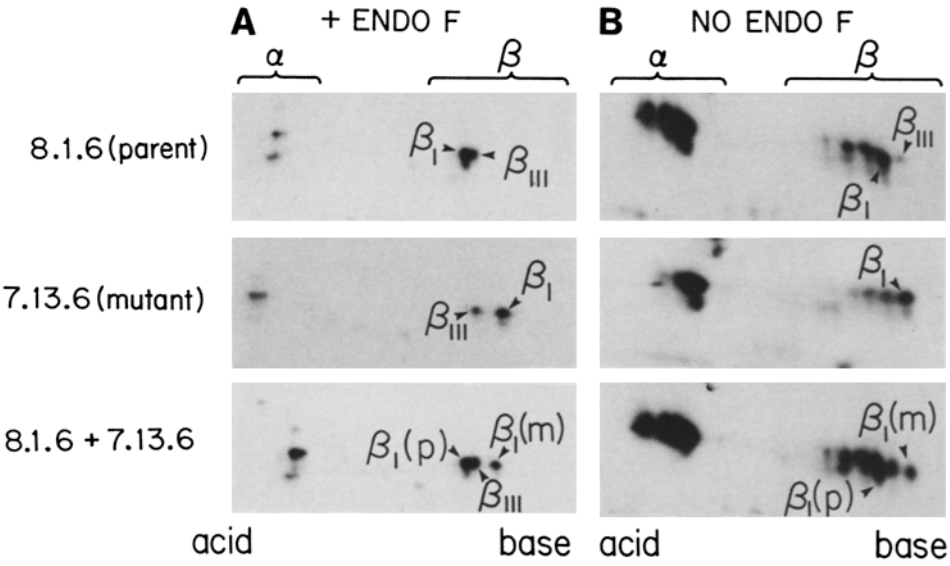

FIGURE 1. (*A*) 2-D SDS-PAGE analysis of Endo F-treated HLA-DR molecules from parent line 8.1.6 and mutant 7.13.6. The designations of 8.1.6 β chains as β_I and β_{III} are based on comparisons of limited NH_2-terminal amino acid sequencing with the published DR3β_I and β_{III} gene sequences (14 and T. Cotner, unpublished results). In both 7.13.6 and 8.1.6 gels, the β_I spot is more abundant than the β_{III} spot; the β_I spots are also marked by a weaker spot of the same pI but lower molecular weight, which most likely represents a proteolytic breakdown product. In the 8.1.6 gel, the β_I spot obscures the more basic β_{III} spot. To compare the 7.13.6 and 8.1.6 patterns, the β chain spots are aligned by the β_{III} spots, which cofocus in the 8.1.6/7.13.6 mixture. The different positions of the DRα spots in the different gels are due to differential stretching of the gels. However, the 8.1.6/7.13.6 mixture, being internally controlled for stretching, demonstrates that the DRα species from 7.13.6 cofocuses with that of 8.1.6, whereas the 7.13.6 β_I chain is more basic than that of 8.1.6. *p*, parental; *m*, mutant. (*B*) 2-D SDS-PAGE analysis of non-Endo F-treated HLA-DR molecules from 8.1.6 and 7.13.6. The β_I chain pattern from 8.1.6 includes the prominent, nonsialated βI spot and a more acidic glycosylation ladder consisting of three spots of increasing acidity. A fifth spot, the nonsialated β_{III} chain, is visualized to the basic side of the β_I spot; its glycosylation ladder is obscured by the β_I spots. In 7.13.6, the nonsialated β_I chain and its glycosylation ladder are shifted to the basic side (relative to 8.1.6) and overlap the β_{III} spots. In the 8.1.6/7.13.6 mixture the nonsialated β_I chain of 7.13.6 and a single glycosylation intermediate are visualized on the basic side of the nonsialated 8.1.6 β_I chain. Because each sialyation contributes 1 acidic charge, this pattern suggests that the 7.13.6 β_I chain has undergone a 2 charge basic shift in pI compared with 8.1.6.

amounts on the cell surface, possibly because the mutant β_I chains associate less efficiently than 8.1.6 β_I chains with the DRα chain.

To evaluate DR3-restricted antigen presentation in 7.13.6, we tested the ability of 7.13.6 to stimulate proliferation of antigen-specific DR3β_I-restricted T cells derived from normal DR3 donors. DR3β_I-restricted T cells were identified as cells that respond to antigen presentation by 8.1.6 but not by an 8.1.6-derived mutant that has lost DRβ_I expression (mutant 9.4.3). As shown on Table II, mutant 7.13.6 is unable to stimulate ND-PPD_1, a DR3β_I-restricted T cell clone specific for PPD. However, the mutant DR3 molecules of 7.13.6 function as effectively as those of 8.1.6 in stimulating a DR3β_I-restricted TT-specific clone CT-TT, a second DR3β_I-restricted PPD-specific clone, ND-PPD_2, and DR3β_I-restricted T cells specific for

TABLE I

Cell Surface Radioimmune Binding Analysis of Mutant 7.13.6 and 8.1.6 with a Panel of Anti-DR Antibodies

	Antibody			
Cell line	16.23	CD6B1	7.3.19	VI.15
		%		
8.1.6	100*	100	100	100
7.13.6	29 ± 4	45 ± 4	66 ± 2	47 ± 6

* Binding ratio is 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. cpm bound by 9.22.3 ranged from 2 to 9% of cpm bound by 8.1.6.

C. albicans (JN-CA). Stimulation by 7.13.6 of a hepatitis B surface antigen-specific clone, which is restricted by non-DR-restricting elements (BJ-HBsAg) is also equivalent to 8.1.6 levels (Table II). These data indicate that the mutation in 7.13.6 specifically affects the presentation of PPD to clone ND-PPD$_1$ and does not result in a generalized abnormality of antigen processing or presentation.

The 7.13.6 defect in stimulation of ND-PPD$_1$ appears to result from the structural change in HLA-DR3 molecules rather than the change in the level of HLA-DR3 molecules on the cell surface based on the following observations. Doubling the number of 7.13.6 cells used as APC does not overcome the defect (data not shown). In addition, a different 8.1.6-derived *DR*3β$_1$ structural gene mutant, 7.25.6, which, like 7.13.6, expresses ~50% of 8.1.6 levels of DR3 (see Materials and Methods), stimulates ND-PPD$_1$ at a level equivalent to 8.1.6 (Table II). This indicates that reductions in HLA-DR3 expression of the magnitude seen in 7.25.6 and 7.13.6 do not of themselves result in reduced stimulation of ND-PPD$_2$ T cells. These data, as well as the ability of 7.13.6 to stimulate other DRβ$_1$-restricted T cells normally, suggest

TABLE II

Stimulation of HLA-restricted Antigen-specific T Cells by Mutants and by 8.1.6

	[^{3}H]TdR T cell incorporation				
APC	ND-PPD$_1$	ND-PPD$_2$	CT-TT	JN-CA*	BJ-HBsAg
			cpm × 10^{-3}		
8.1.6	25.5	14.1	5.5	28.2	61.7
7.13.6	0	20.4	5.3	30.3	63.4
7.25.6	24.0	18.4	ND	34.7	ND
9.4.3	0	0	0	19.8	ND
9.22.3	0	0	0	2.6	62.0
6.1.6	ND	ND	ND	0	0.4

Data are net antigen-specific cpm from the median of triplicate assays from a representative experiment. SEM ≤10%. Relative responses (response to mutant / response to 8.1.6) were comparable (SEM ≤ 10%) in 2-4 independent experiments.

* JN-CA is a polyclonal T cell line, specific for *C. albicans*; as shown, its response is primarily DR3 restricted when assayed with 8.1.6 as APC.

that the functional alteration in mutant 7.13.6 derives from the structural change in its DR3β_1 chain.

To more precisely identify the structural basis for the cell surface antibody binding and functional changes observed in 7.13.6, we determined the site of the mutational lesion in its *DR*β_1 gene. We used the PCR to amplify the entire *DR*α and *DR*β coding region of cDNAs from 7.13.6 and 8.1.6 and subjected the PCR products to dideoxy sequence analysis. The *DR*α gene from 7.13.6 is identical to that of 8.1.6 (data not shown). However, as shown on Fig. 2, the *DR*β_1 gene differs from that of 8.1.6 by a single base change, a guanine → adenine transition in the first nucleotide of the codon for amino acid 9. The mutation changes the encoded residue from glumatic acid to lysine which is consistent with the two charge basic shift in pI of the mutant β_1 chain observed on 2-D gel analysis. Thus, it appears that a single amino acid change underlies the functional alterations in the 7.13.6 mutant. The location of this mutation in the NH_2-terminal domain of β chain (the β1 domain) is not surprising; this domain contains most allelic variant amino acid positions in the HLA-DR molecule (13), and 7.13.6 was selected for altered reactivity with a DR3-specific antibody.

The evidence from mutant 7.13.6 implicates the first hypervariable region (amino acid positions 9–13) of the β1 domain of the DRβ_1 chain as important for the stimulation of some DR3-restricted T cells. The third hypervariable region (62–78) is also involved in presentation of soluble antigens (6). Recently, a model of the binding domain of the class II molecule has been proposed, based on the crystal structure of HLA class I molecule A2 and on shared features of primary structure of class I and II molecules (6). In this model, the NH_2-terminal portions of the α1 and β1 domains (including the first hypervariable region) are folded into β-pleated sheets and form the bottom of an antigen-binding cleft. The side chains of polymorphic residues 9, 11, and 13 of the first hypervariable region point into the antigen-binding site. The COOH-terminal portions of the α1 and β1 domains (including the third hypervariable region) are α helical structures that form the sides of the cleft. Side chains of some residues in the helices point out and are available for interaction with antibodies and TCRs. Position 9, which is altered in 7.13.6, is on the floor of the cleft in this model, but its side chain is also in close proximity to the presumed α-helical portion of the DRα chain. This model suggests at least three alternative mechanisms for the functional alteration in 7.13.6. The glutamic acid at position 9 in normal DR3 molecules may interact directly with an immunogenic peptide derived from PPD. Mutations at this site might therefore interfere with antigen binding. Alternatively, the substitution at this site may induce a local conformational change in the

	8	9	10
	Leu	Glu	Tyr
8.1.6	TTG	GAG	TAC
7.13.6	---	A--	---
	Leu	Lys	Tyr

FIGURE 2. The DR3β_1 nucleotide coding sequence of 7.13.6. The numbers refer to the amino acid positions. 7.13.6 differs from 8.1.6 by a G → A transition in codon 9 of the β_1 chain. Independent evidence verifying this result was obtained by amplifying and sequencing a segment of genomic DNA spanning the second exon of the DRβ_1 genes of 7.13.6 and 8.1.6 (data not shown). The DRβ_1 gene of the DRw9 specificity also has a lysine at position 9, but differs from mutant 7.13.6 at positions 10–13 (15).

α helix of the DRα chain affecting a contact residue for particular TCRs. Thirdly, the 7.13.6 mutation in the $DR\beta_1$ chain may perturb its interaction with the NH_2-terminal region of the DRα chain, changing the tertiary structure of the mutant dimer. These changes might ablate either peptide binding or T cell recognition. Evidence obtained in binding studies of appropriate peptides to purified mutant DR3 molecules from 7.13.6 should help distinguish among these hypotheses.

Summary

Activation of T lymphocytes by immunogenic peptides bound to HLA molecules is a central event in the generation of an immune response. To determine the sites on HLA molecules involved in this process, we isolated mutant EBV-transformed B cell clones that express altered HLA-DR3 molecules. One mutant has lost the ability to stimulate a T cell clone specific for a mycobacterial protein, but retains the ability to stimulate other antigen-specific T cells. The DNA sequence of the complete DRα and β coding regions revealed a single nucleotide change resulting in a glutamic acid to lysine substitution at amino acid 9 in the first hypervariable region of the DRβ chain. These results are discussed in relation to a recently proposed model of class II molecule structure.

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