Identification of HLA-DR1 β Chain Residues Critical for Binding Staphylococcal Enterotoxins A and E

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Summary

Superantigens are thought to make external contacts with major histocompatibility complex (MHC) class II molecules and with the Vβ portion of a T cell antigen receptor (TCR), thereby stimulating entire families of T cells. The precise mapping of superantigen binding sites on class II molecules may provide valuable information on how TCR and MHC molecules interact. Two bacterial superantigens, staphylococcal enterotoxins A and E (SEA/SEE) bind well to most HLA-DR alleles, but poorly to HLA-DRw53. The sequences responsible for this binding were localized to the putative α helix of the DR β chain by measuring toxin binding to a panel of chimeric class II molecules expressed on transfected cells. Binding of SEA/SEE to the DRw14 (Dw9) molecule suggested that the conserved histidine 81 in the β chain of most DR molecules was important, whereas the tyrosine 81 in the DRw53 β chain was detrimental for high-affinity binding. To prove this, reciprocal point mutations were introduced in the DR1 and DRw53 β chains. Mutation of histidine 81 in the DR1 β chain to tyrosine reduced SEA/SEE binding, but did not prevent recognition of two DR1-restricted peptides by six of eight antigen-specific T cell lines. Conversely, introduction of histidine at position 81 in the DRw53 β chain restored normal levels of SEA/SEE binding. These data suggest that a binding site of SEA and SEE lies on the outer face of the β chain α helix, pointing away from the antigen-binding groove.

The class II molecules of the MHC include the HLA-DR, -DQ, and -DP antigens in humans, and the IA and IE antigens in the mouse. They are polymorphic cell surface heterodimers consisting of a noncovalently-associated ~35,000-dalton α chain and a ~29,000-dalton β chain (1, 2). Class II molecules function by binding small (9–12 amino acid) peptides derived from the processing of exogenous macromolecular antigens and subsequently presenting them to $CD4^+$ T lymphocytes. Little is known about the physical interaction of class II molecules with their principal ligands, the TCR variable regions and the T cell coreceptor CD4. Models have been proposed predicting the way that MHC molecules may interact with CD4 and TCR (3, 4), but due to the low avidity of these interactions, no direct binding data have been reported.

Certain bacterial toxins are also ligands for class II. These include the *Staphylococcus aureus* enterotoxins (SEA,[1] SEB, $SEC_{1,2,3}$, SED, and SEE) and the toxic shock syndrome toxin (TSST-1). The staphylococcal toxins are potent polyclonal activators of T cells (5, 6). There is no requirement for processing of the toxins similar to typical antigens. Proteolysis of the toxins abrogates their activity (7). The activation of T cells results in proliferation, cytolytic activity, and secretion of IL-2 and IFN-γ. T cell populations activated by the staphylococcal toxins are polyclonal, yet each superantigen appears to preferentially stimulate T cells bearing TCR representing one (or a few) particular Vβ family (8, 9). Direct binding of many of the toxins to class II has been demonstrated: SEA and SEB to HLA-DR (7); SEA to HLA-DR, -DQ, and -DP (10); and TSST-1 to HLA-DR and -DQ, but not to HLA-DP (11). The K_d's for the interaction of the various toxins with class II molecules determined were in the range of 10^{-6} to 10^{-8} M.

The term superantigen has been used to describe substances with the ability to activate large numbers of T cells in a class II–dependent but unrestricted fashion (8). The staphylococcal toxins are the best studied superantigens, but there is a growing number of microbial products that fit this category. These include the *Mycoplasma arthriditis* mitogen (12) and the streptococcal M protein (13). Other superantigens have been partially characterized from group A streptococcus and Gram-negative bacteria. The Mls (minor lymphocyte system) in mice

[1] *Abbreviations used in this paper:* Mls, minor lymphocyte system; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin.

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has aspects of a superantigen-mediated phenomenon (6). Particular Mls determinants are associated with specific endogenous murine retroviruses (14–16). In one case, the Mls response was duplicated by transfection of lymphocytes with DNA derived from an open reading frame in the 3′ retroviral LTR (17). Other retroviral proteins have been suggested to act as superantigens (18).

The molecular mechanism by which the toxins interact with class II is poorly understood. The toxins appear to bind outside the antigen-binding groove of class II. Preincubation of HLA-DR–expressing cells with a saturating amount of TSST-1 neither prevented nor diminished the binding of a known antigenic peptide (19). Furthermore, an alanine-scanning mutagenesis of the α-helical portion of the IA^k α chain failed to identify a residue that was essential for SEB, SEC1, or exfoliative toxin action, whereas many mutants affected the recognition of specific antigenic peptides by T cells (20). The conclusion that the toxins make external contacts with class II molecules is consistent with the observation that residues of the Vβ region of the TCR that are important for toxin action are postulated to lie on an exposed face of the receptor (21). The precise mapping of superantigen binding sites on class II molecules would provide valuable information about how MHC and TCR interact.

Several clues to the nature of the toxin binding site(s) have been derived from the hierarchy of interaction that exists for many of the isotypes and alleles of class II. The α1 domain of HLA-DR1 was shown to be essential for the high-affinity binding of TSST by producing a series of homologue scanning mutants with sequences derived from the DR or the DP molecules (19). The importance of the DR α chain in TSST binding was confirmed by testing mouse/human hybrid class II molecules (22). The mouse IE molecule, although homologous to DR, does not bind TSST. Cells expressing class II consisting of the DR α chain in combination with the IE β chain bound TSST, and cells expressing an IE α/DRβ heterodimer did not. Several toxin binding sites may exist on class II molecules. TSST and SEB bind independent of each other to HLA-DR1 or HLA-DQw1 (23). In addition, SEA is an effective competitor of SEB binding to HLA-DR, and SEB did not inhibit the binding of SEA, even at a 1,000-fold excess (7). SEA can also inhibit TSST binding, and, again, the converse is not true (D. Karp, unpublished results). This suggests the possibility of several, possibly overlapping, binding sites for the staphylococcal toxins on class II.

The HLA-DRw53 molecule was found to be ineffective in the binding of SEA and SEE when compared with HLA-DR1 (24). This molecule is composed of the nonpolymorphic DR α chain and the DRw53 β chain (*DRB4*0101* allele). This suggested a binding site for SEA and SEE in the β chain, a conclusion supported by peptide inhibition experiments (25). The present investigation uses molecular techniques to identify residues critical for binding of SEA and SEE. Mutant human class II molecules containing sequences from DR1 and DRw53 localize the binding of intact toxins to the putative α-helical region of the β1 domain. Site-directed mutagenesis has identified position 81 of the β chain as a critical residue in the interaction with these two toxins.

Materials and Methods

L Cells Expressing HLA-DR Mutants. Expressible cDNA for the β chain of DR1 was cloned into the vector RSV.3 as described (26). This construct has unique SacI and HindIII restriction endonuclease sites in the DR leader sequence and 3′ untranslated region, respectively. These sites are conserved in cDNA for many DRβ alleles including DRw53 and DRw14(Dw9). The plasmid, RSV.3 DR1β, was digested with SacI and HindIII, and the DR1 β chain coding sequence replaced by cDNA from DRw53 (provided by Dr. Robert Karr, Monsanto Corp., St. Louis, MO) and DRw14 β chains (provided by Dr. Jack Gorski, Blood Research Institute, Milwaukee, WI), digested with the same enzymes.

The cDNA for DR1 and DRw53 β chains were also cloned into the vector pBluescript SK (Stratagene Inc., La Jolla, CA) to create pSKDR1β and pSKDRw53β, respectively. A unique ScaI site occurs at codon 59 of the β chain sequence. Digestion of pSKDR1β and pSKDRw53β with ScaI and ClaI produced three fragments: a ClaI-ScaI fragment containing NH_2-terminal cDNA sequences, a ScaI-ScaI fragment including COOH-terminal cDNA sequences and a portion of the vector, and a ScaI-ClaI fragment having the remainder of the vector. These fragments were separated by gel electrophoresis and appropriate fragments were isolated and religated. This created two chimeric cDNA molecules. DR 153.59 had codons 1–59 derived from DR1 and the remainder from DRw53, DR 531.59 had codons 1–59 from DRw53, and the remainder from DR1.

To create the pair of mutants having β1 domains (codons 1–94) derived from either DR1 or DRw53, and β2 domains (codons 95–237) derived from the other allele, the technique of synthesis by overlap extension was used (27). Oligonucleotides were synthesized corresponding to conserved regions of the DR β chain cDNA sequence in the leader region (codons 1–6, sense direction), the cytoplasmic tail (codons 224–230, antisense direction), and the COOH-terminal portion of the β1 domain (codons 88–94, both directions). These oligonucleotides were used to amplify β1 and β2 domain sequences from DR1 and DRw53 β chain cDNA using PCR. Amplified products were isolated by agarose gel electrophoresis, and purified with GeneClean (Bio101, Inc., Vista, CA). DNA corresponding to the β1 domain of DR1 was combined with DNA for the β2 domain of DRw53, and oligonucleotide primers from the leader and cytoplasmic regions. After a second amplification by PCR, the full-length products were isolated by gel electrophoresis, purified, and digested with SacI and StuI. These fragments were ligated to fragments of RSV.3 DR1β and RSV.3 DRw53β digested with these same enzymes. This created DR 153.94 (having the β1 domain of DR1 and β2 domain of DRw53) and DR 531.94 (having the β1 domain of DRw53 and β2 domain of DR1).

Point mutations in the β chain cDNA were made in a similar manner. Oligonucleotide primers corresponding to codons 78–84 (both directions) of the DR1 and DRw53 β chains were synthesized such that codon 81 of the DR1 sequence would be a tyrosine and codon 81 of the DRw53 sequence would be histidine. These primers were used with the primers from the leader and cytoplasmic sequences described above to create full-length cDNA having the single amino acid change. Fragments of these cDNA were used to replace sequences from RSV.3 DR1β or RSV.DRw53β, as appropriate. The authenticity of the point mutations, as well as the chimeric cDNA sequences, was verified by determining the sequence of the the entire β chain coding region of each clone before use in transfection experiments.

The plasmid, RSV.5(neo) DRα (28) has an expressible cDNA for the nonpolymorphic DR α chain under the control of the RSV LTR, as well as the bacterial neomycin resistance gene under the

control of the SV40 early region promoter. This plasmid was cotransfected with an expressible wild-type or mutant β chain cDNA into L cells as described (28). Transfected cells were selected and maintained in DME plus 10% FCS containing G418 (0.5 mg/ml, active drug, Gibco-Bethesda Research Laboratories, Gaithersburg, MD). Surviving cells from each transfection were pooled and stained with the anti-DR mAb, L243. A FACStar Plus® flow cytometer (Becton Dickinson and Co., Mountain View, CA) equipped with an Automatic Cell Deposition Unit was used to clone transfected cells on the basis of surface expression of DR molecules.

Toxin Binding Assay. SEA, SEB, SEE, and TSST were were all purchased lyophilized from Toxin Technologies (Madison, WI). They were dissolved in sterile water at a concentration of 1 mg/ml and diluted to 10 μg/ml in PBS containing 1% BSA and 0.1% gelatin just before use. 5×10^5 L cells were incubated at 4°C with the various toxins for 2 h. The cells were washed, then incubated with toxin-specific polyclonal rabbit antiserum (Toxin Technologies), diluted 1:100 in PBS/BSA/gelatin, and incubated on ice for 1 h. Cells were washed again and incubated for 30 min on ice with FITC-labeled goat anti-rabbit IgG. Bound toxin was determined by microcytofluorimetry. Controls for each cell type were incubated with antitoxin and fluoresceinated antibody without prior toxin binding. Each cell type was simultaneously analyzed for L243 binding. Data for toxin binding are corrected for different levels of class II expressed by different transfectants as follows: $(PCF_{toxin}/PCR_{control}) - 1]/(PCF_{L243}/PCF_{GAMIG})$. Where PCF_{toxin} is the peak fluorescence value in the presence of toxin, $PCF_{control}$ is the value in the absence of toxin, PCF_{L243} is the peak fluorescence of the anti-DR reagent, PCF_{GAMIG} is the fluorescence of the second antibody alone. The toxin/control ratio is reduced by 1 to reveal the fractional increase in fluorescence due to toxin binding.

T Cell Lines. Fresh PBL were obtained from a normal HLA-DR1 volunteer. 13×10^6 cells were seeded in 10 ml of IMDM supplemented with 2 mM L-glutamine, 10% human serum, and 5 μg/ml of either influenza matrix peptide M1(18–29) or hemagglutinin peptide H3(307–318). After 10 d, 10^5 live cells were transferred into wells of a 24-well plate containing 2 ml of IMDM supplemented with glutamine, 10% human serum, and 10^6 feeder cells. Feeder cells were prepared by incubating autologous PBL in IMDM supplemented with glutamine and 10 μg/ml of either peptide for 3 h, irradiated (2,000 rad), and washed. After 6 d, 10^6 live cells were restimulated in a volume of 5 ml with $3–4 \times 10^6$ irradiated feeder cells prepared as above. After five additional days, responder T cells were assayed for their ability to lyse target B cells incubated with peptide. Both the M1- and H3-stimulated population of T cells displayed peptide-specific target cell lysis. 2 d later, T cells were seeded at 10 cells/well in three round-bottomed 96-well plates. Each well contained 0.2 ml IMDM supplemented with glutamine, human serum, and 25 U/ml rIL-2 (Cellular Products, Buffalo, NY), and 10^5 irradiated and peptide-pulsed autologous feeder cells. The first addition of exogenous IL-2 occurred at this cloning step. After 12 d, growing T cells were tested for their ability to kill target cells in a peptide-specific manner. Lines that displayed specificity were expanded by repeated stimulations at 5–6-d intervals with irradiated peptide-pulsed feeder cells, and addition of 5 U/ml rIL-2 1 d after restimulation. Based on their cloning efficiency, these lines are either clonal or oligoclonal.

Cytotoxicity Assays. Cytolytic activity was measured by standard chromium release assays. The HLA-hemizygous B cell line, 45.1 (29), was used as a human DR1-positive target. About $0.5–1 \times 10^6$ cells were incubated with 10 μg/ml of either matrix or hemagglutinin peptide in 0.5 ml of RPMI 1640 supplemented with glutamine, 40 μg/ml gentamycin, and 5% bovine serum (Hyclone Laboratories Inc., Logan, UT). After 1 h, 50 μCi $Na[^{51}Cr]O_4$ was added, and the cells incubated another 3 × h. After two washes, 5×10^3 live target cells were added to various numbers of effector cells in V-bottomed 96-well plates. For staphylococcal toxin–mediated cytotoxicity, 45.1 cells were incubated with either 0.1 μg/ml SEA or 1 μg/ml SEE, as described above for peptide. When trypsinized L cells were used as target cells, they were incubated with 100 μg/ml of antigenic peptide and labeled as described above. 2.5×10^3 L cells were added to V-bottomed wells, and incubated 6 h with effector cells. E/T ratios of 10:1, 2.5:1, and 0.625:1 were used for B cell targets, and 20:1, 5:1, and 1.25:1 for L cells. Supernatants were harvested and counted. Percent specific lysis was calculated as 100× [(experimental − spontaneous release)/(maximum − spontaneous release)]. Each point represents mean determinations from triplicate wells. Maximum and spontaneous release were determined by incubating targets in 1% Triton X-100 or medium, respectively.

T Cell Proliferation Assays. Purified T cells were prepared from peripheral blood of normal volunteers as previously described (30). After rosetting with SRBC and treatment with L-leucine methyl ester, the cells were passed through nylon wool. Non-T cells were depleted by panning with mAb L243 (anti–HLA-DR), 63D3 (anti-CD14), and B73.1 (anti-CD16). L cell transfectants were treated with mitomycin C (Sigma Chemical Co., St. Louis, MO), 100 μg/ml, at 37° for 1 h and washed extensively. T cells (6×10^4) and L cells (2×10^4) were co-cultured in round-bottomed 96-well plates with various dilutions of SEA or SEE in RPMI 1640 containing 10% heat-inactivated FCS. After 50 h at 37°C, the cells were pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol), and cultured for an additional 16 h. Cells were harvested on glass fiber filters, and incorporated radioactivity was quantitated by liquid scintillation counting.

Results

Binding of SEA and SEE to DR1 Is Controlled by the β1 Domain. Mouse L cells were transfected with cDNA for the nonpolymorphic DR α chain and β chains from the DR1 (*DRB1*0101*) or DRw53 (*DRB4*0101*) alleles, or chimeric β chains having sequences from both DR1 and DRw53. These cells were tested for their ability to bind the staphylococcal enterotoxins, SEA, SEB, SEE, and TSST (Fig. 1 *A*). As shown previously (24), there was a marked difference between DR1 and DRw53 in the binding of SEA and SEE. For these two toxins, the level of binding to DRw53 (corrected for expression of class II) was 1–5% of that seen with DR1. In contrast, there was no significant difference in the binding of either SEB or TSST to DR1 or DRw53. The lower level of binding of these two toxins is consistent with their greater reported dissociation constants (31).

Since the DR α chain is common to both DR1 and DRw53, the sequences responsible for the high-affinity interaction with SEA and SEE must lie in the β chain. To isolate the region of the β chain containing these sequences, a series of homologue scanning mutants were constructed. The first pair of these mutants combined the membrane distal β1 domain of one allele (amino acids 1–94) with the β2 domain, transmembrane, and intracytoplasmic portions (amino acids 95–237) of the other. L cells expressing class II molecules comprised of these mutant β chains in association with

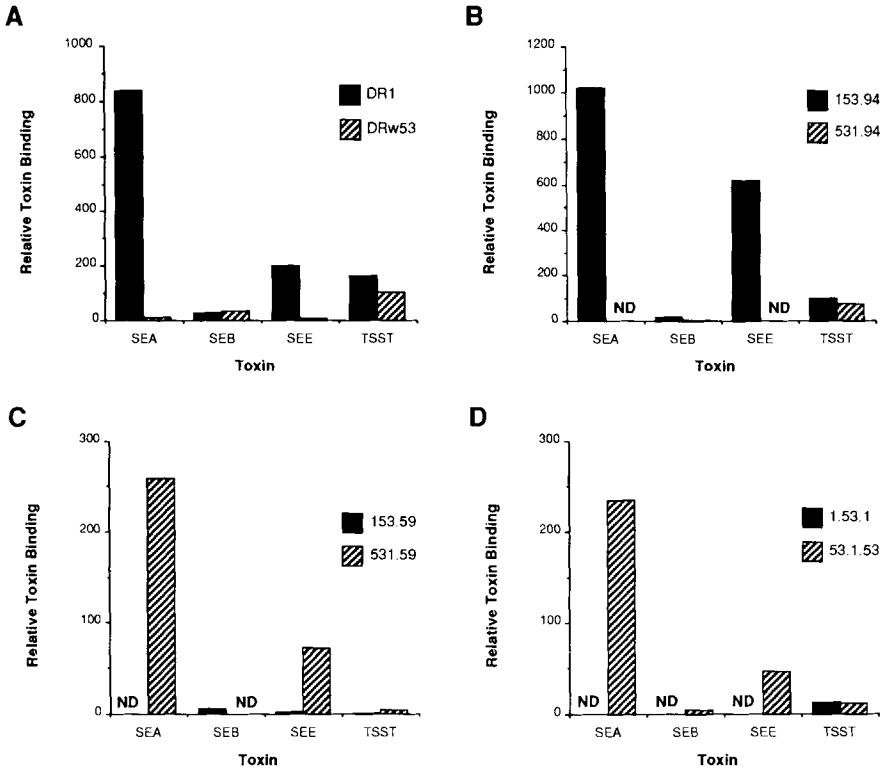

Figure 1. Binding of staphylococcal toxins to wild-type and chimeric HLA-DR molecules. Mouse L cells were stably transfected with HLA-DR1, HLA-DRw53, or DR1/w53 chimeras and tested for their ability to bind SEA, SEB, SEE, and TSST. In each panel, the *y*-axis is the relative binding of the individual toxins as measured by microcytofluorimetry. It is a dimensionless quantity as defined in Materials and Methods. (*A*) *Solid bar*, HLA-DR1; *hatched bar*, HLA-DRw53. (*B*) *Solid bar*, DR 153.94, a chimera having the β1 domain of DR1 and the β2 domain of DRw53; *hatched bar*, DR 531.94, a chimera having the β1 domain of DRw53 and the β2 domain of DR1. (*C*) *Solid bar*, DR 153.59, a chimera having residues 1–59 of DR1 and the remainder of the β chain from DRw53; *hatched bar*, DR 531.59, a chimera with residues 1–59 from DRw53 and the rest of the β chain from DR1. (*D*) *Solid bar*, DR 1.53.1, a double chimera with amino acids 59–94 derived from DRw53 and the rest of the β chain from DR1; *hatched bar*, DR 53.1.53, a double chimera with the residues 59–94 from DR1 and the other residues from DRw53. ND, None detected.

the DR α chain were tested for toxin binding (Fig. 1 *B*). Only the chimera DR 153.94, which expresses the β1 domain of DR1, was able to bind SEA and SEE. The reason for the increased binding of SEE to this mutant relative to the wild-type is unknown. The mutant DR 531.94, expressing the β1 domain of DRw53, did not bind these two toxins, although it did bind SEB and TSST.

The next pair of mutants had the first 59 amino acids derived from one allele and the remainder of the molecule derived from the other. Residues 1–59 are predicted to form the β sheet "floor" of the antigen-binding groove (32). Thus, the mutant DR 153.59 had DR1-derived residues in the β sheet region, and DRw53-derived residues in the putative α-helical portion of the antigen-binding domain, as well as in the β2 domain. The mutant, DR 531.59, had the β sheet from DRw53 and DR1 residues in the remainder of the molecule. This latter molecule was able to bind SEA and SEE, whereas DR 153.59 was not (Fig. 1 *C*). This suggests that the residues responsible for the high-affinity binding of these two toxins lie in the region of residues 59–94. This was tested directly with a final pair of homologue mutants created from the first two. DR 1.53.1 had DR1-derived residues in the β sheet and β2 domains, and DRw53-derived residues in the α-helical portion of the β chain. DR 53.1.53 had the α-helical portion (amino acids 59–94) derived from DR1, and the remainder of the β chain was derived from DRw53. As expected, only DR 53.1.53 bound SEA and SEE (Fig. 1 *D*).

In each pair of homologous mutants, high-affinity binding of SEA/SEE was determined by sequences in the α helix of the DR1 β chain, although other β chain sequences appeared to affect the quantitative level of this binding. For example, in Fig. 1, compare *A* and *B* to *C* and *D*. The highest binding was seen when the entire β1 domain of DR1 was present (DR1 wild-type and the 153.94 mutant). When the DR1 α helix was present in conjunction with the DRw53 β sheet (mutants 531.59 and 53.1.53), there was reduced binding. Either there is participation of sequences outside the α helix in direct SEA/SEE contact, or an influence on the conformation of the α helix by amino acid residues that are distant in the β chain primary sequence but nearby in the tertiary structure.

Residue 81 Is Critical for SEA and SEE Binding. A comparison of the DR1 and DRw53 β chain amino acid sequences from residues 59–96 is shown in Fig. 2. There are five differences between these two alleles in this region. Positions 70, 74, 86, and 96 are all highly variable among the known β chain sequences and are presumably responsible for differences in antigen presentation among DR alleles. Previous reports have documented SEA binding by DR alleles differing from DR1 at one or more of these positions (10, 24). In contrast, the occurrence of tyrosine at position 81 in the DRw53 β chain is unique to this allele. All other DR β chains, as well as the β chains of HLA-DQ, HLA-DP, I-E, and I-A (except A^u_β [33]), have histidine at this position. The DRw14 (Dw9) β chain (*DRB1*1401*) is thought to be the result of a gene conversion event between the DRB1 and DRB4 loci (34). The region coding for the β sheet portion of the β1 domain has nucleotide sequence similarity to several DRB1 alleles including DRw11, w12, w13, w17, and w18. However, the sequence in the α helix appears to be derived from the DRw53 allele except that there is a histidine at position 81. The β2 domain of this allele appears to be derived from

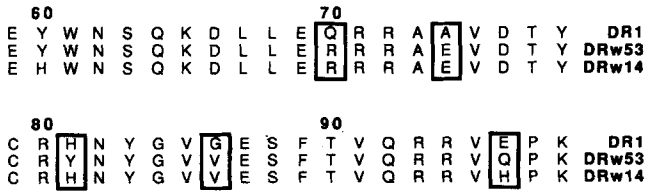

Figure 2. Comparison of amino acid sequences of HLA-DR1, -DRw53, and DRw14 (Dw9). The protein sequence (single-letter amino acid code) from residue 59–96 is shown. Boxed residues are the allelic differences between DR1 and DRw53 in this region.

DRB1 as well. An L cell transfectant expressing the DRw14 (Dw9) class II molecule was made and found to bind SEA and SEE (Fig. 3). Thus histidine 81 was the most likely residue controlling SEA and SEE binding.

To test the role that residue 81 may play in the binding of SEA and SEE to HLA-DR, a pair of point mutant β chains were synthesized. The first was a DR1 β chain with tyrosine at position 81; the second was a DRw53 β chain with histidine at position 81. L cells expressing these mutant DR β chains in association with the wild-type DR α chain were tested for toxin binding (Fig. 3). The mutant, DR1-81HY lost most, but not all, of the SEA and SEE binding seen with the wild-type DR1 molecule. More significantly, the mutant DRw53-81YH bound both toxins at levels comparable with other DR alleles. Therefore, the introduction of histidine at position 81 of the DR β chain restored SEA and SEE binding to a DR β chain which otherwise would be unable to bind these toxins.

Superantigen-induced T Cell Proliferation. The ability of point-mutated DR molecules to stimulate T cells in the presence of SEA or SEE was tested (Fig. 4). As shown previously (24), DRw53 was much less efficient than DR1 in the SEA-induced stimulation of purified T cells. Both the mutant DRw53 with histidine at position 81, and the mutant DR1 with tyrosine at position 81, were intermediate in the ability

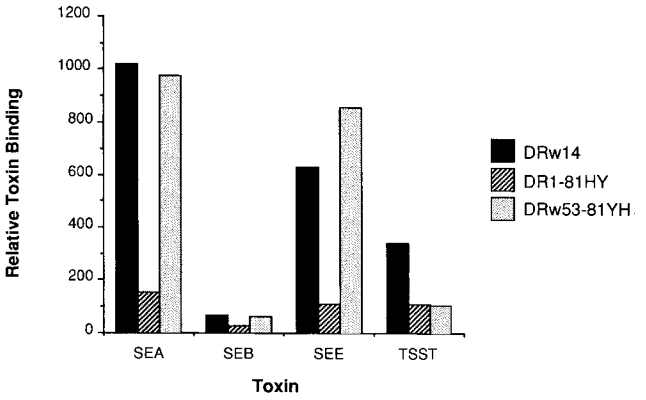

Figure 3. Binding of staphylococcal toxins to DRw14 and to DR1 and DRw53 point mutants. L cells stably expressing the indicated class II molecules were tested for toxin binding as in Materials and Methods. *Solid bar*, DRw14; *hatched bar*, DR1-81HY; *shaded bar*, DR53-81YH.

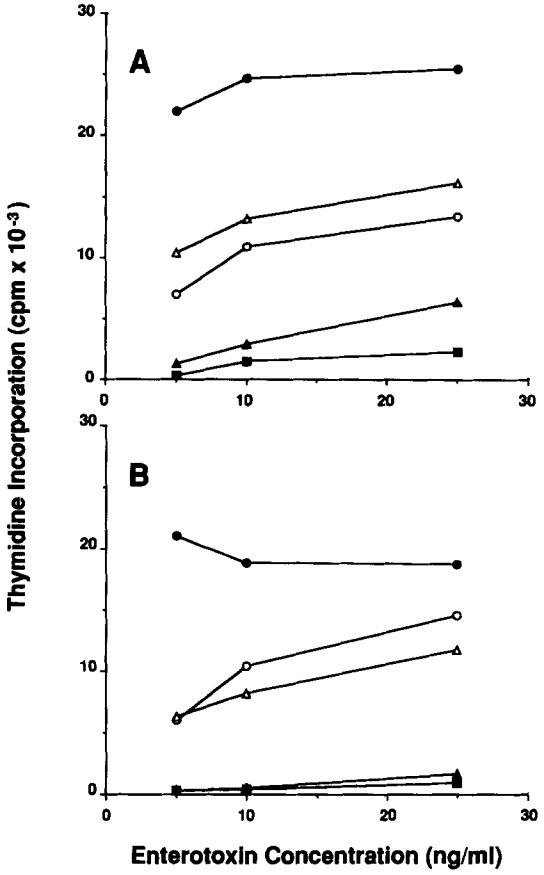

Figure 4. Enterotoxin-induced T cell proliferation. Highly purified T cells were incubated with untransfected L cells or L cells expressing wild-type or mutant DR molecules, and the indicated concentrations of SEA or SEE. The induced T cell proliferation was measured by [^{3}H]thymidine incorporation. Each point represents the mean of triplicate determinations. (*A*) Proliferation in response to SEA. (*B*) Proliferation in response to SEE. (■) DAP-3 (untransfected L cells); (●), L cells expressing DR1; (▲), L cells expressing DRw53; (○), L cells expressing the DR1-81HY point mutant; (△), L cells expressing the DR53-81YH point mutant.

to stimulate T cells in the presence of SEA. The similarity in the ability of these two mutants to cause T cell proliferation is unlike their wide disparity in the ability to bind toxins at saturation (Fig. 3). This is similar to previous data showing that HLA-DP can cause marked T cell proliferation in the presence of TSST although direct binding cannot be demonstrated (11). T cell proliferation is therefore a more sensitive assay for the low level of toxin–class II interaction that results from the residual binding seen with DR1-81HY.

It is not clear why there is submaximal T cell stimulation by the mutant DRw53-81YH, despite a high relative toxin binding (Fig. 3). It is known that allelic differences in class II that do not affect toxin binding result in variation in T cell stimulation by superantigens (24). It is possible that structural features distinct from those responsible for SEA/SEE binding determine the ability of this allele to interact with T cell receptors when bound to these toxins.

The DR1-81HY Mutation Does Not Prevent T Cell Recognition. It is possible that the mutation of histidine to tyrosine

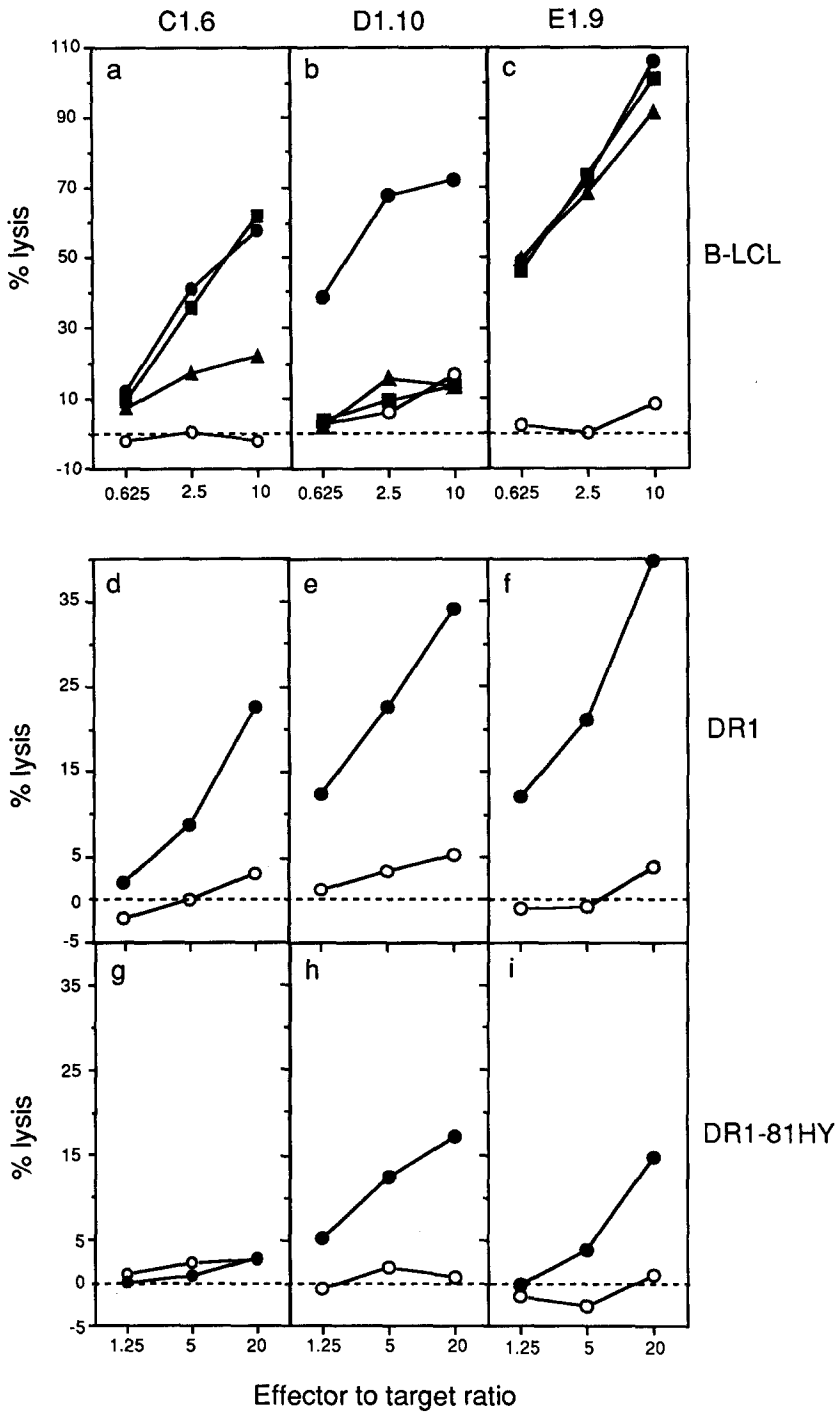

Figure 5. Cytotoxic T cell assays. Three DR1-restricted T cell lines specific for the hemagglutinin peptide H3(307–318) were tested for their ability to lyse a B cell line (B-LCL, *a–c*), a DR1-expressing L cell (DR1, *d–f*), and a DR1-81HY-expressing L cell (DR1-81HY, *g–i*). Specific lysis was measured at the indicated E/T ratios. Target cells were either untreated (○), incubated with peptide (●), incubated with SEA (■), or SEE (▲).

in DR1 abrogated the ability to bind SEA and SEE, not by selective disruption of the toxin binding site, but by causing more extensive changes in the conformation of the class II molecule. The L cells expressing the class II point mutants DR1-18HY and DRw53-81YH were compared with cells expressing wild-type DR1 or DRw53 for their ability to bind the mAbs SG465, L243, and SG250. The first two antibodies react with determinants that require both the α and β chains of human class II (35) or HLA-DR, respectively. SG520 is specific for a monomorphic determinant on the β chain (36). There was no difference in the relative levels of staining among these three antibodies for any of the cell lines tested (data not shown), suggesting that the conformation of the mutants was not grossly altered.

One of the most critical ways to evaluate the functional conformation of an MHC molecule is to test its ability to present peptide antigen. This requires that the ability to bind the particular peptide be preserved and that the peptide-MHC combination result in a recognizable TCR ligand. A panel of DR1-restricted human T cell lines specific for peptides de-

Table 1. *Summary of Staphylococcal Enterotoxin-dependent Cytotoxicity and Reactivity to DR1-81HY in a Panel of Human T Cell Lines*

Line	Specificity	Reactivity		
		SEA*	SEE*	DR1-81HY + peptide‡
B3.10	M1 18–29	0.08 (0.011)	0.47 (0.042)	+
F3.1	M1 18–29	ND	ND	+
H3.12	M1 18–29	0.75 (0.233)	0.98 (0.007)	+
C3.5	M1 18–29	0.42 (0.255)	0.83 (0.247)	–
C1.6	H3 307–318	0.89 (0.167)	0.44 (0.067)	–
E1.9	H3 307–318	0.92 (0.042)	0.88 (0.057)	+
C3.4	H3 307–318	ND	ND	+
D1.10	H3 307–318	0.0 (0.085)	0.05 (0.156)	+

* Reactivity to toxin expressed as lysis of targets incubated with toxin divided by the lysis of targets incubated with 10 μg/ml antigenic peptide (SD).
‡ Lysis of DR1-81HY transfectants incubated with antigenic peptide was scored positive when it was >40% of the lysis obtained with the wild-type DR1 transfectant in the same conditions.

rived from the matrix (M1) and hemagglutinin (H3) proteins of influenza were established. Of the 10 lines, eight were able to recognize peptide presented by transfected mouse L cells. Transfected L cells are in general recognized much less efficiently than human B cell lines by human T cells. Those eight lines were tested for their ability to recognize peptide presented by L cells expressing the DR1-81HY molecules.

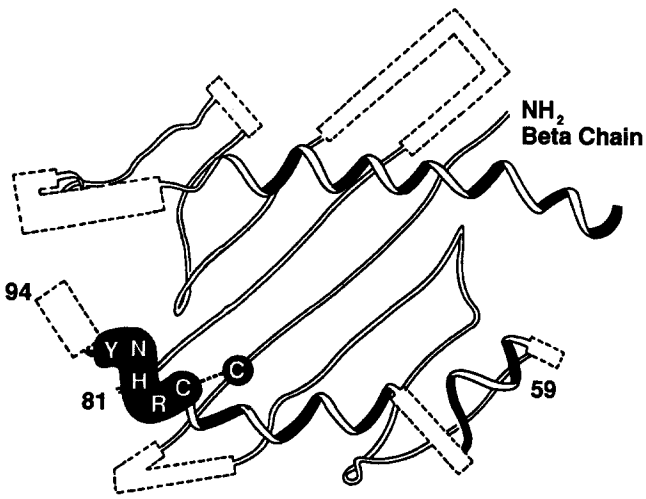

Figure 6. Location of residue 81 on the class II structural model. The proposed three-dimensional structure of the antigen-binding domain of a class II molecule is shown (adapted from reference 32; reprinted from *Nature (Lond.)* 1988, 332: 845–850, by copyright permission of Macmillan Magazines, Ltd.). The position of the conserved pentapeptide, C-R-H-N-Y is indicated, as is the disulfide bond linking cysteine 79 in the α helix with cysteine 15 in the β strand below.

Despite the fact that these transfectants expressed one-third to one-half the level of DR molecules present on the DR1-transfected L cells (not shown), most T cells were able to recognize peptide presented by the DR1-81HY molecule (Fig. 5 and Table 1). Six of the eight T cell lines (three each of the anti-M1 and anti-H3) were able to lyse the transfectant expressing DR1-81HY after incubation with the relevant peptide. Thus, the failure of this class II molecule to bind SEA and SEE is not due to a significant conformational change in the β1 domain. However, the finding that two of the lines did not respond to DR1-81HY when pulsed with peptide suggests that the particular amino acid at residue 81 can dictate recognition by some T cell receptors.

The binding of SEA and SEE to the region of the β chain surrounding position 81 of DR1 may prevent an interaction with those T cell receptors that are sensitive to changes in this portion of the class II molecule. To study this, the M1- and H3-specific T cell lines were tested for their ability to become activated by SEA, and SEE bound to DR1. The 45.1 B cells were incubated with the toxins, labeled with chromium-51, washed, and then used as target cells for the T cell lines. Four of the six lines evaluated responded by lysing the target cells in the absence of antigenic peptide (e.g., Fig. 5, *a* and *c*). This included the two lines that required histidine at position 81 for recognition of peptide in the context of DR1. One of the lines, D1.10, was not stimulated at all by the toxins (Fig. 5 *b*). This clone was able to recognize the H3 peptide regardless of the presence of histidine at position 81. The final T cell clone, B3.10, was stimulated by SEE but not SEA. Thus, while the interactions of certain TCR and SEA/SEE with class II molecules can be sensitive to changes in the same region of the DR β chain, these interactions are functionally distinct.

Discussion

Superantigens such as the SEs are potentially useful reagents for the study of MHC/TCR interactions. A first step in understanding how superantigens trigger T cells, and whether the topology of MHC/TCR is maintained when T cells respond to superantigens, is to define how these molecules bind to class II MHC molecules. The experiments reported here document the identification of a specific amino acid residue critical for the binding of native SEA and SEE to HLA-DR. The ability of class II molecules to bind these two toxins and subsequently stimulate T cells was dependent on the presence of histidine at position 81 of the β chain. This residue is part of a conserved sequence, C-R-H-N-Y, in the putative α-helical portion of the β1 domain. It is found in all reported class II β chain sequences of humans and mice except the HLA-DRw53 and I-A^u alleles. The single replacement of the tyrosine at position 81 of the DRw53 β chain by histidine was sufficient to restore normal binding of SEA and SEE, whereas most of the binding to DR1 was lost by the replacement of histidine by tyrosine.

The ability of the natural and mutated class II molecules to stimulate T cells in the presence of SEA and SEE was similar qualitatively to the binding data. However, the mutant

DRw53-81YH was still less potent than DR1. Conversely, DR1-81HY was still more potent than DRw53. These results are consistent with the possibility that polymorphic MHC residues contacting the TCR contribute to the T cell response in conjunction with the Vβ/superantigen contacts. Clonal T cells respond differently to the same toxin bound to different alleles of HLA-DR (24). This would be in agreement with the view that superantigens strengthen rather than disrupt the class II/TCR interaction (6).

The full extent of the binding site of SEA and SEE cannot be predicted from the data presented here. However, these experiments argue strongly that necessary contact residues are in the conserved sequence at positions 79–83 of the β chain. First, although the single amino acid substitutions described here may cause local changes in secondary structure, it would be unusual for them to cause global conformational changes affecting a distant binding site. Second, there was no difference in the reactivity of the two point mutants with a panel of mAbs to either monomorphic determinants located on the β chain alone or combinatorial determinants requiring both the α and the β chains. Lastly, a majority of T cell lines specific for peptides derived from influenza and restricted by DR1 were capable of responding to these peptides presented by the mutant DR1-81HY. The interaction of T cell receptor with class II and peptide is complex, involving five separate polypeptide chains. It is therefore improbable that the mutation that caused a loss of SEA and SEE binding activity would also cause significant conformational changes in the rest of the class II molecule, particularly in the antigen-binding groove.

It is not surprising that SEA and SEE have similar class II binding characteristics. They are 82% similar in amino acid sequence and cross-react serologically (37, 38). There are significant differences in their secondary and tertiary structures predicted by circular dichroism and fluorescence quenching (39). The NH_2-terminal portion of SEA (residues 1–45) has been suggested to be responsible for binding to class II molecules (25). This region has extensive primary sequence similarity to SEE. In addition, both proteins are predicted to contain similar α helix and β sheet structures in this region. Other regions of these two toxins have much less similarity in the arrangement of putative helix, sheet, and β turn structures. The functional consequence of this is the ability of certain T cell receptors to distinguish between SEA and SEE (Table 1, clone B3.10).

The staphylococcal toxins SEB and TSST bind to distinct sites on HLA-DR (23). SEA will compete with both of these toxins for binding to L cells transfected with DR1 (D. Karp, unpublished observations), suggesting that all three toxins bind to the same general region of class II, although with distinct contact points. The identification of histidine 81 as a critical element for SEA and SEE binding forms the starting point for further investigations into the nature of other toxin binding sites, with the aim of understanding how superantigens as MHC/TCR coligands (40) achieve their potent effects.

The current model of the three-dimensional structure of class II places residues 79–83 of the β chain on a turn of α helix with residue 81 facing away from the antigen-binding groove (Fig. 6) (32). This is appropriate if this sequence is involved in binding staphylococcal toxins. This portion of the helix also overlies the NH_2-terminal end of the α chain, allowing a possible role for both chains in the toxin–class II interaction. There are strong theoretical and experimental reasons to model class II based on the class I crystal structure. However, it is not known how primary sequence differences between class I and class II will ultimately be reflected in the class II secondary or tertiary structure. For example, the region of the α helix discussed above is predicted to be β turn by both Garnier et al. (41) and Chou-Fasman algorithms (42). The probability of a β turn is much larger in the class II β chain sequence than in the class I α2 sequence. Obviously, there are other constraints on the structure in this region (including the disulfide bond between the α helix and the β strand beneath). A β turn–like structure in this area would be an attractive feature for a protein-protein interaction such as toxin binding. The residues at positions 79–83 of the class II β chain comprise the five amino acids with the highest degree of conservation across isotypic, allelic, and species boundaries. A potential physiologic role of this structural feature in the interaction with other ligands is not known.

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Note added in proof: Another report on the SEA binding site in HLA-DR has been published (43).

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