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Localization of Binding Sites of Staphylococcal Enterotoxin B (SEB), a Superantigen, for HLA-DR by Inhibition with Synthetic Peptides of SEB

JACK L. KOMISAR,* SHEILA SMALL-HARRIS, AND JEENAN TSENG

Department of Experimental Pathology, Division of Pathology, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100

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Staphylococcal enterotoxins are major causes of food poisoning and toxic shock syndrome. Their ability to bind to major histocompatibility complex (MHC) class II molecules has been suggested to be the first step in the mechanism whereby they cause illness. By flow cytometric analysis, the sites of interaction of staphylococcal enterotoxin B (SEB) with HLA-DR molecules were probed in the present study by inhibiting the binding of biotinylated SEB to a human T-cell line (HUT-78) with synthetic peptides of SEB. Five peptides of SEB gave significant inhibition of binding: a peptide containing amino acids 9 to 20 [SEB(9–20)], SEB(30–38), SEB(61–70), SEB(90–114), and SEB(169–181). One peptide, SEB(39–51), enhanced binding. Among the inhibitory peptides, SEB(90–114), a peptide spanning the entire disulfide loop, showed the most efficient inhibition of binding. Peptides SEB(9–20) and SEB(39–51) include amino acid residues that have been identified by previous mutation studies (J. W. Kappler, A. Herman, J. Clements, and P. Marrack, J. Exp. Med. 175:387–396, 1992) as being important in binding to MHC class II. Amino acids lining the α5 groove of SEB have also been postulated to be involved in binding to MHC class II molecules. However, only two of the residues that line the α5 groove of SEB, His-12 and Tyr-17, are on peptide SEB(9–20) that inhibits binding. These results confirm previous studies that implicated the amino-terminal portion of the molecule in binding to MHC class II molecules and further indicate an important role for residues in other regions, particularly the disulfide loop.

Staphylococcal enterotoxins (SEs) are a major cause of food poisoning and can also cause toxic shock (32). They are all protein molecules with molecular masses of about 28 to 30 kDa and a single disulfide loop and contain many small regions of homology scattered throughout the molecules (32). Overall, the toxins fall into two groups. One group, consisting of staphylococcal enterotoxin B (SEB), SEC_1, SEC_2, and SEC_3, has 66 to 98% amino acid sequence identity, while the other group, consisting of SEA, SED, and SEE, has 53 to 81% identity (3). There is 29 to 36% identity between the two groups (3). Despite high degrees of homology, the question as to whether the homologous or nonhomologous regions are the active sites that are responsible for inducing disease has not been definitely answered.

Recently, SEs have been shown to be superantigens (9). They bind to major histocompatibility complex (MHC) class II antigens (13, 15, 30, 34). Binding of staphylococcal toxins to MHC class II molecules delivers various signals to the cells, such as comitogenic and activation signals, and stimulates cytokine gene expression, phospholipase cγ1 activation, and calcium mobilization (10, 14, 19, 26, 35, 47). After binding, the class II-SE complexes are then presented to and stimulate T cells bearing certain T-cell-receptor Vβ elements (32). Binding of enterotoxins to MHC class II antigens is necessary for T-cell activation in several systems (7, 12, 41, 52). It has been suggested that activation of both the antigen-presenting cells and T cells results in production of large amounts of cytokines that then cause illness (18, 31). Thus, the first step in the pathogenesis of SEs is believed to be the binding of SEs to MHC class II antigens. SEs bind to MHC class II antigens outside of the antigen groove to which conventional antigens bind (11). Several studies to determine the sites on the SEs that bind to human MHC class II molecules have been performed (17, 18, 20, 27, 37). Grossman and coworkers (18, 20) found that reduction and alkylation or mutation of the disulfide loop of SEA has only small effects on the binding of SEA to human MHC class II antigens and that reduction and alkylation of the disulfide loop of SEB has little effect on class II-mediated monocyte stimulation. Pontzer and coworkers (37) found that a peptide of SEA containing amino acids 1 to 45 [SEA(1–45)] and SEA(1–27) block the binding of SEA to Raji cells, and Griggs and coworkers (17) found that SEA(39–66), SEA(62–86), and SEA(121–149) do also. Kappler and coworkers (27) studied SEB mutant molecules generated by random mutations and their resulting effectiveness in T-cell mitogenesis. They identified four regions that affect T-cell proliferation: residues 9 to 23, 41 to 53, 60 to 61, and 91 to 93. The region of residues 9 to 23 appears to be involved in interactions with T-cell-receptor Vβ chains and binding to MHC class II molecules. The region of residues 41 to 53 appears to be involved in MHC class II binding, while residues 60 to 61 appear to be involved in Vβ interactions. Thus, the active sites involved in binding to MHC class II antigens seem to be located in the N-terminal portion of SEB. However, further studies using different methods are required to confirm these findings because each of the techniques used to map structure-function relationships have their own limitations.

A straightforward way of studying the binding sites of SEs to MHC class II antigens is to use living MHC class II-bearing cells and small synthetic peptides in competitive inhibition studies. This approach offers the advantage that the conforma-

* Corresponding author. Mailing address: Walter Reed Army Institute of Research, 14th and Dahlia Sts., N.W., Washington, DC 20307-5100. Phone: (202) 576-3053. Fax: (202) 576-0947.

TABLE 1. Peptides of SEB used in this study

Peptide	Residues	Sequence
1	1–9	ESQPDPKPD
2	9–20	DELHKSSKFTGL
3	21–30	MENMKVLYDD
4	30–38	DNHVSAINV
5	39–51	KSIDQFLYFDLIY
6	50–60	IYSIKDTKLGN
7	61–70	YDNVRVEFKN
8	71–80	KDLADKYKDK
9	81–92	YVDVFGANYYYQ
10	90–114	YYQCYFSKKTNDINSHQTDKRKTCM
11	114–123	MYGGVTEHNG
12	123–134	GNQLDKYRSITV
13	135–142	RVFEDGKN
14	142–150	NLLSFDVQT
15	150–162	TNKKKVTAQELDY
16	161–169	DYLTRHYLV
17	169–181	VKNKKLYEFNNSP
18	181–194	PYETGYIKFIENEN
19	194–207	NSFWYDMMPAPGDK
20	207–220	KFDQSKYLMMYNDN
21	220–229	NKMVDSKDVK
22	229–239	KIEVYLTTKKK

tions of the SEs are not altered and the binding of SEs to living cells remains physiological. The present study used synthetic SEB peptides to locate the regions of SEB that are involved in binding to MHC class II antigens on a human T-cell line, HUT-78. This cell line expresses HLA-DR4,4 (16). Flow cytometry was used to analyze the inhibition of binding. Five peptides of SEB gave significant inhibition, namely, SEB(9–20), SEB(30–38), SEB(61–70), SEB(90–114), and SEB(169–181), and one, SEB(39–51), enhanced binding. This result confirmed previous reports that place the binding sites of enterotoxins at the N-terminal portion of the molecule. Furthermore, it also implies that some other regions, particularly the disulfide loop, may also be directly involved in binding of SEs to MHC class II antigens.

MATERIALS AND METHODS

SEB peptides. The peptides of SEB were synthesized by Macromolecular Resources, Fort Collins, Colo., by standard methods (48) using solid-phase tert-butyloxycarbonyl chemistry. The sequences of the SEB peptides made are shown in Table 1. Each peptide is highly pure, showing a single peak in reversed-phase high-performance liquid chromatography with a C18 column, 0.1% trifluoroacetic acid, and a gradient of 0 to 50% acetonitrile. The peptides were dissolved at a concentration of 10 mg/ml in dimethyl sulfoxide and further diluted to microgram-per-milliliter concentrations in phosphate-buffered saline (PBS)–bovine serum albumin (BSA)–azide for assays of inhibition of MHC class II binding. Control tubes contained an equivalent amount of dimethyl sulfoxide without peptide.

SEB. SEB was purified by the method of Schantz and coworkers (42) and was obtained in lyophilized form from the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md. The SEB preparation was the same as that used in previous studies (28, 51). It was relatively pure and showed an intense SEB band (29 kDa) and two very faint bands (10 and 17 kDa), which were the sizes expected for proteolytic fragments of SEB, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (28). The SEB was biotinylated by the method of Hsu and coworkers (23) by using *N*-hydroxysuccinimidobiotin.

Binding of SEB to HLA-DR on HUT-78 cells. The HUT-78 human leukemic T-cell line (ATCC TIB 161) was obtained from the American Type Culture Collection, Rockville, Md., and was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were harvested, washed in PBS-BSA-azide (0.2% BSA, 0.1% sodium azide) and resuspended at 10^7 cells per ml in the same solution. Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labeled monoclonal antibodies to HLA-DR were obtained from Becton Dickinson Immunocytometry Systems, Mountain View, Calif.

To quantify SEB binding to MHC class II, it is necessary to determine whether the amount of binding is proportional to the fluorescence intensity. If this relationship holds, the amount of staining for HLA-DR should be proportional to the intensity of staining for SEB. To determine this, the HUT-78 cells were doubly stained with FITC-labeled anti-HLA-DR and biotinylated SEB and then with avidin-PE as follows. HUT-78 cells were incubated with 50 μl of a 4-μg/ml concentration of biotinylated SEB and 50 μl of FITC-conjugated anti-HLA-DR (25 μg/ml) for 15 min on ice. The cells were washed with PBS-BSA-azide and incubated for an additional 15 min on ice with 50 μl of a 2.5-μg/ml concentration of PE-avidin (Molecular Probes, Eugene, Oreg.). The stained cells were analyzed in a FACScan flow cytometer (Becton Dickinson). Compensation was set on green-only (i.e., FITC-labeled anti-HLA-DR stained cells only) and red-only (i.e., cells stained with biotin-SEB followed by PE-avidin) controls. A gate was set around the major cell population to exclude small and damaged cells. Ten thousand cells were analyzed. Cells doubly stained with SEB and anti-HLA-DR served as an indication of binding of SEB to HLA-DR on HUT-78 cells.

Inhibition of binding to HUT-78 cells. Inhibition of SEB binding to HLA-DR on HUT-78 cells by peptides of SEB was analyzed and quantified by flow cytometric analysis. In the initial experiments to screen for inhibitory peptides, 50 μl of biotinylated SEB, at a concentration of 0.5 μg/ml, and 50 μl of peptide in various concentrations (0.2 to 1.0 mg/ml) were mixed in a tube (12 by 75 mm). In follow-up experiments to verify the inhibition (shown in Fig. 6), the signal was increased by using 100 μg of biotinylated SEB per ml. One hundred microliters (10^6 cells) of HUT-78 cell suspension was then added, and the mixture was incubated on ice for 15 min. The cells were washed two times with 3 ml of PBS-BSA-azide, and then 50 μl of FITC-avidin (Vector Laboratories, Burlingame, CA) at 30 μg/ml in the initial experiments or 50 μl of PE-avidin at 5 μg/ml (in the follow-up experiments) was added to stain the cells in the pellet. The cells in the pellet were resuspended and incubated for 30 min on ice and then washed with 4 ml of PBS-BSA-azide. The cells were fixed with 1 ml of 1% ultrapure formaldehyde (Polysciences, Warrington, Pa.) and kept at 4°C in the dark until they were examined by flow cytometry. Ten thousand cells were examined in each test. Cells were examined within 24 h of staining. Data files of stained cells were analyzed in a FACScan flow cytometer by using Becton Dickinson's LYSIS II program. Median fluorescence intensity values calculated by the program were used as the measure of binding of SEB to HUT-78 cells; the percent inhibition by the peptides was then calculated. Since the fluorescence was displayed on a 4-decade logarithmic graph, representing 1,024 channels, a 256-channel difference between two points represented a 10-fold difference in fluorescence intensity. Therefore, the channel difference was divided by 256, and the result was taken as the log of the fold difference between two points. The antilog was then taken, and the percentage of control

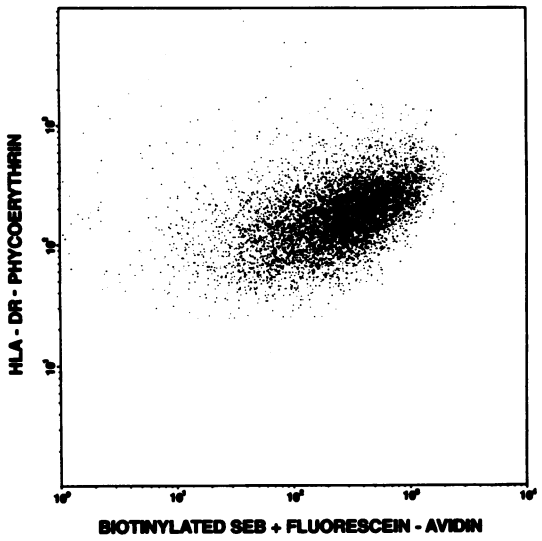

FIG. 1. Dot plot of fluorescence of HUT-78 cells stained with PE-labeled anti-HLA-DR and labeled with 4 μg of biotinylated SEB per ml followed by FITC-avidin as described in Materials and Methods.

fluorescence was calculated. Because of this conversion from logarithmic to linear values, the mean fluorescence intensity of uninhibited samples shown in Fig. 6 does not add up to exactly 100%.

In the follow-up experiments to check the inhibitory peptides, the percent inhibition values from 17 replicate tubes in three experiments [except for peptide SEB(90–114), in which there were 12 replicate tubes from two experiments] were compared with those of uninhibited tubes by using Student's *t* test for groups, two-tailed, with separate variances. The tests were calculated by using the computer program Statpal (Statpal Associates, Montpelier, Vt.).

RESULTS

To determine whether there is a correlation between the amount of HLA-DR on the cells and the intensity of SEB staining, HUT-78 cells were doubly stained by biotinylated SEB and anti-HLA-DR reagents. As shown in Fig. 1, all of the HLA-DR-positive cells bound SEB.

To locate the regions of SEB molecule that are involved in SEB binding to HUT-78 cells, peptides of SEB were used to inhibit this binding. The amino acid sequences of the peptides of SEB used in the inhibition tests are shown in Table 1. As shown in Fig. 2, several of the peptides of SEB inhibit binding of SEB to HUT-78 cells. These includes peptides SEB(9–20), SEB(30–38), SEB(61–70), SEB(90–114), and SEB(169–181). SEB(90–114) showed the highest inhibition. SEB(9–20) contains some of the amino acids in region 1, residues 9 to 23, described by Kappler and coworkers (27), which these workers found to be involved in T-cell mitogenesis and binding of HLA-DR to SEB. Peptide SEB(39–51) also coincides with residues 41 to 53, which was shown to be the region involved in binding to HLA-DR.

To further determine the efficiency of binding inhibition, peptides of SEB were titrated. Figures 3 and 4 show the titration of five peptides that inhibit binding. Peptide SEB(90–114) inhibits binding at a concentration as low as approximately 60 μg of peptide per ml. Peptides SEB(9–20), SEB(30–38), SEB(61–70), and SEB(169–181) showed moderate inhibition at higher concentrations. However, peptide SEB(39–51) at a low concentration significantly inhibited binding but repeatedly enhanced binding at higher concentrations (Fig. 5). This may be due to hydrophobic interactions that link SEB to a hydrophobic region of HLA-DR. This peptide has 6 hydrophobic residues out of a total of 13 residues, or 46%, which is a higher percentage than any of the other peptides. The three-dimensional structure of an SEB–HLA-DR complex shows that hydrophobic residues 44, 45, and 47 protrude from a loop between strands 1 and 2 of the SEB molecule and fit into a predominantly hydrophobic depression in the HLA-DR1 molecule (25).

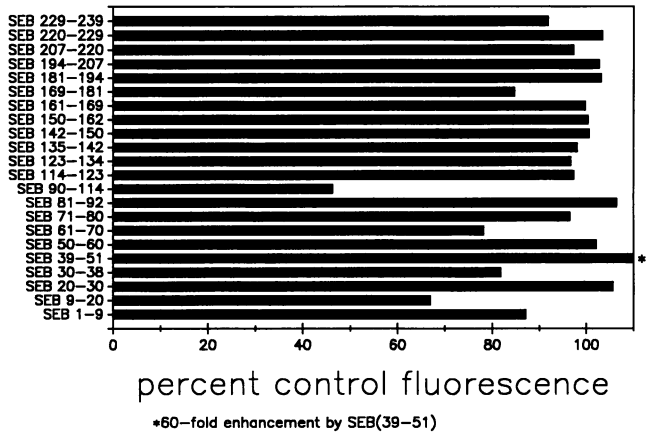

FIG. 2. Plot of the inhibition of binding of biotinylated SEB to HUT-78 cells by 22 synthetic peptides of SEB used at a concentration of 1 mg/ml. The inhibition is expressed as the percentage of control fluorescence of biotinylated SEB detected by FITC-avidin.

To verify that the five identified peptides were indeed inhibitory, a series of confirmatory experiments was done. The biotin-SEB concentration was raised to 100 μg/ml to increase the signal. The results are shown in Fig. 6. All five peptides gave significant inhibition of binding of biotin-SEB.

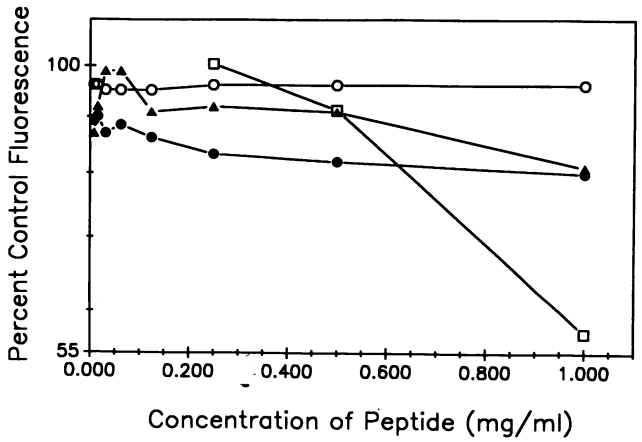

FIG. 3. Results of titration of four peptides to determine their ability to inhibit the binding of biotin-SEB to HUT-78 cells. Symbols: ●, peptide SEB(9–20); ○, peptide SEB(30–38); □, peptide SEB(61–70); ▲, peptide SEB(181–189).

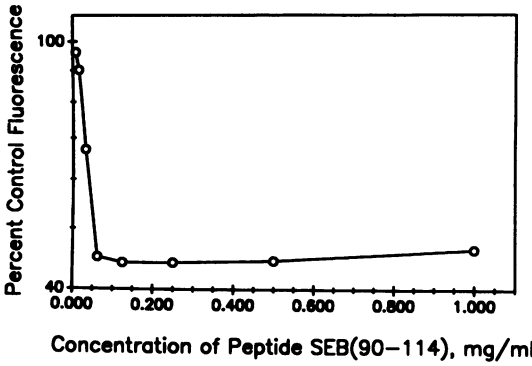

FIG. 4. Results of titration of peptide SEB(90–114) mixed with biotin-SEB. This peptide gave the greatest inhibition.

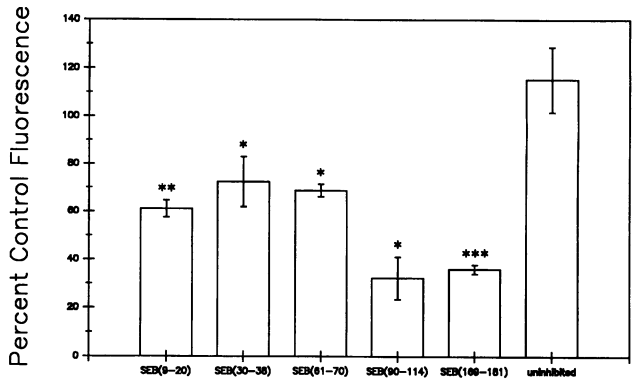

FIG. 6. Plot of the five inhibitory peptides showing the standard error of 17 replicate tubes in three experiments [except for peptide SEB(90–114), which was tested with 12 tubes in two experiments]. In these experiments only, the concentration of biotin-SEB was 100 μg/ml. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

DISCUSSION

SEB binds to HUT-78 cells on a single class of binding sites with a K_d of 10^{-6} M. (29). Scholl and coworkers (43) found that SEB bound to a heterodimer on HUT-78 cells. This heterodimer had two chains, with molecular masses of 35 and 31 kDa, which comigrated with the α and β chains of HLA-DR, the human MHC class II antigens to which enterotoxins bind most strongly (22, 44). We found that the intensity of staining with FITC-labeled anti-HLA-DR was correlated with the intensity of staining with biotinylated SEB plus PE-avidin. The cells that stained more brightly with the anti-HLA-DR also stained more brightly with biotinylated SEB.

Several groups have identified sites on enterotoxins that are involved in binding to MHC class II antigens. Kappler and coworkers (27), by using mutated SEB molecules, identified two regions that are important in the binding of SEB to HLA-DR, one comprising residues 14 and 23 and one comprising residues 41 to 53. Mutations in residues 14 (S→L), 17 (F→S), and 23 (N→S) of SEB reduced binding 100-fold (27). In agreement with Kappler and coworkers (27), we found inhibition of SEB binding by peptide SEB(9–20). Region 1 of Kappler and coworkers, residues 9 to 23, was identified as being involved in both T-cell-receptor and MHC binding. However, the three-dimensional structure of an SEB–HLA-DR1 complex (25) did not show these residues to be involved in the SEB-DR1 interface. Peptide SEA(1–45) inhibits binding of SEA to HLA-DR and to murine I-Aβ^b-bearing cells (37). Peptide SEB(39–51), which enhanced binding at most of the concentrations used, includes some residues of Kappler's region 2, residues 41 to 53, which are involved in MHC binding only. This peptide includes residues 43 to 46, which are involved in hydrogen bonds to HLA-DR1 (25). Residue Ile-190, identified by Swaminathan and coworkers (50) as a possible MHC-binding residue, and residues 210 to 217, identified by Jardetzky and coworkers (25) as being on a contact site, are on peptides that did not inhibit binding. Peptides SEB(30–38), SEB(61–70), SEB(90–114), and SEB(169–181) inhibit binding but had not been previously identified as being important in the binding of SEB to MHC class II. However, Jardetzky and coworkers (25) confirmed that residues 92 to 96 of the disulfide loop and residues Arg-65, Glu-67, and Lys-71 were in the contact region with SEB.

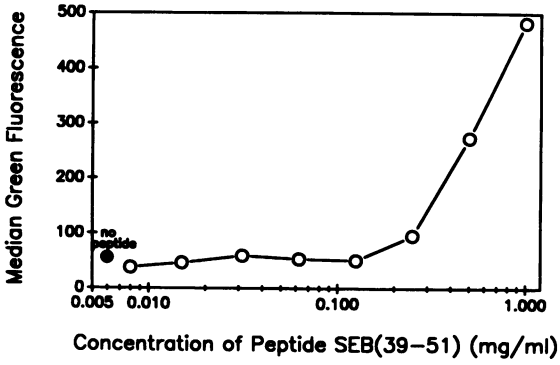

FIG. 5. Results of a typical experiment showing the titration of peptide SEB(39–51) mixed with biotin-SEB. Increasing concentrations of this peptide increased the binding of biotin-SEB to HUT-78 cells.

The strongest inhibition was obtained with a peptide spanning residues 90 to 114, which includes the disulfide loop (residues 93 to 113) of the SEB molecule. The use of this peptide at 1 mg/ml reduces binding to near-background levels. Part of this region is poorly defined in the electron density map and resulting three-dimensional model of SEB (50), presumably because it is flexible. This very flexibility, however, might facilitate binding to MHC class II. Grossman and coworkers (18) found that an intact disulfide bond was not necessary for binding of SEA to MHC class II antigens and was not necessary for class II-mediated monocyte stimulation by SEB. Apparently, the residues of the loop will bind to MHC class II antigens without an intact disulfide bond. This is consistent with the ability of a linear synthetic peptide to inhibit binding of the SEB molecule. The superantigen staphylococcal toxic shock syndrome toxin-1 (TSST-1) is 20 to 30% homologous to the SEs (3). TSST-1, which lacks a disulfide bond, is still capable of binding to MHC class II antigens and stimulating T-cell proliferation. However, SEB and TSST-1 bind to different sites on HLA-DR (8).

We found significant inhibition with peptides SEB(30–38) and SEB(61–70) in addition to peptides SEB(9–20) and SEB(90–114). One of these, SEB(61–70), corresponds to residues 64 to 73 of SEA, one of the four peptides of SEA that inhibit the binding of SEA to HLA antigens on Raji cells (17). The four peptides were SEA(1–45), SEA(39–66), SEA(62–86), and SEA(121–149) (17). Peptide SEB(61–70), which inhibits binding, straddles peptides 60 to 70 and 70 to 80 of SEA. However, Griggs and coworkers (17) found no inhibition of binding by peptides that included the residues of the disulfide

loop of SEA. The disulfide loop of SEA is 11 residues long, while the disulfide loop of SEB is 21 residues long, and there is little homology between them. Therefore, it would not be surprising to find that they do not have the same importance in binding to MHC molecules. SEA competes with SEB for binding to HLA-DR, but the reverse is not true, suggesting either that SEA binds to more than one site or that the binding sites for SEA and SEB to HLA-DR overlap but are not identical (8). Swaminathan and coworkers (50), who determined the three-dimensional structure of SEB, suggested that the α5 groove, which lies on the same side of the molecule as the residues identified by Kappler and coworkers (27), is important for binding of SEB to MHC class II (residues 14 to 23 within region 1 and region 2, residues 41 to 53) and is also involved in binding. They identified 22 residues that line the α5 groove: His-12, Phe-17, Met-24, Tyr-28, Asp-161, Arg-165, Glu-191, Phe-196, Trp-197, Tyr-198, Asp-199, Met-201, Phe-208, Lys-212, Tyr-213, Met-215, Met-216, Tyr-217, Asp-219, Lys-221, Val-223, and Val-228. Among these are 16 residues that are conserved among all the SEs for which the sequences had been published at the time (SEA, SEB, SEC_1, SEC_2, SEC_3, SED, and SEE). In our hands, only two of those residues, His-12 and Tyr-17, are contained in the peptides that inhibited binding of SEB. It may be that binding to MHC class II involves a large number of low-affinity interactions, only some of which can be detected by mutation or peptide inhibition experiments. Jardetzky and coworkers (25) identified 19 residues in several regions of SEB involved in the interaction between HLA-DR1 and SEB. This may explain why most of the peptides studied in the present work gave only partial inhibition of binding.

There have been a large number of studies to locate the active sites of SEs that are responsible for inducing disease. However, the results are controversial. When SEB is digested with proteolytic enzymes and is reduced and alkylated, two fragments are formed: one fragment (11 kDa) has the N-terminal end, while the other (17 kDa) has the C-terminal end. Some investigators have localized the mitogenic activity of SEs to the N-terminal portion (5, 6, 27, 38), while others have abolished mitogenic functions by deleting 9 residues from the C-terminal portion (33). Binek and coworkers (4) found mitogenic activity in the 17-kDa C-terminal fragment of SEB produced by trypsin and found none in the 12-kDa N-terminal fragment. Several studies have also localized MHC class II binding to the amino terminus (17, 27, 37, 39), but Pontzer and coworkers (36) and Hedlund and coworkers (21) have observed MHC class II binding to C-terminal portions (residues 121 to 149 and 107 to 233, respectively) of SEA. One possible explanation for the discrepancies is the different methods used. For example, several workers have suggested that deletion mutations of SEs could alter the conformation of the molecules (24, 33).

Some structure-function studies of enterotoxins have focused on mitogenesis and the ability of the molecules to cause emesis in monkeys. These activities may be dissociated from one another. For example, certain formaldehyde treatments of SEB can abolish its emetic activity even though the molecules retain some mitogenicity (45). Another example is provided by SEC_1, which is 66% homologous with SEB (3). Treatment of SEC_1 with trypsin results in two major peptides (46). The amino-terminal 6.5-kDa fragment has mitogenic activity, while the carboxy-terminal 22-kDa fragment has no mitogenic or emetic activity but does cause diarrhea (46). A superantigen related to the enterotoxins, TSST-1, causes mitosis but is only weakly emetic (2).

The three-dimensional structure of TSST-1 has been reported recently (1, 40). There are sequences in SEB involved in MHC class II binding that have no structural counterpart in TSST-1 (1), but TSST-1 residues 39 to 68, which compete for binding with the native molecule, include structures that are similar to the β3, α3, and β4 loops of SEB (1). This points to some similarity with SEB, since in the present study, peptide SEB(61–70), which is located primarily on β3, inhibits binding in the present study.

The ability of enterotoxins to stimulate T-cell proliferation and their ability to bind to MHC class II antigens are both thought to be involved in their ability to cause illness (19, 49). Therefore, understanding the binding of enterotoxins to MHC class II may help us control two types of cell activation, either of which may be involved in disease.

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