Localization of a Site on Bacterial Superantigens That Determines T Cell Receptor β Chain Specificity

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Summary

A defining characteristic of superantigens is their ability to stimulate T cells based predominantly on the type of variable segment of the T cell receptor (TCR) β chain (Vβ). The Vβ specificity of these toxins most likely results from direct contact between the toxin and the TCR, although the low affinity nature of this binding has prevented direct assessment of this interaction. To identify important functional sites on the toxin, we created chimeric enterotoxin genes between staphylococcal enterotoxins A and E (SEA and SEE) and tested the Vβ specificity of the chimeric toxins. This approach allowed us to identify three amino acid residues in the extreme COOH terminus of these toxins that are largely responsible for their ability to stimulate either human Vβ5- or Vβ8-bearing T cells, or mouse Vβ3 or Vβ11. We also found that residues in the NH_2 terminus were required for wild-type levels of Vβ-specific T cell activation, suggesting that the NH_2 and COOH ends of these superantigens may come together to form the full TCR Vβ contact site. SEA and SEE also differ with respect to their class II binding characteristics. Using the same chimeric molecules, we demonstrate that the first third of the molecule controls the class II binding phenotype. These data lead us to propose that for SEA and SEE, and perhaps for all bacterial-derived superantigens, the COOH and NH_2 termini together form the contact sites for the TCR and therefore largely determine the Vβ specificity of the toxin, while the NH_2 terminus alone binds major histocompatibility complex class II molecules. The predominant role of the COOH terminus of bacterial superantigens in determining Vβ specificity resembles current models being proposed for virally encoded superantigens, suggesting that these molecules may demonstrate some structural relationship not seen at the amino acid level.

Superantigens are a family of T cell mitogens that are unique in two respects: first, they require the presence of MHC class II molecules to effectively activate T cells; and second, the T cells that respond to them share one or a few variable segments of the TCR Vβ chain (1–6). One class of proteins that demonstrates such characteristics are exotoxins derived from *Staphylococcus aureus* and *Streptococcus pyogenes* (staphylococcal enterotoxin [SE][1] A through E, toxic shock toxin [TSST-1], and streptococcal pyrogenic exotoxins A and C). A second superantigen family consists of the proteins encoded by the 3′ LTR of mouse mammary tumor viruses (MMTVs). Superantigen activation of T cells in a Vβ-specific manner, with only limited regard for the make up of the other variable segments of the TCR (Dβ, Jβ, Vα, Jα), has been presumed to be due to direct contact between the toxin and the TCR Vβ chain. Indeed, the contact site on TCRs by bacterial and viral superantigens has been mapped and shown to lie outside the site of the TCR thought to contact conventional peptide antigen/MHC complexes (7, 8). Several years ago we and others demonstrated direct binding between bacterial superantigens and MHC class II molecules (9–11). This binding reaction is of moderately high affinity, especially with SEA and HLA-DR, making it a relatively easy interaction to study functionally and map biochemically (12). However, direct binding between toxins and TCRs has been much more difficult to demonstrate. This reaction, therefore, must be of lower affinity and/or more transient, although direct binding between bacterial superantigen/class II complexes and isolated TCR β chains has been reported (13). As our mechanistic understanding of toxin-mediated T cell activation has increased, it is easy to understand why previous approaches of looking for mitogenic fragments of these molecules failed to provide convincing results. T cell activation by superantigens requires both class II binding and the TCR contact site on the same molecule. Therefore, a reductionist approach, finding smaller and smaller mitogenic fragments,

[1] *Abbreviations used in this paper:* GAM, goat anti-mouse; SE, staphylococcal enterotoxin; SOE, splicing by overlap extension.

is precluded in analysis of SE-mediated T cell activation, unless a single fragment were to possess both activities.

Besides the in vitro activities most often studied, such as T cell proliferation, Vβ-specific T cell activation, and MHC class II binding, the SEs and TSST-1 cause a number of in vivo effects, such as fever production (14), emesis (15), and toxic shock (16, 17), that may reflect one or several of these in vitro activities. Consequently, it would be of substantial interest to construct mutant SEs that completely lacked, for example, the ability to activate T cells yet retained the ability to bind class II molecules and then test for the correlative loss of in vivo activities. To construct such mutations, one first must map the regions of the toxin that directly mediate these in vitro activities and then create discrete mutations that effectively eliminate one activity while preserving others.

Recently, Kappler et al. (18) advanced a structure-function model of the related enterotoxin SEB. Using a random mutagenesis approach, these investigators reported that mutations affecting both MHC class II binding and TCR interaction/activation clustered to the NH_2 terminus of the molecule. These data led them to propose that these two critical immunological functions are intertwined at the NH_2-terminal end of the molecule. If this model is correct then hopes of constructing mutants with selective loss of one of these functions may be misfounded. However, previous data from our laboratory had suggested that class II binding and TCR contact activities were dissociable (19, 20). Therefore, we sought to map these two activities using a different genetic approach to determine whether they are indeed intertwined or not.

To map functional regions of bacterial superantigens, we took the approach of constructing intragenic SEA/SEE chimeras. SEA and SEE, despite their high degree of structural similarity (21, 22), demonstrate reciprocal patterns of Vβ-specific T cell activation (23), as well as distinct class II binding affinities (24, 25), making them good candidates for this type of study. By testing the chimeric molecules for Vβ specificity, or class II binding affinity, we hoped to localize the region of one or both molecules that mediates these functions. In this report, we use this approach to map the region of SEA and SEE that controls their Vβ specificity, and to separate this region from that determining class II binding affinity.

Materials and Methods

Mice. 8–12-wk-old male B10.BR/SgSnJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). T cells were purified from spleen cells by lysis of RBC and adsorption of B cells onto goat anti-mouse (GAM) Ig–coated magnetic beads (Advanced Magnetics, Cambridge, MA) as described (20). Purified T cells (2×10^6/ml) were stimulated with recombinant toxins and irradiated (2,000 rad) syngeneic spleen cells (10^6/ml) for 4 d. Viable nonadherent cells were isolated by lympholyte-M gradients, washed in HBSS, and analyzed by flow cytometry with a panel of FITC-labeled, Vβ-specific mAbs (Pharmingen, San Diego, CA). Forward angle and 90° light-scatter patterns were used to restrict the analysis to blast cells.

Antibodies. The following murine mAbs were used for isolation of purified human T cells: L227 (IgG1) and L243 (IgG2a), anti–human class II monomorphic determinant; 9.3F10 (IgG2a), anti-HLA DR/DQ; OKM1 (IgG2b), anti-CD11b; LM2/1.6.11 (IgG1), anti–human MAC-1. All cell lines were obtained from the American Type Culture Collection (Rockville, MD). The mAbs L227, L243, and LM2/1.6.11 were used as ascites to stain cells at a 1:1,000 dilution. OKM1 was used to stain cells as a tissue culture supernatant at a 1:20 dilution. Antibodies specific for human TCR Vβ segments were from the Diversi-T Anti–Human TCR Antibody Kit (T Cell Diagnostics, Cambridge, MA).

Construction of Chimeric Enterotoxins. Construction of chimeric enterotoxin genes and site-directed mutagenesis were accomplished by the technique of splicing by overlap extension (SOE) (26, 27). The coding regions of the *ent*A (SEA) and *ent*E (SEE) genes were subcloned from the plasmids pMJB117 (22) and pMJB46 (21), respectively, into the phagmid vector pBluescript II SK(-); these plasmids were designated pMV2 and pMV5, respectively (20). All oligonucleotide primers were obtained form Midland Certified Reagent Co. (Midland, TX). To construct a chimeric enterotoxin gene, internal PCR primers were designed to provide at least 12 bp of priming homology on the two different toxin genes and ~15 bp of overlapping homology with each other. The two fragments of the genes were amplified using the SOE primers and flanking primers homologous to the reverse primer (sense orientation) and M13-20 primer (antisense orientation) sites on the pBluescript vector. Primary PCR reactions consisted of 1 μM of each primer, 1 μg of template plasmid, 10 μl of 10× PCR buffer, 200 μM of each dNTP in a total volume of 100 μl. For the first several chimeras, 2.5 U Taq polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT) was used along with the 10× buffer (Perkin-Elmer Cetus Corp.) with a final Mg^{2+} concentration of 2.5 mM. For the remaining chimeras, 2.5 U of PFU polymerase (Stratagene, La Jolla, CA) per reaction was used with 10 μl of the manufacturer's 10× buffer no. 2 (200 mM Tris-Cl, pH 8.8, 100 mM KCl, 60 mM $[NH_4]_2SO_4$, 15 mM $MgCl_2$, and 1% triton X-100), as PFU polymerase was found to introduce far fewer polymerase errors than Taq polymerase. PCR reactions were overlayed with 100 μl light white mineral oil and denatured at 95°C for 5 min, the polymerase was then added, and reactions were subjected to 27 cycles of amplification (1 min at 95°C; 2 min at 55°C, and 3 min at 72°C) on a thermocycler (Perkin-Elmer Cetus Corp.). The products of the primary PCR reaction were separated on a 1.0% agarose gel, and the amplified products excised from the gel and melted in three volumes of 6 M NaI, 100 mM $NaPO_4$, 22.2 mM dithiothreitol, pH 6.0. The DNA was isolated from melted agarose on SpinBind cartridges (FMC Bioproducts, Rockland, ME) according to the manufacturer's instructions. Typically, two cartridges were used to purify the products from one PCR reaction. Each cartridge was eluted with 60 μl of water, the eluates from the same reaction were pooled, and the DNA was precipitated with 0.1 vol of 3 M sodium acetate and 2.5 vol ethanol. The toxin fragments were resuspended in 20 μl of water and quantitated at OD_{260}. In the secondary PCR reaction, 1 μg of each of the toxin fragments to be spliced together were mixed in a 100-μl PCR reaction along with 1 μM of reverse primer and M13-20 primer each, 200 μM of each dNTP, 10 μl of 10× buffer, 2.5 U of polymerase, and subjected to 32 amplification cycles as described above except that the annealing temperature was lowered to 45°C. The products of the secondary PCR reaction were phenol extracted, ethanol precipitated, and resolubilized in 32 μl of water, supplemented with 4 μl of appropriate 10× enzyme buffer, and digested with 40 U (4 μl) of either BamHI or HindIII (Gibco BRL, Gaithersburg, MD). Digestions were carried out for 2 h at 37°C with phenol extraction and ethanol precipi-

tation between digestions. After completion of the second restriction digestion, the products were separated on a 1.0% agarose gel, and the band corresponding to the spliced and digested product was excised from the gel. This product could be distinguished from the predigested material on the basis of size because of the absence of the flanking DNA fragments. The digested fragment was purified from the agarose gel on one SpinBind cartridge, eluted in 80 μl of water, and ethanol precipitated. The fragment was resolubilized in 15 μl of water, quantitated at OD_{260}, and ligated into a BamHI-HindIII–digested pBluescript II SK(-) vector at a 10:1 insert-to-vector ratio. 0.5–5 μl of the ligation reaction was used to transform XL-1 blue–competent cells (200236; Stratagene) according to the manufacturer's instructions. White colonies were screened for appropriate insert and orientation by bacterial PCR reactions (28) using the flanking primers (reverse and M13-20 primer), as well as one of the internal SOE primers used to construct the chimera. Clones displaying PCR products of appropriate size were chosen for further analysis. All of the chimeric/mutant toxins were sequenced in full and found to have no unintended mutations, or rarely, silent mutations. DNA sequencing was carried out on single-stranded templates using a Sequenase dideoxy termination sequencing kit (U.S. Biochemical Corp., Cleveland, OH) as previously described (20, 29).

Nomenclature. Three types of recombinant toxins were made: chimeric enterotoxin, region-specific exchange mutants, and point mutations. Chimeric enterotoxins were designated according to the composition of the NH_2- and COOH-terminal regions followed by the amino acids at the chimeric junction; e.g., SEE-SEA 70-71 indicates a toxin with SEE sequences from amino acids 1–70 and SEA sequences from amino acids 71–233. All amino acid numbering is according to the SEA sequence (21). Region-specific mutations are designated by indicating the substituted stretch of amino acids in parenthesis after the toxin of origin; e.g., SEA-SEE (136–149) indicates that the amino acids 136–149 of SEE are substituted into the SEA molecule at the analogous position. Point mutations are indicated by designating the toxin, amino acid position, and amino acid mutation; e.g., SEA 200 G→D represents a mutation of glycine to aspartic acid in the SEA molecule at amino acid 200.

Production of the Chimeric and Mutant Toxins. 2 liters of 2× YT media (16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter) containing 100 μg/ml ampicillin was inoculated with 75 ml of confluent overnight bacterial cultures. The cultures were grown to an OD_{600} of 0.9 and supplemented with isopropyl-β-D-thiogalactopyranoside (Pierce Chemical Co., Rockford, IL) to a final concentration of 1 mM. The cultures were returned to the incubator and grown with vigorous aeration for 2–4 h. At the end of the incubation period bacteria were pelleted at 4,000 rpm for 20 min at 4°C, and resuspended in 20 ml of ice-cold SET (20% sucrose, 1 mM EDTA, and 20 mM Tris-Cl, pH 7.5). The resuspended bacteria were incubated at 4°C for 15 min, pelleted at 4,800 rpm in a rotor (SS34; Sorvall, Wilmington, DE) and resuspended in 10 ml of 1 mM EDTA, rocked at 4°C for 45 min, and repelleted at 7,000 rpm for 15 min. The supernatant, hereafter referred to as the shockate, was removed and filtered (0.45 μm). For toxins consisting mostly of SEA-derived sequences, the shockate was diluted 1:2 in PBS, pH 7.2, and purified on an anti-SEA Sepharose column as previously described (20). For chimeric/mutant toxins consisting mostly of SEE-derived sequences, the shockate was concentrated to 1.5 ml, first on a Centriprep 10 and then on a Centricon 10 (Amicon Corp., Danvers, MA). The sample was fractionated on a Superdex 75 gel filtration column in 200 mM ammonium bicarbonate using an FPLC system (Pharmacia LKB Biotechnology, Piscataway, NJ). The column was calibrated with native SEA to determine the fractions that would contain the eluting chimeric toxin. The semipurified toxin was concentrated to 3 ml on a Centricon 10 and desalted into PBS on a DG-10 column (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. Total protein content was determined by the bicinchoninic acid assay (BCA) (Pierce Chemical Co.). Toxins prepared by affinity chromatography ran as single bands at 28 kD on Coomassie blue–stained SDS-PAGE gels. Typical yield from 1 liter broth (10 ml periplasmic shockate) was ~100 μg.

Vβ Analysis of Toxin-activated Human T Cells. PBMC were obtained from freshly isolated buffy coat packs derived from healthy donors (Gulf Coast Blood Center, Houston, TX). T cells were purified from the PBMC by negative selection using GAM Ig–conjugated magnetic beads (Advanced Magnetics). Briefly, PBMC were stained with a cocktail of anti–class II and anti–monocyte mAb consisting of L227, L243, 9.3F10, OKMI, and LM2/1.6.11 for 30 min at 4°C. The cells were washed once and incubated with GAM Ig magnetic beads at a ratio of 200 × 10^6 cells/12-ml equivalents of GAM Ig beads. The GAM Ig beads were washed four times in HBSS/2%FCS before use. The cells and beads were incubated for at least 2 h with gentle agitation at 4°C. After one round of negative selection, the cells typically consisted of >96% $CD3^+$ cells. Purified T cells (10^6/ml) and autologous irradiated (1,000 rad) unfractionated PBMC (2.5 × 10^6/ml) were cultured in 5 ml of assay media composed of RPMI, 10% heat-inactivated human AB^+ serum, 100 μg/ml gentamicin, 1% antibiotic-antimycotic mixture, 2 mM L-glutamine, 5 mM Hepes (all components, except serum, from Gibco Laboratories, Grand Island, NY) in six-well dishes (3506; Costar, Cambridge, MA). All affinity-purified recombinant toxins were added to a final concentration of 0.5 μg/ml and the gel filtration–purified recombinant toxins were used at a final concentration of 5 μg/ml (found to be optimal for each of the preparations in preliminary experiments). Native SEA and SEE (Toxin Technology, Sarasota, FL) were added at a final concentration of 0.5 and 0.25 μg/ml, respectively. The cultures were incubated for 3 d at 37°C in a humidified incubator with 5% CO_2. Viable cells were then isolated from the culture on an isolymph gradient and recultured in 5 ml of assay media supplemented with 18 ng/ml rIL-2 (R&D Systems, Minneapolis, MN) or 50 U/ml rIL-2 (Amgen Biologicals) overnight. The following day, 0.5 × 10^6 cells were stained with either the indicated anti-Vβ antibody (T cell Diagnostics) followed by GAM Ig(G,M) FITC (Fluoricon; Baxter Healthcare Corp., Mundelein, IL), anti-CD3 (Leu-4 FITC; Becton Dickinson & Co., Mountain View, CA), or goat anti–mouse Ig (G,M) FITC alone. Cell staining was carried out for 60 min at 4°C in PBS/1%FCS/10 mM Hepes/0.025% (wt/vol) sodium azide in a total volume of 200 μl. In the initial phase of this study, cells were analyzed with the entire panel of anti-Vβ antibodies in the Diversi-T TCR α/β screening panel. After finding no perturbation in the percent positive blasts for any of the antibodies except for Vβ5(*a*) and Vβ8, the latter part of the study involved staining the T cell blasts with only the anti-Vβ5(*a*) (clone 1C1, isotype IgG1) and anti-Vβ8 (clone 16G8, isotype IgG2b) antibodies. The cells were analyzed by flow cytometry on an Epics Profile (Coulter Corp., Hialeah, FL) by gating on the blast cell population only. Except where indicated, all chimeric/mutant toxins were tested on three different donors in the same experiment. Data are expressed as the mean value for percent positive cells ± SEM.

Class II Binding Assay. The ability of selected chimeric toxins to bind MHC class II molecules directly was assessed using an HLA-DR1-transfected fibroblast cell line, D.5-3.1 (kindly provided by E. Long, National Institutes of Health, Bethesda, MD). The assay was performed essentially as described (19), except the assay was

carried out in HBSS/2% FCS and the cells were stained with a pretitered amount of SEA-FITC.

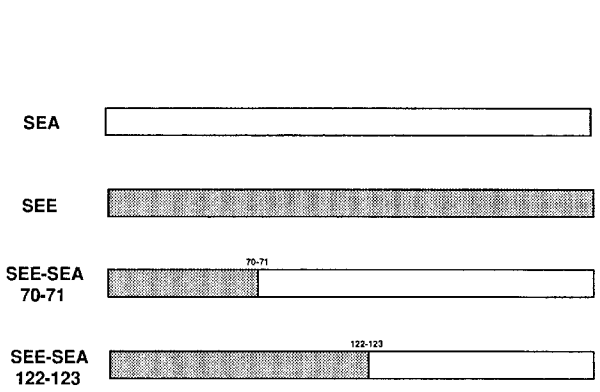
Proliferation Assay. Selected chimeric toxins were assessed for their ability to stimulate T cell proliferation by incubating dilutions of purified chimeric toxins with 8×10^4 PBMC in round-bottomed 96-well plates. The cells were cultured for 66 h, with 1 μCi [^{3}H]TdR (2 Ci/mmol; DuPont Co., Wilmington, DE) added per well for the final 18 h of culture. DNA was harvested onto glass fiber filters, and incorporation of [^{3}H]TdR was assessed by liquid scintillation counting.

Results

Vβ Specificity of SEA and SEE Maps to the COOH-terminal Half of the Molecules. Previous results from our laboratory showed that the central disulfide loop region, highly conserved among the enterotoxins, plays an important role in their mitogenicity (19), but does not itself contain the Vβ contact site (20). Therefore, we began by constructing two chimeric molecules that were designed to localize the region determining the Vβ specificity of these two enterotoxins to one side of the loop region. Both of these chimeras were designed with SEE sequences at the NH_2-terminal portion and SEA sequences at the COOH-terminal portion. The chimeric junctions were chosen to be well upstream and downstream of the cysteine loop, which in SEA is formed by cysteines 96 and 106. Analysis of the Vβ specificity of these two chimeras indicated that they both closely resembled the stimulatory phenotype of SEA (Fig. 1). Thus, the region of the molecule responsible for Vβ specificity mapped to the COOH-terminal half of these molecules, downstream of amino acid 123. Interestingly, T cell responses to these chimeras exhibited slightly lower levels of Vβ5 and slightly elevated levels of Vβ8 compared with responses to native SEA.

To define more precisely the region of the toxins that mediates Vβ specificity, we created a series of chimeric toxins in which the junction lay progressively further downstream from the 122-123 junction. These were constructed in the opposite orientation from the initial two to take advantages of the high Vβ8 signal of SEE. For chimeric toxins containing progressively fewer SEE sequences at the COOH terminus, the percentage of Vβ8$^+$ T cell blasts decreased while that of Vβ5$^+$ T cells increased (Fig. 2 *A*). Some chimeric toxins showed an unexpected phenotype, stimulating significant levels of both Vβ5$^+$ and Vβ8$^+$ T cells. The most striking example of this was the chimera SEA-SEE 187-188, although it was also seen with SEA-SEE 162-163 and to a lesser degree with SEA-SEE 142-143. T cell blasts stimulated with SEA-SEE 187-188 contained approximately equal percentage of Vβ5- and Vβ8-bearing T cells, although both levels were below those seen with wild-type SEA and SEE, respectively. As in the two chimeras shown in Fig. 1, although the COOH terminus largely dictated the Vβ specificity of chimeras SEA-SEE 142-143 and 160-161, the percentage of the Vβ8$^+$ blasts was ~50% that of native SEE. As expected, the reciprocal constructs of these two chimeras stimulated predominantly Vβ5$^+$ T cells (Fig. 2 *B*), confirming the importance of the COOH terminus in Vβ specificity. However, when the SEA-SEE chimeric junction was placed at amino acids 219-220, the chimera was phenotypically indistinguishable from native SEA. Because the 160-161 chimera possessed the most downstream junction that provided a clean reciprocally stimulating phenotype, we concluded that the region largely responsible for determining Vβ specificity of these two toxins lay downstream of amino acid 161 and upstream of 219.

Amino Acid Differences between SEA and SEE in the Vβ-determining Domain Cluster to Four Regions. Fig. 3 shows an amino acid comparison between SEA and SEE in the COOH-terminal half of the molecule. Amino acid differences cluster to four regions, which we designated regions 1–4. Because regions 1–3 contained the greatest number of differences, we initially exchanged these regions between the two toxins to test the contribution of each to Vβ specificity independently of the other regions. Exchanging region 1, 2, or 3 from SEA into SEE or the reciprocal constructions showed no pertur-



	Percent Positive Blasts	
	Vβ5	Vβ8
SEA	5.5 ± 0.6	0.7 ± 0.1
SEE	1.6 ± 0.1	14.7 ± 3.1
SEE-SEA 70-71	4.3 ± 0.0	1.9 ± 0.4
SEE-SEA 122-123	4.4 ± 0.0	1.4 ± 0.3

Figure 1. The region of SEA and SEE that determines Vβ specificity is located in the COOH-terminal half of the molecule. Chimeric enterotoxins with indicated amino acid junctions were constructed and used to stimulate purified human T cells for 3 d in vitro. Responding cells were stained with mAbs specific for either human Vβ5 or Vβ8, and the blast cells were analyzed by flow cytometry for percentage of positive staining cells. In this and subsequent figures, unless otherwise noted, data shown are the mean of three analyses on different donors in one experiment ± SEM. Background staining was not subtracted. Responding cell populations were >98% CD3 positive and <0.3% positive when stained with GAM Ig FITC alone.

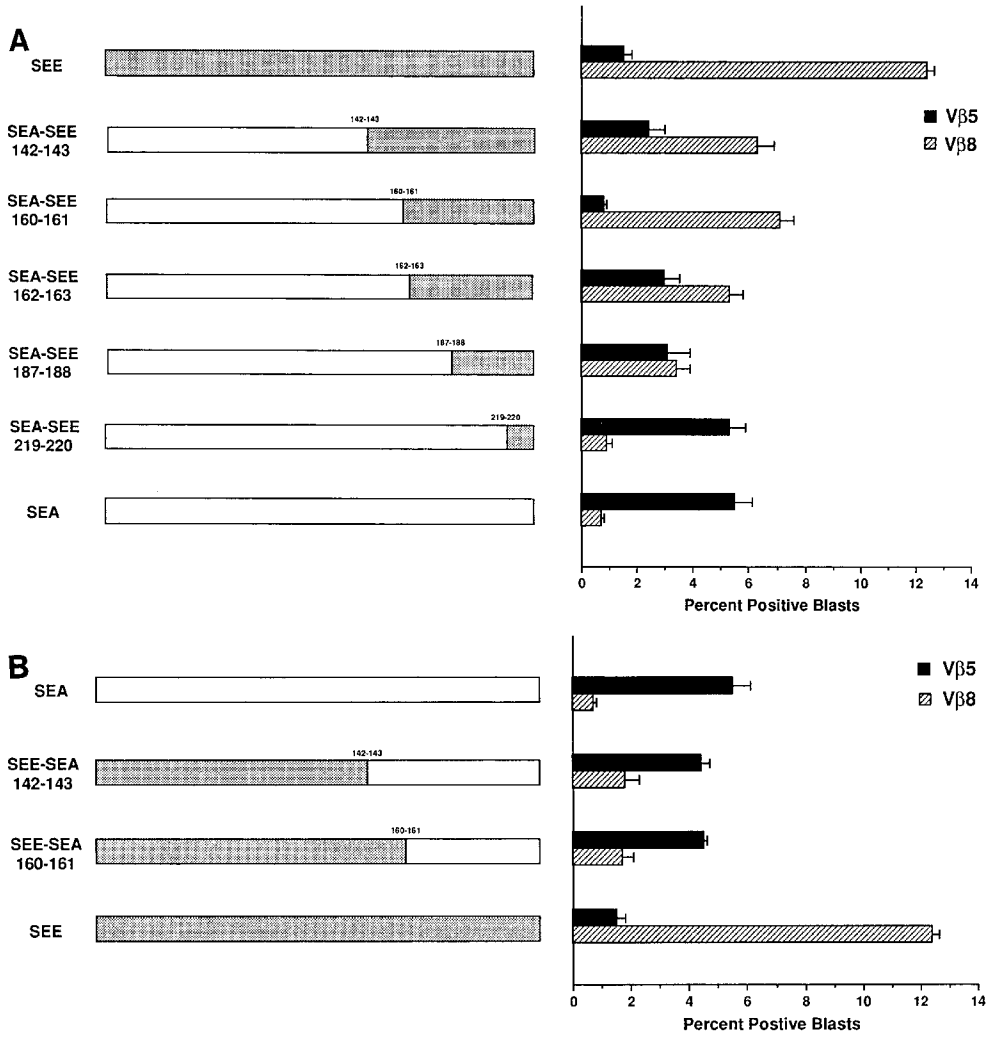


Figure 2. Chimeric enterotoxins with progressively downstream junctions further define the Vβ-determining region of SEA and SEE. All chimeras were analyzed in three different donors in one experiment, except for SEA-SEE 160-161, which is the result of six analyses in one experiment.

bation of the Vβ stimulatory phenotype compared to the native toxin (Table 1). The one exception was SEA-SEE(136–149), which stimulated Vβ8$^+$ T cells significantly above background, although this increased stimulation of Vβ8$^+$ T cells was not accompanied by a concomitant decrease in Vβ5 stimulation. Moreover, this mixed phenotype was not observed with the reciprocal construct SEE-SEA(136–149). Additionally, we mutagenized the internal four amino acids in region 3 to alanines but again saw no effect on the Vβ stimulatory phenotype (SEA-Ala[190–193]; Table 1). Importantly, in all of these mutants, in contrast to the chimeras shown in Figs. 1 and 2, we observed percentages of Vβ5 or Vβ8 equivalent to those seen in the wild-type toxins. From these data we concluded that neither region 1, 2, nor 3 alone was sufficient to transfer Vβ specificity from one toxin to the other. It was possible that more than one region was required to transfer specificity. For example, regions 2 and 3 together may convert the Vβ stimulatory phenotype of one toxin to another.

Region 4 Alone Can Reciprocally Transfer Most of the Vβ Stimulatory Phenotype between SEA and SEE. To investigate the possibility that the region required to transfer Vβ specificity is larger than either region 1, 2, or 3 alone, we took the approach of constructing double chimeric molecules. We started with SEA-SEE 160-161, because this is the most downstream chimera that did not show a mixed phenotype and appended the COOH terminus of SEE from amino acid 196 to the end of the molecule. These terminal 38 amino acids contain only six amino acid differences between SEA and SEE. Three

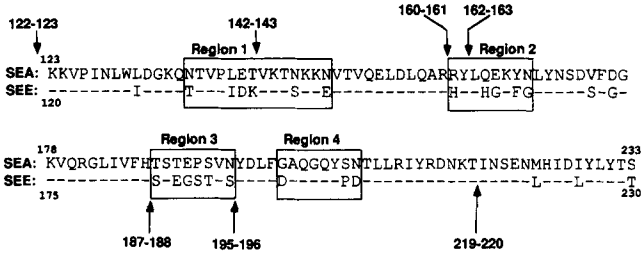


Figure 3. Amino acid comparison of SEA and SEE in the Vβ-determining domain. Sequence differences cluster to four regions. Numbering used to designate chimeric joints is according to the SEA molecule (21). SEA and SEE contain the same number of amino acids in the immature, unprocessed protein. Mature SEA, however, contains three extra amino acids due to differential processing of the NH_2 terminus.

Table 1. *Mutations Exchanging Regions 1, 2, and 3 between SEA and SEE Do Not Affect the Vβ Stimulatory Phenotype*

Chimera/Toxin	Parental toxin	Region mutated	Region mutated to:	Percent positive blasts Vβ5	Vβ8
SEA	-	-	-	4.9 ± 0.6	1.0 ± 0.2
SEA-SEE(136–149)	SEA	1	SEE	4.5 ± 0.5	4.1 ± 1.2
SEA-SEE(161–168)	SEA	2	SEE	6.4 ± 0.8	0.7 ± 0.1
SEA-SEE(188–195)	SEA	3	SEE	5.1 ± 0.1	0.4 ± 0.0
SEA-Ala(190–193)	SEA	3	Alanine	5.4 ± 0.6	0.5 ± 0.1
SEE	-	-	-	1.5 ± 0.3	12.4 ± 0.2
SEE-SEA(136–149)	SEE	1	SEA	1.1 ± 0.3	11.4 ± 0.4
SEE-SEA(161–168)	SEE	2	SEA	0.9 ± 0.2	12.7 ± 0.5
SEE-SEA(188–195)	SEE	3	SEA	0.4 ± 0.0	11.7 ± 2.2

of these amino acid differences had already been ruled out as playing a role in Vβ specificity by the chimera SEA-SEE 219-220 and the other three differences were encompassed by region 4. We were surprised to find that in these constructs, the flanking regions of the molecule dictated the Vβ specificity. For instance, SEE-SEA(161–195) was phenotypically indistinguishable from native SEE (Fig. 4), ruling out a complex Vβ-determining site comprised of some combination of residues in regions 1–3.

These chimeric molecules led us to conclude that the amino acids that determine the Vβ specificity lie downstream of amino acid 196. To test this hypothesis, we constructed the chimera SEA-SEE 195-196 as well as reciprocal region 4 exchange mutants. These mutants again demonstrated that the COOH-terminal region, specifically the three amino acid dimorphisms in region 4, dictated the Vβ specificity of these toxins. As with previous chimeras, however, reduced levels of Vβ8 and Vβ5 were now observed. The effect of these three amino acid differences on the Vβ phenotype of the toxin was especially dramatic when comparing SEA-SEE(200–207) to SEA (Fig. 4). Mutation of these three amino acids in region 4 elevated levels of Vβ8 and depressed Vβ5 to background levels. The phenotype of the reciprocal constructions shown in Fig. 4 demonstrated that mutation of 200–207 in SEE to the SEA sequence could also depress Vβ8 stimulation and elevate levels of Vβ5 (data not shown). These data demonstrate that analogous sites in SEA and SEE control stimulation of Vβ5 and Vβ8, suggesting that one site on the molecule controls stimulation of all Vβs, rather than multiple TCR contact sites each controlling the stimulation of one Vβ.

All Three Amino Acid Dimorphisms in Region 4 Are Required for Complete Transfer of Vβ Specificity. To test the importance of each of the three amino acid differences in region 4, we mutated each individually. We chose to mutate the SEA molecule to the SEE sequence in these positions because, collectively, these three differences showed the most complete transfer of specificity when mutated from the SEA to the SEE sequence. Mutation of any of these amino acids individually failed to transfer specificity completely, as judged by elevation of Vβ8 and depression of Vβ5 (Fig. 5). Interestingly, each of the three individual mutations showed a distinct pattern. For the Ser→Pro 206, Vβ8 levels were slightly

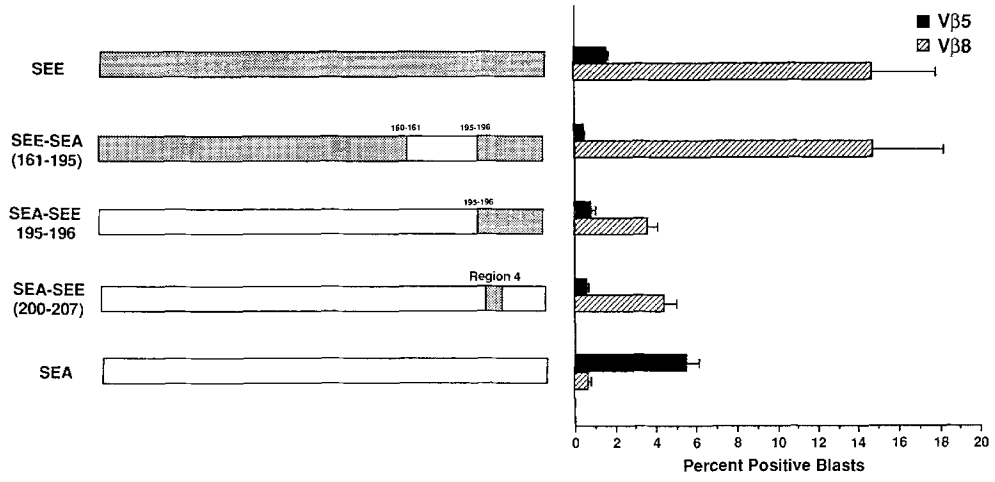


Figure 4. Region 4 alone mediates Vβ specificity in SEA and SEE. Reciprocal exchange of region 4, containing three amino acid differences (Fig. 3), inverted the Vβ stimulatory phenotype of the toxin. Note that levels of responding Vβ8+ T cells were not achieved unless both the NH_2- and COOH-terminal regions of the molecule were derived from one toxin. Responding cell populations were typically >97% CD3 positive with a background <0.3% positive when stained with GAM Ig FITC alone. Background staining was not subtracted.

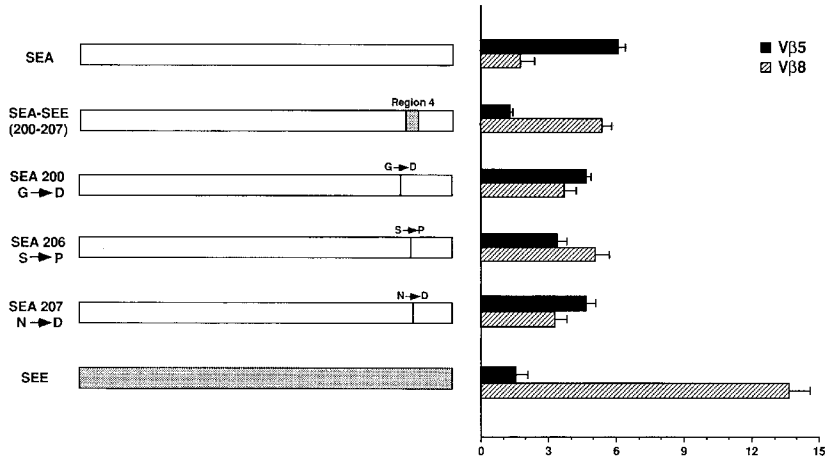


Figure 5. All three amino acid dimorphisms in region 4 are required for full transfer of Vβ specificity. Each of the three amino acid mutations alone, all contained within region 4 (see Fig. 3), failed to transfer specificity from SEE to SEA as efficiently as all three together.

elevated above those of Vβ5, whereas for the Gly→Glu 200 and Asn→Glu 207, a reciprocal pattern was seen. This pattern was seen with T cells from all three of the individuals tested.

NH_2-terminal Residues Are Required for Wild-Type Levels of Vβ8 Expression. When we compared the levels of $V\beta8^+$ T cells stimulated by SEE-SEA(161–195) and SEA-SEE 195-196 (Fig. 4), it was evident that sequences located upstream of amino acid 161 could complement the level of Vβ8 expression from 25 to 30% of that of native SEE to full wild-type levels. To map the sequences required to complement Vβ8 expression to wild-type levels, we superimposed the region 4 of SEE on the chimera, SEE-SEA 70-71 (Fig. 6). The data demonstrated that the region of the toxin responsible for complementing Vβ8 levels back to those seen with native SEE was located in the first 70 amino acids. The effect of region 4 on the Vβ stimulatory phenotype was particularly dramatic when comparing SEE-SEA 70-71 and SEE-SEA 71-71/SEA-SEE(200–207). Substitution of the SEE region 4 onto the SEE-SEA 70-71 chimera not only decreased Vβ5 levels to background, but also increased Vβ8 to wild-type levels.

The NH_2-terminal Region Determines Class II Binding Phenotype. SEA and SEE bind class II molecules with different affinities and most likely at slightly different sites. Because two reports have suggested that the site on the toxins that interacts with class II molecules is located in the NH_2-terminal region (18, 30), we tested the two chimeras, SEA-SEE(200–207) and SEE-SEA 70-71/SEA-SEE (200-207), for class II binding phenotype. The data demonstrate that SEA-SEE(200–207) bound class II molecules in a fashion similar to SEA, whereas SEE-SEA 70-71/SEA-SEE (200–207) bound class II molecules similar to SEE (Fig. 7). Thus, the NH_2-terminal region of SEA and SEE determined their characteristic abilities to bind MHC class II molecules, consistent with previous reports. Furthermore, class II binding assays of SEA-SEE 142-143, 160-161, 187-188, 219-220, and SEE-SEA 70-71 indicated that the chimeric nature of these molecules did not perturb their ability to bind class II molecules (data not shown).

Proliferative Capacity of Selected Chimeric Toxins. Our data have shown that chimeric toxins that contained NH_2 and COOH regions from different toxins stimulated lower percentages of $V\beta5^+$ or $V\beta8^+$ T cells than that seen with the wild-type toxins or with toxins that had both NH_2- and COOH-terminal regions derived from a single toxin. To in-

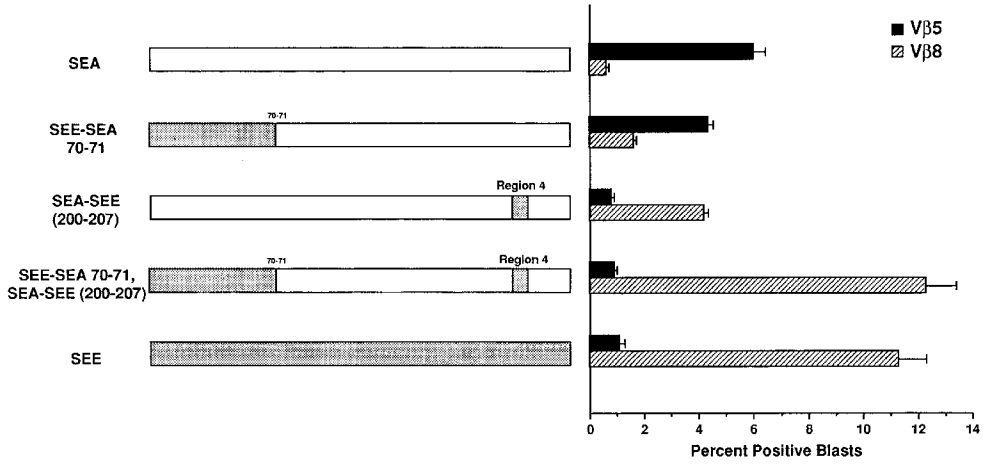


Figure 6. Residues upstream of amino acid 70 restored wild-type levels of Vβ8 stimulation to those seen with native SEE.

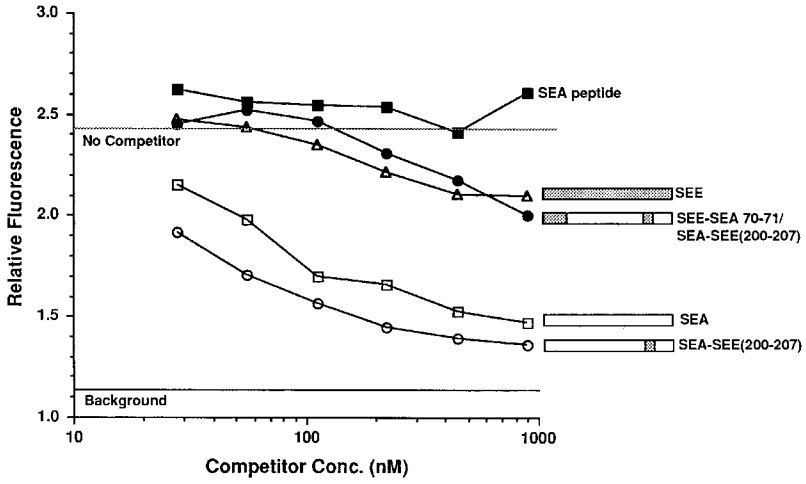


Figure 7. NH_2-terminal 70 amino acids determine class II binding characteristics of SEA and SEE. SEA-SEE(200–207) competed with FITC-SEA for binding to DR1-transfected L cells similarly to wild-type SEA derived from *E. coli*. SEE-SEA 70-71/SEA-SEE(200–207) competed with FITC-SEA poorly for binding to DR1 similarly to wild-type SEE. Data are expressed in linear fluorescence units and are from one experiment representative of two done.

vestigate whether this reduced level of Vβ-specific stimulation was due to decreased mitogenic potency of the chimeras, we tested the ability of SEA-SEE(200–207) and SEE-SEA 70-71/SEA-SEE (200–207) to stimulate T cell proliferation (Fig. 8). The chimeric molecules were equally potent T cell mitogens, comparable to wild-type SEA, ruling out the possibility that the reduced level of Vβ-specific stimulation seen in some chimeras, such as SEA-SEE(200–207), was due to decreased ability to stimulate T cell proliferation. The molecular basis of the greater potency of wild-type SEE, paradoxical in view of its much lower avidity for MHC class II, remains unexplained.

Stimulation of Mouse T Cells with Chimeric Enterotoxins Maps Vβ Specificity to Region 4. It is possible that the staphylococcal enterotoxins contain several sites that contact the TCR, each controlling one type of Vβ-specific T cell response. This model might explain why all toxins stimulate T cells that bear several types of Vβ segments. The alternate hypothesis is that one site on the toxin crossreacts with several different Vβ segments. Mapping more than one set of Vβ-specific reciprocal stimulating activities to region 4 would support the latter model. Therefore, we tested selected chimeric toxins from our panel for their ability to stimulate T cells from B10.BR mice and assessed the phenotype of the responding cells with Vβ-specific mAbs. Many of the initial chimeras demonstrated mixed patterns of Vβ-specific T cell activation as was seen with human T cell responses (data not shown). However, mouse T cell responses to the region 4 exchange mutants as well as SEE-SEA 70-71/SEA-SEE(200–207) clearly demonstrated that Vβ3- or Vβ11-specific T cell responses in B10.BR mice also converge upon region 4 (Table 2).

Discussion

We used the approach of constructing chimeric enterotoxin genes to map important functional activities on these molecules. In construction, we took advantage of the fact that the two enterotoxins most closely related structurally, SEA and SEE, differ with respect to two important immunological parameters. First, SEA stimulates human T cells that bear Vβ5 and not Vβ8, whereas SEE shows the opposite stimulatory phenotype (23). Second, SEA binds class II with a relatively high affinity, whereas SEE binds class II molecules with an affinity so low as to be almost undetectable (24, 25). Given these dimorphisms and their high degree of structural similarity, we assumed that chimeric molecules should re-

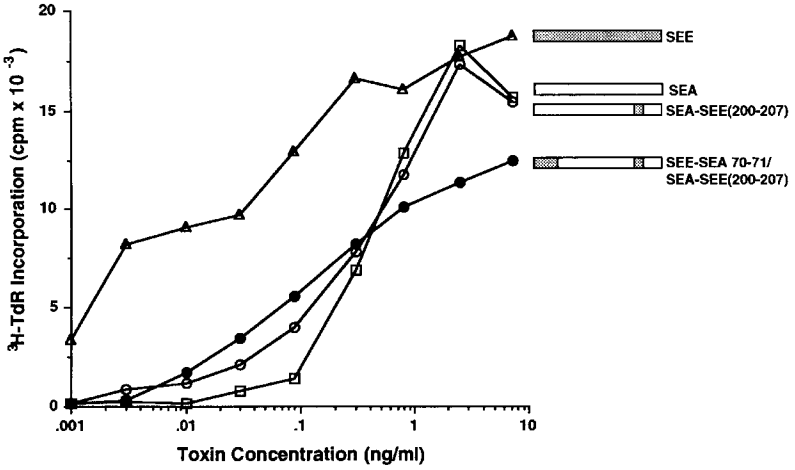


Figure 8. Chimeric enterotoxins are not impaired in their ability to stimulate T cell-proliferative responses. PBMC (8×10^4) were incubated with threefold dilutions of purified SEA-SEE(200–207) and SEE-SEA(70-71)/SEA-SEE(200–207) in round-bottomed 96-well plates. Proliferation was assessed on day 2 with an 18-h [^{3}H]TdR pulse. SEMs of triplicate responses were <10% of the mean for all points shown.

Table 2. *Vβ Analysis of B10.BR T Cell Responses to Chimeric Enterotoxins*

Toxin	Percent positive blasts		
	Vβ3	Vβ11	Vβ9
SEA	30	12	1
SEE	3	56	1
SEA-SEE(161–195)	32	14	1
SEE-SEA(161–195)	3	60	1
SEA-SEE 195–196	22	20	1
SEE-SEA 195–196	44	6	1
SEA-SEE(200–207)	19	20	1
SEE-SEA(200–207)	44	6	1
SEE-SEA 70-71/ SEA-SEE(200–207)	6	65	1

B10.BR splenic T cells were stimulated with chimeric enterotoxins in vitro for 4 d as previously described (20). Concentrations of the toxins used were 1 μg/ml for GF-purified toxins and 0.1 μg/ml for AP-purified toxins. Cells were stained with mAbs specific for the indicated Vβ segment, and the blast cell population was analyzed by flow cytometry. All T cell blasts were >97% positive for CD3. Data were from one experiment.

tain these two distinct phenotypes, allowing them to be mapped genetically. This approach has several advantages. It allows one to study the enterotoxins with all functions intact, obviating the problems inherent in biochemical or genetic fragmentation of the molecule. More importantly, functions can be studied by changing them to new functions instead of knocking them out, providing a higher degree of surety that identified regions of interest are directly involved in the observed phenotypic changes.

Using this approach, we identified a three-amino acid dimorphism between SEA and SEE that is responsible for the differential stimulation of human Vβ5 or Vβ8 and mouse Vβ3 or Vβ11 T cells. These three amino acids are located near the COOH-terminal end of the molecule (last 15% of the linear sequence), and all three dimorphic amino acids play a role in preferential stimulation of one specific TCR Vβ segment versus another. Although these three amino acid differences determine most of the Vβ specificity, we cannot say how many of the surrounding amino acids that are identical between the two toxins are involved in direct contact with the TCR. Thus, this area of the toxin may resemble a type of framework/complementarity-determining region arrangement in which some relatively constant residues in the toxin strengthen the interaction with the TCR, whereas other polymorphic residues may confer specificity. We can say that the upstream boundary of the site must lie distal to amino acid 195 (i.e., the end of region 3) because exchanging region 3 between SEA and SEE had no effect on the stimulatory phenotype of the two toxins.

In addition to mapping a Vβ-determining site to the COOH terminus, we used these chimeric molecules to map the region of the toxin that interacts with class II molecules to the first 70 amino acids of the NH_2 terminus. These data are consistent with work from other laboratories suggesting that class II binding activity lies near the NH_2 terminus. For example, using a synthetic peptide approach, Pontzer et al. (30) reported that peptides derived from the NH_2 terminus block SEA binding to class II molecules. Also, Kappler et al. (18) showed that mutations located in the NH_2 terminus could drastically reduce the class II binding activity of SEB. The class II binding assays (Fig. 7) also support previous reports that SEA and SEE bind class II molecules with different affinities (24). Interestingly, we have mapped a second activity to the first 70 amino acids of SEE that when combined with the Vβ8-specific contact site at the COOH terminus stimulated wild-type levels of Vβ8. When this NH_2-terminal site of one toxin was forced to interact with the Vβ-determining region of the other, as in the chimeras, wild-type levels of appropriate Vβ-specific stimulation were not achieved. These data most likely indicate that both the NH_2- and COOH-terminal ends of SEA and SEE contribute to the site on the toxins that contacts the variable segment of the TCR β chain. Thus, the amino acids 200–207 might be located adjacent to NH_2-terminal amino acids in the three-dimensional structure of the molecule, forming a conformational determinant that is responsible for TCR contact and Vβ specificity. Recent studies of the crystal structure of SEB demonstrates that both NH_2- and COOH-terminal ends of this superantigen are closely apposed and contribute to a shallow pocket predicted to contact the TCR (31). Other amino acids predicted to lie in this binding site are derived from the middle part of the molecule. However, since this middle region of the protein is highly conserved between SEA and SEE, these residues, though located in the TCR binding cleft, are not the ones responsible for Vβ5 or Vβ8 discrimination.

Our data also support the SEB structure-function model advanced by Kappler et al. (18). These authors reported that randomly introduced mutations localized both the class II–binding and TCR contact sites to the NH_2 terminus of SEB. We have also mapped to the first 70 amino acids of SEA and SEE two activities, one that controls class II binding and a second that contributes to Vβ specificity. These data are compatible with the suggestion that the class II–binding and part of the Vβ-contact sites of bacterial superantigens are closely associated and perhaps intertwined in the NH_2 terminus of these molecules. However, preliminary data from our laboratory indicated that this second Vβ contact site in the NH_2 terminus can be completely separated from the site on the molecule that determines class II binding characteristics, suggesting that the class II binding site and both Vβ contact sites are distinct and separable on the primary structure of the molecule (J. Lamphear and R. Rich, unpublished observations).

Bacterial superantigens typically demonstrate specificity for two or more TCR Vβ segments. Our data support the conclusion that these toxins contain one Vβ contact site that crossreacts with several different Vβs. This contrasts to a

second possibility in which there may be two or more TCR contact sites on the enterotoxins, each interacting with one Vβ. Two human Vβ-stimulating activities specific for Vβ5 and Vβ8 mapped to the same region of SEA/SEE, as did stimulation of mouse Vβ3 and Vβ11. Human Vβ8 and mouse Vβ11 are ancestral homologues, whereas the human Vβ5 family and mouse Vβ3 are not related evolutionarily (32). Thus, our data have mapped three distinct Vβ stimulatory activities to region 4 (amino acids 200–207), which suggests, but does not prove, that all Vβ-specific activation is controlled by this COOH-terminal region or a combination of the NH_2- and COOH-terminal regions. Analysis of the Vβ usage by chimeric toxin-activated T cell blasts using quantitative PCR should indicate whether stimulation of all Vβs are controlled by the COOH terminus or only a subset.

Lastly, our model is somewhat similar to that now being proposed for MMTV-encoded endogenous superantigens. The localization of polymorphisms between the different viral superantigens suggests that the COOH terminus of these superantigens controls their Vβ specificity as well (33). A broadly similar design between these two families suggests either a very old relationship and a case of divergent evolution or a striking case of convergent evolution. Our demonstration of the utility of creating chimeric superantigens to map functionally significant regions of SEA and SEE suggests that this approach may be more broadly applicable to other superantigens.

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Note added in proof: Since submission of this manuscript, Irwin et al. (34) have published similar conclusions concerning the importance of residues 200, 206, and 207. In their numbering system, these amino acid positions are denoted 214, 220, and 221, respectively.

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