Mutations Defining Functional Regions of the Superantigen Staphylococcal Enterotoxin B

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Summary

Staphylococcal enterotoxin B (SEB) is both a superantigen and toxin. As a superantigen, SEB can bind to major histocompatibility complex (MHC) class II molecules to form a ligand for α/β T cell receptors bearing particular Vβ elements. As a toxin, SEB causes rapid weight loss in mice sometimes leading to death. We show here that both of these functions map to the NH_2-terminal portion of the toxin. Three regions were identified: one important in MHC class II binding, one in T cell recognition, and one in both functions. These results support the conclusion that the toxicity of SEB is related to massive T cell stimulation and release of cytokine mediators and show that the residues interacting with MHC and the T cell receptor are intertwined.

Staphylococcus aureus produces a set of exotoxins harmful to humans and other species (1–4). These include a group of enterotoxins (Staphylococcal enterotoxins [SEs][1]), associated with food poisoning; toxic shock syndrome toxin 1 (TSST-1), associated with toxic shock syndrome; and exfoliating toxins (ExF), associated with scalded skin syndrome. Many of these toxins were purified to homogeneity some years ago and most have been cloned and sequenced (5–12). Nevertheless, there still is no consensus on their mechanisms of action in human diseases.

These toxins were shown some time ago to be powerful T cell stimulators (13–15), a capacity that we and others have recently attributed to their properties as microbial superantigens (16–24). As superantigens, each toxin has the ability to bind to class II molecules of the MHC and create a ligand for virtually all T cell α/β antigen receptors containing particular variable elements on the β chain (Vβ). Amino acid differences in the other receptor variable components (Dβ, Jβ, Vα, Jα) do not play a major role in this interaction. Since there are a limited number of Vβs (at least 57 in humans [25] and 22 in mice), the frequency of T cells reactive to each toxin is very high. A recent study (26) has indicated that T cell stimulation occurs by interaction of the MHC-bound toxin with specific residues in the Vβ element lying on the side of the receptor rather than the variable face thought to contact conventional antigen bound to MHC. We have suggested that the pathology of these toxins involves the massive release of bioactive cytokines accompanying this high frequency T cell response in vivo (22, 27).

Although binding of the toxin to class II MHC is a prerequisite for T cell recognition (16, 28–30), the process is much more permissive than that seen with the binding of conventional peptide antigens. Whereas peptide antigens are very dependent on allelic MHC residues for binding, toxin superantigens bind to a wide variety of allelic and isotypic forms of class II MHC in mouse and humans (31–34). Furthermore, T cells rarely recognize peptide antigens bound to allo-MHC molecules, while individual T cell clones can respond to toxins bound not only to various allelic forms of MHC, but also to different class II isotypes and even to xenogenic class II. These observations have suggested that toxin superantigens bind to MHC at a relatively conserved site outside the allelically hypervariable groove thought to bind conventional peptide antigens.

Our purpose in the present study was to perform a mutational analysis of one of the toxin superantigens, SEB, to define regions of the toxin involved in MHC binding and TCR Vβ interaction, and to relate the functions of these regions to the in vivo toxic effects of SEB.

Materials and Methods

Polymerase Chain Reaction (PCR). PCRs (35) were performed using AmpliTaq recombinant Taq polymerase and the DNA Thermal Cycler from Perkin Elmer Cetus (Norwalk, CT). 20–30 cycles were performed with 1-min denaturing and annealing steps, and an ex-

[1] *Abbreviations used in this paper:* SE, Staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin 1.

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tension step of 1 min for syntheses <500 bp and 2 min for those >500 bp. Template concentrations were 1–10 nM and oligonucleotide primer concentrations were 1 μM. The concentration of the dNTPs was 200 μM, except when attempting to introduce mutations, where the concentration of one of the dNTPs was reduced to 20 μM.

SEB Construct. The SEB gene was overexpressed in *Escherichia coli* as follows. A linearized plasmid containing the genomic SEB gene (36) was used as a template in a PCR utilizing oligonucleotide primers that flanked the portion of the gene encoding the mature SEB without the signal peptide. The 5′ primer was 5′-TAGGGAATTCCATGGAGAGTCAACCAGA-3′, which contained an EcoRI site placing the SEB gene in-frame with the LacZ gene when cloned into pTZ18R (Pharmacia Fine Chemicals, Piscataway, NJ). This oligo also had an NcoI site that added an ATG between the lacZ gene fragment and the beginning of the SEB gene so that the SEB gene could be moved easily to other plasmids carrying its own initiation ATG. The 3′ primer, which contained a HindIII site after the termination codon of the SEB gene, was 5′-AGCTAAGCTTCACTTTTTCTTTGTCG-3′. The PCR fragment was digested with EcoRI and HindIII and ligated into EcoRI/HindIII–digested pTZ18R. *E. coli* XL1-Blue (Stratagene, La Jolla, CA) was transformed with the plasmid, a single transformant picked, and the insert in its plasmid (pSEB2) sequenced to check that it had no mutations. Upon induction this construct led to overproduction of mostly cytoplasmic SEB (~10 μg/ml of broth). However, rather than producing a LacZ/SEB fusion protein, the bacteria produced a protein with the same apparent molecular weight as secreted SEB from *S. aureus* (Fig. 1 *a*). Either the LacZ portion of the fusion protein was cleaved in vivo from the majority of the SEB or the ATG introduced between LacZ and SEB was for some reason a more efficient translation initiation site than that of LacZ. In the course of these studies, this construct was modified to introduce silent base changes which produced useful restriction sites in the 5′-end of the gene.

Generation of SEB Mutants. SEB mutants were prepared in two ways. First, to introduce random mutations along the entire length of the SEB gene, the SEB construct was prepared again, but the PCR was performed with the concentration of either dATP or dTTP reduced 10-fold in order to increase the error rate of Taq polymerase (37). This reduced the amount of product by ~5-10 fold. The products of the two reactions were combined and cloned into pTZ18R as above and individual transformants screened for mutant SEB as described below (BR mutants). Of ~400 toxin-producing transformants screened, 10 were identified as functional mutants by their reduced ability to stimulate T cells. Low concentrations of dCTP and dGTP were tried as well, but less reduction in product resulted and no mutants were detected in screening ~200 transformants. A second PCR method was used to introduce random mutations in ~60–75 base-defined regions of the SEB gene. The following oligonucleotides (A, B, and C), positioned as shown in Fig. 2, were synthesized with each position containing 1% each of the three incorrect bases: A-, 5′ATTCCCTAACTTAGTGTCCTTAATAGAATATATTAAGTCAAAGTATAGAAATTGATCTATAGA3′; B-, 5′AGCTAGATCTTTGTTTTTAAATTCGACTCGAACATTATCATAATTCCCGAGCTTA3′; C+, 5′CCGGATCCTAAACCAGATGAGCTCCACAAATCTTCCAATTCACAGGCCTGATGGAAAATATGAAAGTTTGTAT3′. These mutant oligonucleotides were used as primers in a PCR reaction with either a vector (A and B) or internal SEB (C) oligonucleotide as the other primer and SEB as a template. Therefore, each molecule of the synthesized SEB fragments was predicted to have two to three random base mutations in the region corresponding to the mutant primer. The mutant fragment was incorporated into the SEB gene, either by using it plus another fragment containing the 3′ portion of the gene as mixed template in a PCR reaction to resynthesize a full-length SEB2 gene as previously described (38, 39), or by digestion with the appropriate restriction enzymes and ligation into pSEB2 from which the corresponding region had been removed.

DNA Sequencing. Plasmid inserts were sequenced directly by the dideoxynucleotide method (40) using Sequenase (U.S. Biochemical Corp., Cleveland, OH) with a modification for double-stranded supercoiled plasmid templates (41). Several oligonucleotide primers were used matching sequences either in the vector or SEB insert.

Anti-SEB mAbs. 10 mAbs specific for at least five epitopes of SEB were produced by standard methods from B10.Q(βBR) immunized multiple times with SEB. The properties of these antibodies will be described in detail elsewhere (Kappler, J. W., and P. Marrack, manuscript in preparation). One of these antibodies, B344.1, was used both for quantitation and immunoaffinity purification of SEB and its mutants in these experiments. B344.1 is an IgG1 that was chosen because initial characterization showed that it had a high affinity for SEB, bound equally well to all of the SEB functional mutants, could detect and immunoprecipitate SEB bound to MHC class II molecules, and did not block T cell recognition of SEB bound to DR (data not shown).

ELISA for SEB. The amount of SEB in preparations was determined in an ELISA. Microtiter wells were coated overnight with a solution of 6 μg/ml natural SEB (Sigma Chemical Co., St. Louis, MO). The wells were then incubated with 25% FCS and washed thoroughly. Various concentrations of known and unknown SEB preparations were added to the wells as inhibitor followed by a constant amount of anti-SEB antibody (polyclonal rabbit anti-SEB [Toxin Technology, Madison, WI] in BR experiments and monoclonal anti-SEB, B344, in BA, BB, and BC experiments). After 1 h, the wells were washed thoroughly, and the bound antibody was detected by standard techniques using alkaline phosphatase coupled either to goat anti–rabbit IgG (Sigma Chemical Co.) or to goat anti–mouse IgG1 (Fisher Scientific Co., Pittsburgh, PA) and *p*-nitrophenyl phosphate as substrate. The OD of the reaction at 405 nm was related to the dose of inhibitor and the concentration of the SEB in the unknown estimated by computer analysis of the data.

Preparation of Recombinant SEB. For initial screening, individual colonies of transformants picked from agar plates were transferred to wells of 96-well microtiter plates containing 100 μl of 2XYT and carbenicillin. A replicate plate was prepared except that the media contain 1 mM IPTG as well. Both were incubated overnight at 37°C with shaking. 50 μl of glycerol was added to each well of the first plate, which was mixed and then stored at −70°C. To prepare SEB-containing lysates, each well of the second plate received 50 μl of HNM buffer (10 mM Hepes, pH 7.0, 30 mM NaCl, 5 mM $MgCl_2$) containing 3 mg/ml lysozyme and 300 μg/ml DNAse I. The plate was incubated at 37°C for 15 min, frozen, thawed three times, and centrifuged to pellet debris. The supernatants were transferred to a new plate and tested for the presence of SEB both by ELISA and T cell hybridoma stimulation. This method produced preparations containing between 0.3 and 10 μg/ml of SEB.

To produce purified mutant SEB, transformants were recovered from the 96-well plate stored at −70°C. Bacteria from overnight cultures (1 vol) containing IPTG were collected by centrifugation, resuspended in a 1:10 vol of HNM buffer containing 1–2 mg/ml lysozyme and 10 μg/ml DNAse I, and frozen and thawed three times. The suspension was centrifuged at 15,000 *g* for 20 min to remove bacterial debris, and the supernatant was harvested and filtered (0.2 μ). The filtrate was passed through a column containing

a 1:50 volume of Sepharose 4B beads coupled with 2–3 mg/ml of a mAb to SEB (B344). The beads were washed thoroughly with PBS and the toxin was eluted with 0.1 M glycine·HCL (pH 2.7) and neutralized with 1 M Na_2CO_3. The SEB was concentrated to 1 mg/ml and its buffer changed to BSS using Centricon10 microconcentrators (Amicon Corp., Denvers, MA). This method yielded 3–10 mg of toxin per liter of bacterial culture. SEB and its mutants produced in this manner were >95% pure as judged by SDS-PAGE, although some proteolytic cleavage was evident (Fig. 1 *a*).

Computer Analysis of Primary Sequence. Computer analysis of the primary sequence of SEB predicting the hydrophilicity (42) and secondary structure (43, 44) of particular regions was performed using MacVector Version 3.5 (International Biotechnology, Inc., New Haven CT).

Results

Initial Production of SEB Mutants. SEB mutants were prepared in two ways, as described in Materials and Methods. Random mutations were introduced by PCR into the entire 319–amino acid toxin (BR series) or into ~20–amino acid defined regions (1–3) using randomly mutagenized oligonucleotide primers (BC, BA, BB series). For primary screening, total lysates were prepared from individual transformants containing a potentially mutant SEB gene. Aliquots of each lysate were tested for the presence of functional toxin by stimulation of murine T cell hybridomas bearing α/β receptors with either Vβ7 or Vβ8.3, using human DR-expressing cell lines as presenting cells. Lysates deficient in stimulating either of these hybridomas were assayed for the presence of SEB protein to rule out mutations affecting the level or the full length of the SEB produced. Plasmids from those producing protein were sequenced to locate the mutation. The sequences of the mutants are shown in Fig. 2.

The Taq polymerase error–induced random mutants (BR) were clustered in three regions (1, 2, 4), all in the NH_2-terminal 93 amino acids of the molecule (except an additional conservative mutation in one case, BR-374, in the COOH-terminal half of the molecule). As predicted by their method of generation, all but one of these mutations involved a nucleotide substitution of A to G or T to C, and only one silent mutation was found elsewhere in their sequences (data not shown). Additional mutants were generated in region 1 or 2 with mutant oligonucleotide C or A (BC, BA mutants). Region 3 was originally discovered as a single mutant (BA-62) involving the last amino acid covered by oligonucleotide A. The mutant had a different phenotype than the other BA mutants (see below). Additional mutants were produced in this region with mutant oligonucleotide B (BB mutants). Mutations in region 4 were eliminated from further analysis, because we felt that interfering with the conserved disulfide forming cysteine at position 93 could have far reaching unpredictable effects. In addition, several mutants were not further characterized either because they involved more than one region (BR-474, BA-72), produced highly degraded toxin (BR-267), or were identical to an already existing mutant (BA-50).

Binding of Mutant SEB to HLA-DR. Since binding to MHC class II is a prerequisite for toxin recognition by T cells, the mutations could have affected either the ability of

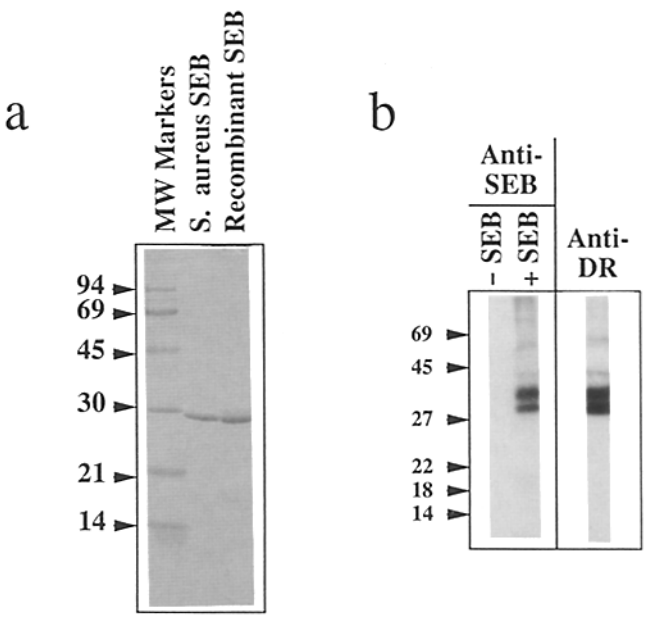

Figure 1. (*a*) Recombinant and natural SEB have the same molecular mass 2 μg of recombinant SEB purified from *E. coli* and SEB purified from *S. aureus* cultures (Sigma Chemical Co.) were analyzed by SDS-PAGE using a 12% gel under reducing conditions. Molecular mass markers (in kD): β-phosphorylase, 94; bovine albumin, 69; ovalbumin, 45; carboxylase, 30; soybean trypsin inhibitor, 21; lysozyme, 14. (*b*) Binding of SEB to DR on LG2 cells. ^{125}I-labeled LG2 cells (53) were incubated with or without 50 μg/ml recombinant SEB for 2 h at 37°C. A cell free lysate was prepared in 1% digitonin and incubated for 4 h at room temperature with Sepharose beads coupled with 3 mg/ml B344 anti-SEB mAb. The beads were washed thoroughly, and the labeled bound material was analyzed by SDS-PAGE under reducing conditions (54) and autoradiography. As a control, beads bearing the anti-DR mAb, L243 (55), were used (1/20 the volume of lysate used with the anti-SEB beads). Molecular mass markers (in kD): bovine albumin, 69; ovalbumin, 45; chymotrypsinogen, 27; soybean trypsin inhibitor, 21; myoglobin, 17; lysozyme 14.

the toxin to bind to DR molecules or the recognition of this complex by the TCR-α/β. To help distinguish these two possibilities, we used the HLA-DR1 homozygous lymphoblastoid line, LG2 (45). SEB binds to DR molecules on LG2 (Fig. 1 *b*). We prepared immunoaffinity-purified toxins and assessed their ability to bind to LG2 using flow cytometry with the same anti-SEB mAb used to purify the SEB and its mutants. The results are shown in Fig. 3.

The binding by four of the region 1 mutants to LG2 was indistinguishable from that of unmutated SEB. The other three mutants were reduced in their binding capacity by ~100-fold. These results suggest that residues between 14 and 23 within region 1 are important in MHC binding. Five of the seven mutations involved residue 23N. In only one case (BR-291, 23N→S) did this mutation reduce MHC binding. These results suggest residue 23N may be important in both in MHC binding and Vβ interaction. Region 2 mutants all bound poorly to LG2, ~1,000 times poorer than SEB, indicating that region 2 defines a stretch of amino acids, especially 44F, important in binding of the toxin to class II MHC. Region 3 mutants were essentially unaffected

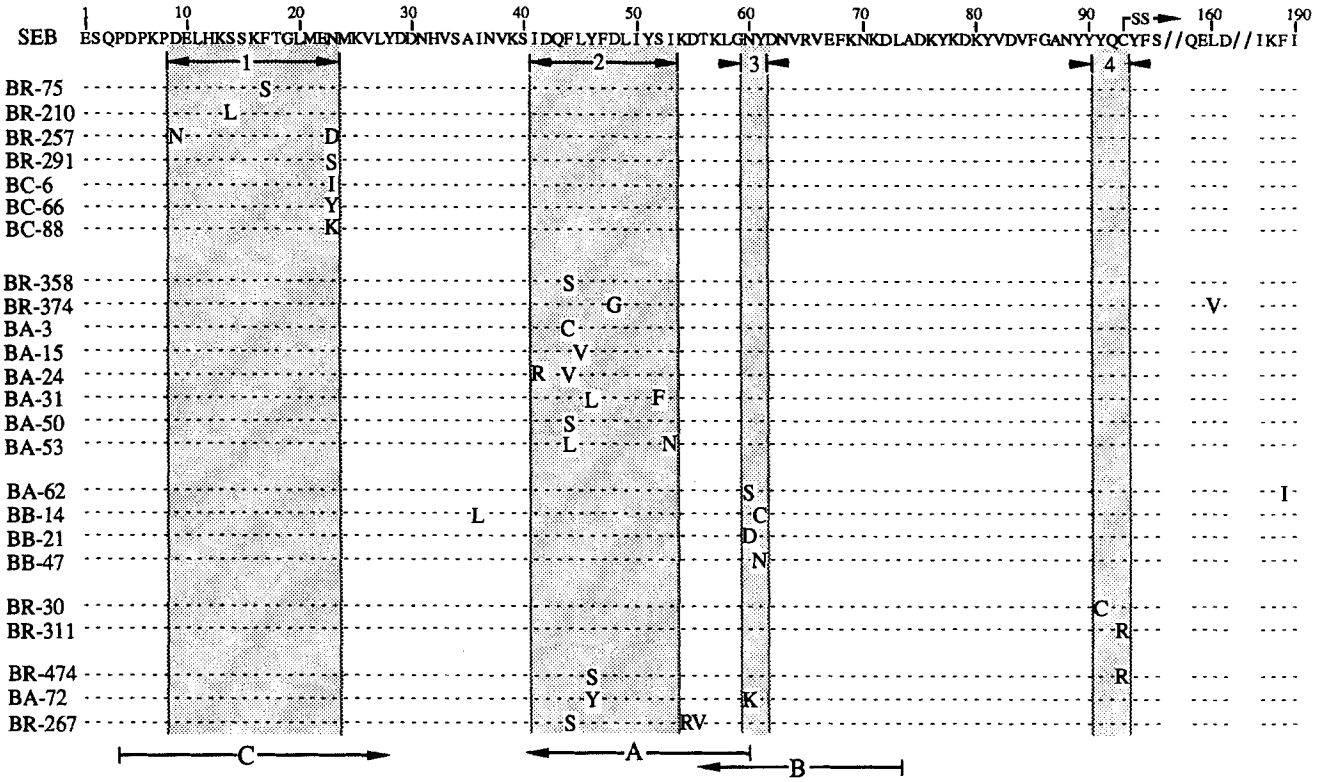

Figure 2. Sequences of the SEB mutants used in this study. Partial amino acid sequence of SEB (8) is shown. The sequence of the SEB mutants generated in this study are shown with a dash, indicating identity to unmutated SEB. Positions of oligo nucleotides used to generate mutants are shown as well. See Materials and Methods for details.

in binding to LG2, strongly suggesting that this two-amino acid region (60N, 61Y) is important in Vβ interaction.

Effect of Mutations on T Cells Bearing Different Vβ Elements. The SEB mutants were originally identified because they stimulated either a Vβ7$^+$ or a Vβ8.3$^+$ T cell hybridoma poorly. To assess the effect of the SEB mutations on T cell recognition in more detail, we retested the purified mutant toxins at various doses on additional T cell hybrid-

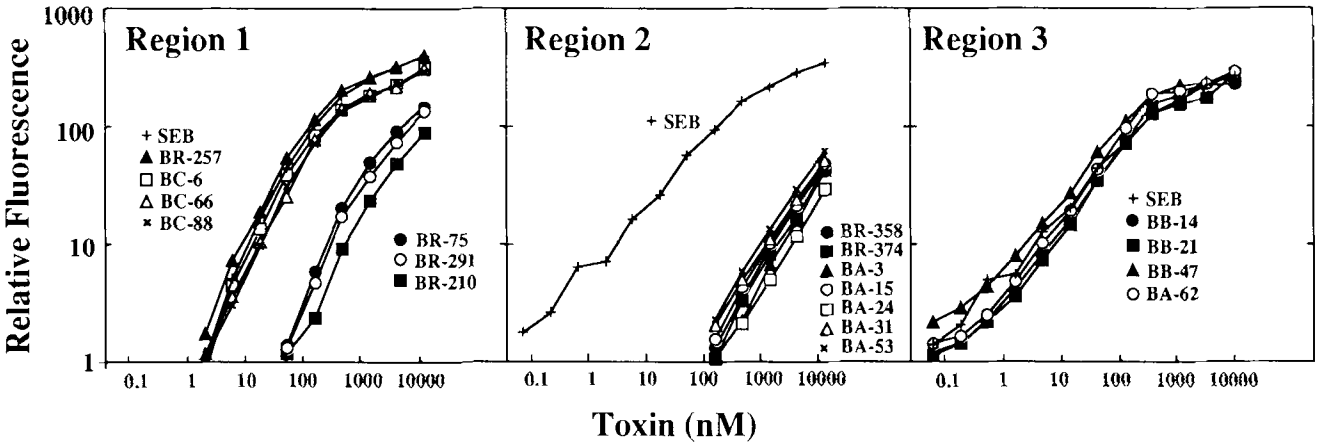

Figure 3. Binding of SEB and its mutants to DR1-bearing LG2 cells. 3 × 10^4 LG2 cells were incubated in 100 μl of tissue culture medium overnight at 37°C with various concentrations of SEB or its mutants. The cells were washed thoroughly and incubated for 30 min at 4°C with ~1 μg/ml of the anti-SEB mAb, B344.1. The cells were washed again and incubated for 15 min at 4°C with fluoresceinated goat anti-mouse IgG1 (Fisher Scientific Co.). The cells were washed again and analyzed for surface fluorescence with a Epics C flowcytometer. The results are plotted as the mean relative fluorescence of the cells corrected for the fluorescence seen with the secondary reagent alone vs. the amount of toxin added. (*Region 1*) (+) SEB, (●) BR-75, (■) BR-210, (▲) BR-257, (○) BR-291, (□) BC-6, (△) BC-66, (×) BC-88. (*Region 2*) (+) SEB, (●) BR-358, (■) BR-374, (▲) BA-3, (○) BA-15, (□) BA-24, (△) BA-31, (×) BA-53. Region 3: (+) SEB, (●) BB-14, (■) BB-21, (▲) BB-47, (○) BA-62.

omas bearing each of the four murine Vβ elements known to recognize SEB (Vβ7, Vβ8.1-3) (16, 20, 24). The results are shown in Figs. 4–6.

Among the region I mutants (Fig. 4), the five involving 23N (BR-257, BR-291, BC-6, BC-66, BC-88) stimulated all of the hybridomas poorly, despite the fact that four of these bound to DR as well as unmutated SEB did. These results indicate that residue 23N is an important amino acid for Vβ interaction, but because the fifth mutant involving this amino acid, BR-291, bound poorly to MHC, this amino acid may influence MHC binding as well. The other two region 1 mutants also stimulated poorly. In the case of BR-75, this may have been due primarily to its poor binding to DR, but the effect of the BR-210 mutation was several orders of magnitude greater on T cell stimulation than on binding to DR. Taken together, these results suggest again that during T cell recognition of SEB bound to DR, the amino acids in region 1 are situated in the trimolecular complex at the junction between Vβ and MHC, so that individual residues may interact with either component.

The mutations in the other regions produced less complicated phenotypes. All of the region 2 mutants were defective in stimulation of all of the T cell hybridomas, regardless of the Vβ element in their receptors (Fig. 5). There were small differences, but in general the effect of mutations on stimulation was about the same as that seen on DR binding. These results were consistent with the conclusion that mutations in region 2 primarily affect DR binding.

The two-amino acid region 3 mutants were the most discriminating (Fig. 6). Despite the fact that random mutants in a 20-amino acid stretch flanking this region were generated, all mutations affecting function were found in these two amino acids. These mutants failed to stimulate the hybridomas bearing Vβ7 and Vβ8.1, but stimulated the Vβ8.2 hybridoma very well and the Vβ8.3 hybridoma about as well as unmutated SEB. Since all these mutant toxins bound DR as strongly as unmutated SEB, this region appears to be essential for toxin interaction with Vβ7 and Vβ8.1, but not Vβ8.2 or Vβ8.3. To insure that this property was not peculiar to these particular hybridomas, we tested the toxins with four other T cell hybridomas: one $V\beta7^+$, two $V\beta8.1^+$, and one $V\beta8.3^+$. The results were indistinguishable from those in Fig. 6 (data not shown).

Requirement for T Cell Interaction for In Vivo Effects of SEB The question of how important the superantigen properties of the bacterial toxins are to their in vivo toxic effects is unresolved. Our previous experiments suggested that the toxicity of SEB in mice was related to its ability to stimulate T cells in a Vβ-specific manner, since the toxic effect of SEB was directly related to the frequency of T cells bearing the relevant Vβ elements (27). However, the ability of some of the *S. aureus* toxins to bind to class II on monocytes and stimulate the production of cytokines such as TNF and IL-1 (46) opens the possibility that direct monocyte stimulation may be sufficient to account for much of the toxin pathology in some situations.

To test this idea, we injected mice with various concentrations of region I mutant BR-257, which binds very well to class II but does not stimulate T cells except at extremely high levels. For controls we used unmutated SEB and mu-

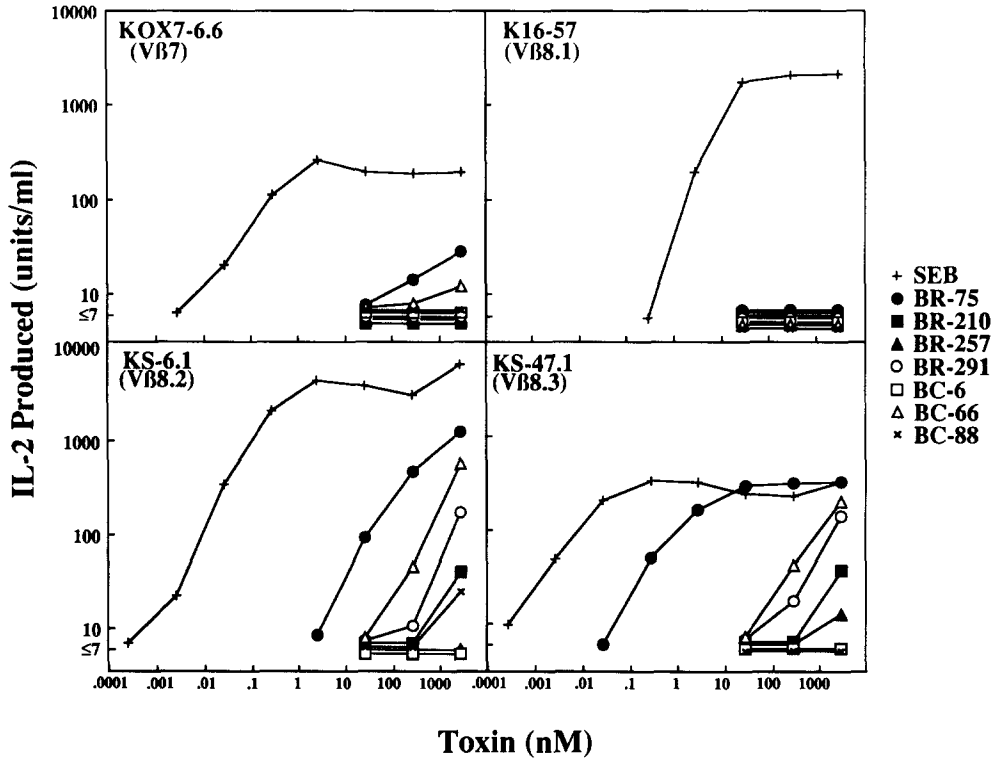

Figure 4. Stimulation of T cells hybridomas by region 1 SEB mutants. Preparations of purified SEB or the region 1 mutants were tested for their ability to stimulate a collection of T cell hybridomas bearing each of the Vβ elements known to recognize SEB: KS-20.15(Vβ7), KS-6.1(Vβ8.2), KS-47.1(Vβ8.3) (16), and K16-57(Vβ8.1) (56). Various concentrations of the toxins were incubated at 37°C overnight with 3 × 10^4 $DR1^+$ LG2 cells in 200 μl of tissue culture medium. 5 × 10^4 T cell hybridomas cells were added in 50 μl, and the mixture was incubated overnight again. The response of the T cell hybridomas was measured as the amount of IL-2 secreted as previously described (57, 58). Symbols are as in Fig. 3.

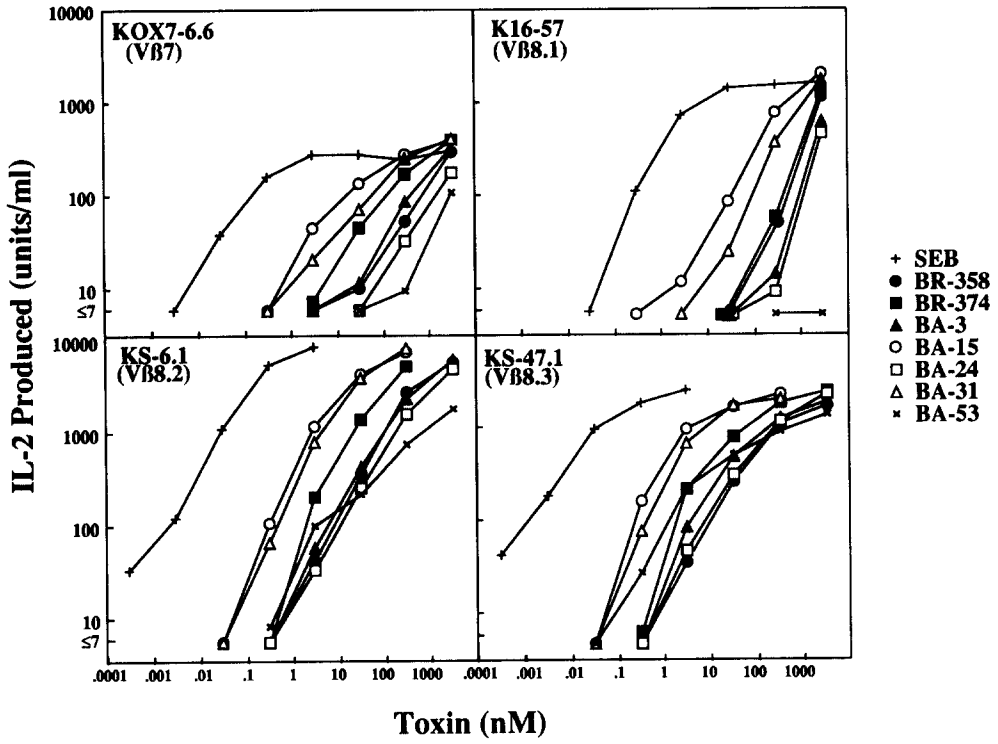

Figure 5. Stimulation of T cells hybridomas by region 2 SEB mutants. Preparations of SEB and region 2 mutants were tested for their ability to stimulate murine T cell hybridomas as in Fig. 5. Symbols are as in Fig. 3.

tant BR-358, which like all of the region 2 mutants, bind very poorly to class II. To minimize the effects of LPS, which might contaminate the preparations, we used C3H/HeJ mice, a strain defective in LPS responsiveness. Since rapid weight loss is one of the most obvious immediate toxic effects of SEB in mice (27), we weighed the mice daily after the injection. The results are shown in Fig. 7. Mice given either 50 or 100 μg of recombinant SEB lost weight rapidly over 3–4 d, and all of the mice were dead by day five. Mice given mutant BR-358 showed no effects and were indistinguishable from

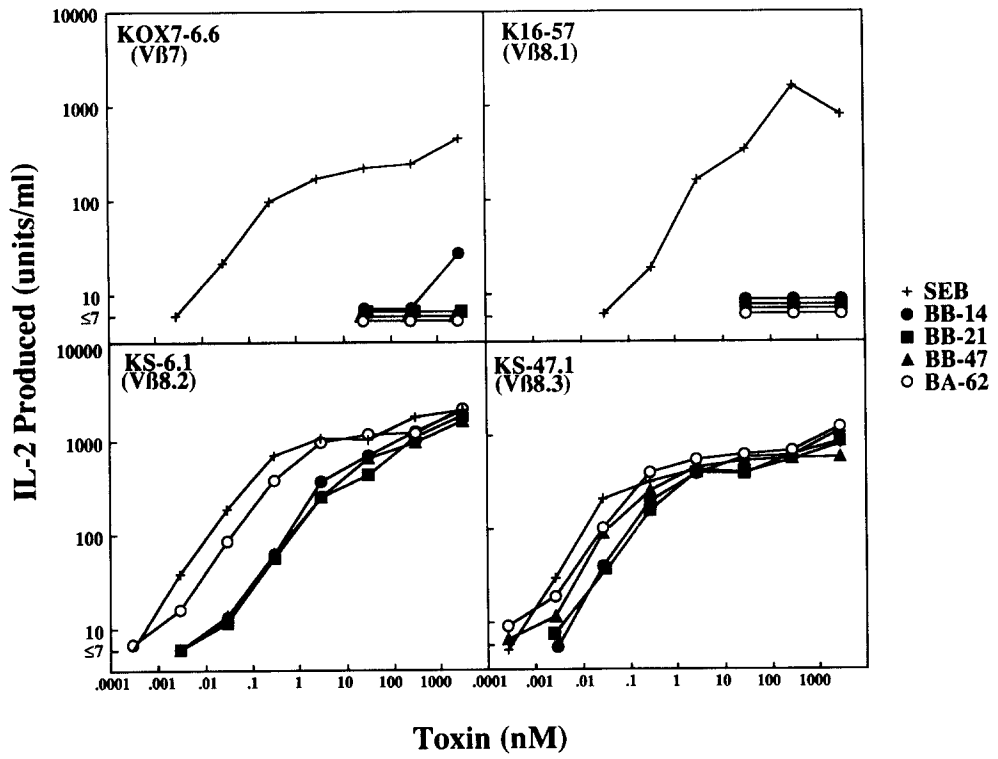

Figure 6. Stimulation of T cells hybridomas by region 3 SEB mutants. Preparations of SEB and region 3 mutants were tested for their ability to stimulate murine T cell hybridomas as in Fig. 5. Symbols are as in Fig. 3.

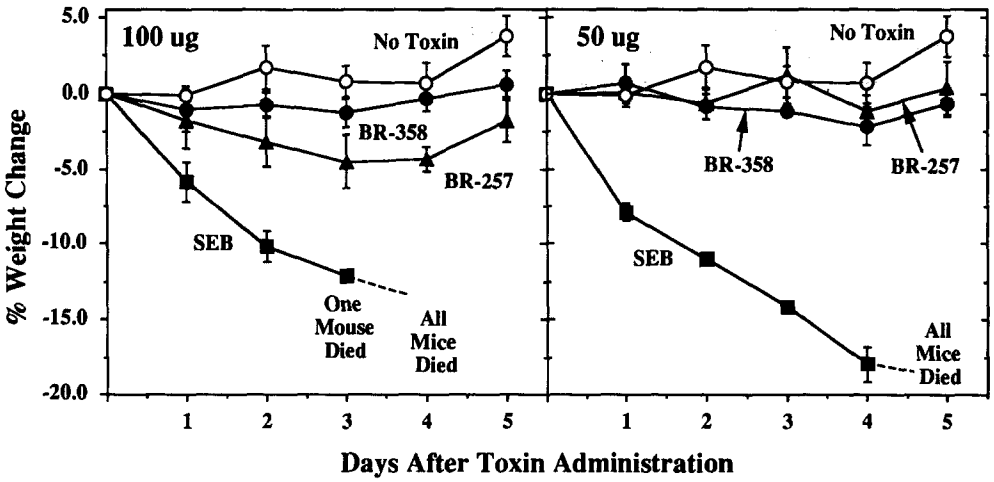

Figure 7. Effects of SEB and its mutants in vivo. Groups of three mice were weighed and then given balanced salt solution (BSS) containing nothing (O) or 50 μg (*left*) or 100 μg (*right*) of recombinant SEB (■) or the mutant SEBs BR-257 (▲) or BR-358 (●). The mice were weighed daily at the same time of day until they died. Results are presented as the average percent change from the starting weight for the surviving mice. One mouse in the 100 μg of SEB group died by day 3 and the other two by day 4. All mice in the 50 μg SEB group died by day 5. None of the mice given BSS alone or the mutant toxins died.

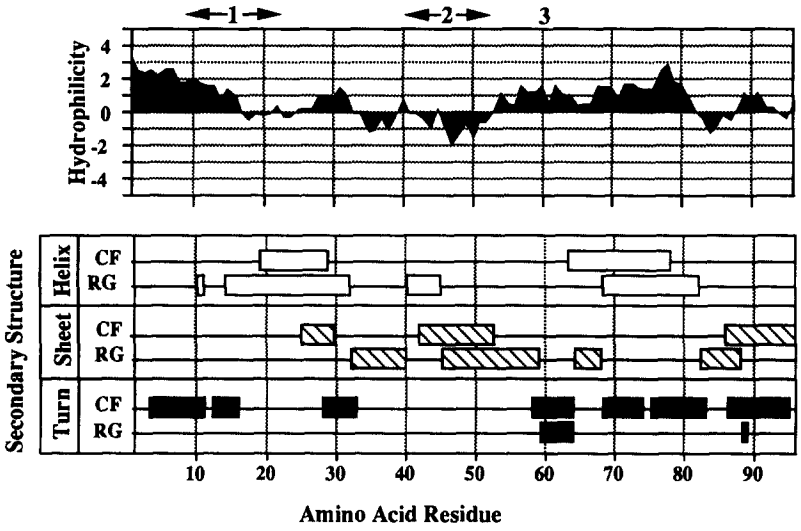

Figure 8. Structural predictions from the primary amino acid sequence of SEB. Predictions of hydrophilicity and secondary structure are shown for the NH_2-terminal 97 amino acids of SEB using MacVector 3.5. The algorithms used for secondary prediction were Chou-Fasman (*CF*) and Robson and Garnier (*RG*). The three mutant containing regions are shown at the top of the figure.

those given BSS alone. Mice given 50 μg of BR-257 were unaffected as well; however, those given 100 μg of BR-257 showed a slight weight loss followed by recovery.

These results confirm that in mice the majority of the toxicity of SEB is dependent on its ability to stimulate T cells, suggesting that T cell–derived lymphokines themselves or those produced by other cells activated by T cells are very important in the mode of action of this toxin. However, the small effect of BR-257 at the higher dose raises the possibility of a contribution from class II–bearing cells directly stimulated by bound SEB without T cell involvement.

Discussion

The results presented here identify the NH_2-terminal portion of SEB as important in both MHC binding and Vβ interaction. Three regions were defined with particular amino acids in each pinpointed. Mutations in the first region (residues 9–23) affected both MHC binding and T cell interaction. The results suggested that 23N was particularly important. When the sequences of the *S. aureus* enterotoxins are aligned for maximum homology (22), this residue is conserved among all of the enterotoxins and toxic shock toxin as well. The mutations in region 2 covered residues 41–53. All drastically reduced the ability of the toxin to bind to MHC class II with a similar effect on their ability to stimulate T cells. About half of the mutations involved 44F. Again, this residue is conserved among all the enterotoxins, indicating that this residue probably plays a critical role in the binding of all of the toxins to MHC. Interestingly, none of the mutations in either region 1 or 2 completely obliterated toxin binding to MHC, and in both cases the T cell–stimulating ability of the mutants could be recovered by adding a large excess of toxin. Perhaps regions 1 and 2 cooperate in the binding of the toxin such that both regions must be mutated to eliminate toxin binding completely. Mutations in the third region (60N, 61Y) did not affect binding of the toxins to MHC, but did affect their interaction with two Vβs, 7 and 8.1. This Vβ-specific effect suggests that these amino acids are important for interaction with some, but perhaps not other, TCR Vβs.

There is at present no solved structure for any of the en-

terotoxins; however, several observations are worth noting from an analysis of the primary sequence of SEB in regions 1–3 (Fig. 8). Region 1 is a hydrophilic to neutral stretch with 23N predicted to lie within an α helix. Region 2 is quite hydrophobic and predicted to be within a β strand. Therefore, the side chains of the amino acids (e.g., 44F) may not all point into the aqueous phase. The two amino acids of region 3 are predicted to lie at the beginning of an extended hydrophilic region on the turn at the end of the β stand containing region 2, and are, therefore, likely to be on the surface of the molecule.

These mutations may affect toxin structure in any of several ways. They may identify amino acids whose side chains make critical contact with either MHC or Vβ. They may cause local perturbations in the toxin secondary or tertiary structure, which in turn displace other amino acids critical in contacting MHC or Vβ. Finally, they may cause gross alterations in the toxin structure amounting effectively to the denaturation of the protein. This third possibility seems unlikely, given the independent effect of many of the mutations on MHC vs. Vβ interaction or on interaction with different Vβ elements. Furthermore, these mutant SEBs continued to bind to most of a panel of 10 anti-SEB mAbs detecting at least four independent epitopes (data not shown). However, discriminating between changes in contact amino acids vs. those maintaining localized structural elements will require a solution to the toxin structure.

Two other studies have suggested that the NH_2-terminal portion of enterotoxins may be important in T cell stimulation. Pontzer et al. (47) have blocked SEA MHC binding and T cell stimulation with synthetic peptides matching SEA NH_2-terminal sequences, and Spero and Molock (48) demonstrated weak T cell–stimulating activity with an NH_2-terminal, but not COOH-terminal tryptic fragment of SEC1. On the other hand, Bohach et al. (49) found that the same C-terminal fragment of SEC1 contained the T cell stimulating activity of the toxin and Blanco et al. (50) found amino acids critical to the functions of toxic shock syndrome toxin in the COOH-terminal half of the molecule. At the moment, we cannot completely reconcile these latter results with our own data. Our results certainly argue strongly that the COOH-terminal part of SEB cannot be sufficient for its superantigen activity. However, our results do not exclude a role for amino acids in the COOH-terminal half of the protein, although they indicate that random point mutations do not easily reveal these amino acids.

Recently, the 3′ LTRs of mouse mammary tumor viruses have been found to encode a superantigens (vSAG) (51, 52). These proteins are of similar molecular weight to the bacterial toxins. Comparison of the sequences of vSAGs with different Vβ specificities has strongly suggested that the COOH terminus of these proteins determine Vβ specificity. However, this observation is probably not relevant to mapping the Vβ interaction site on the bacterial toxins, since there is no sequence homology between the two types of superantigens. Furthermore, these proteins may interact with MHC in fundamentally different ways, since the toxins bind as soluble proteins, while the evidence indicates that vSAGs are type II integral membrane proteins with their NH_2 terminus cytoplasmic and their COOH terminus extracellular (Choi et al., manuscript in preparation).

Systemic exposure to any of the *S. aureus* enterotoxins or other exotoxins can be associated with fatal shock in humans, mice, and other species. Each of these toxins has ability to bind to class II MHC, but each interacts with a different set of Vβ elements. This observation a priori argues strongly that the ability to stimulate T cells is an important and conserved feature of the mode of action of these toxins, and that it is necessary that stimulation involves a large set of T cells regardless of the specificities of the T cells involved. We have suggested that the massive release of cytokines accompanying this T cell stimulation is largely responsible for the toxin pathology (27), but the ability of toxins to bind to class II molecules on macrophages may be sufficient to induce the symptoms of these toxins through the direct stimulation of macrophage cytokine production without the involvement of T cells.

The results presented here are consistent with the former view in that mutations affecting either Vβ or MHC binding eliminated the fatal toxic effects of SEB injected intraperitoneally in mice. SEB and the other enterotoxins, however, are most often encountered by ingestion, which in humans usually results in a less serious disease involving a short episode of vomiting and diarrhea. At present we do not know that these symptoms are also dependent on the T cell–stimulating properties of SEB, and it is possible that this is a separate function of the toxin mapping elsewhere in its structure. If this were the case, our findings would focus attention of the COOH-terminal half of the molecule. Unfortunately, we can not test this idea in mice since there is at present no rodent model for this disease.

Lastly, our results indicate that the stretches of the staphylococcal toxins that bind MHC and Vβ are intertwined in the primary sequence of the toxins rather than separated into two distinct domains. This close association of the Vβ and MHC binding sites of SEB suggests that for successful recognition the TCR may be forced into intimate contact with both the toxin and MHC component of the complex. Perhaps this explains why occasional individual TCRs bearing the appropriate Vβ element nevertheless fail to recognize toxin bound to a particular allelic or xenogeneic form of class II MHC (16), since in these cases interaction between residues in Vβ or the other receptor variable elements and polymorphic residues of the MHC molecule may interfere with Vβ/toxin interactions.

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