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Biochemical and Mutational Analysis of the Histidine Residues of Staphylococcal Enterotoxin A†

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The goal of this study was to examine the role of histidine residues in the biological activities of staphylococcal enterotoxin A (SEA). Carboxymethylated SEA was unable to stimulate murine T-cell proliferation but was resistant to monkey stomach lavage fluid degradation, suggesting that native conformation was intact. Site-directed mutagenesis of the histidine residues of SEA was subsequently performed. SEA-H44A (SEA with histidine 44 replaced with alanine), SEA-H44D, SEA-H50A, SEA-H50D, SEA-H114A, SEA-H114D, SEA-H187A, and SEA-H187D retained superantigen and emetic activities, whereas SEA-H225A and SEA-H225D were defective in the ability to stimulate T-cell proliferation. These mutants were unable to compete with SEA for binding to Raji cells, suggesting that the defect in SEA-H225A and SEA-H225D is due to impaired major histocompatibility complex class II binding. SEA-H225D provoked an emetic response in monkeys only if fed at high doses, while SEA-H225A did not provoke an emetic response at low or high doses. In comparison, SEA-H61A and SEA-H61D were defective in emetic activity but not in the ability to stimulate murine T-cell proliferation. Overall, these studies show that the carboxy-terminal histidine at residue position 225 of SEA is important for both the superantigen and emetic activities of this enterotoxin. Histidine 61 appears to be important for emetic activity but not for superantigen activity, consistent with the hypothesis that the two activities are separable in staphylococcal enterotoxins.

Staphylococcal enterotoxins (SEs) induce emesis and are the causative agents of staphylococcal food poisoning (5). Five serological groups of SEs, SEA through SEE, have been characterized, with SEC having multiple subtypes. The genes for each of these toxins have been cloned. On the basis of the homology between their predicted amino acid sequences, SEs can be divided into two groups, one with SEA, SED, and SEE and the other with SEB and the SECs (reviewed in reference 6). A recently identified staphylococcal exotoxin which induces shock in rabbits, called SEH, shares considerable amino acid sequence identity with SEA, SED, and SEE (33); a separate study showed that a protein also designated as SEH, with the same 35 amino-terminal residues, is emetic (41).

In addition to causing emesis, the SEs are members of a group of potent immunological activators called superantigens (29). The hallmark activity of a superantigen is the ability to stimulate T cells in a manner dependent on the Vβ sequence of the T-cell receptor (TCR). Like conventional exogenous antigens, superantigens must be presented to T cells by a class II major histocompatibility complex (MHC) molecule. Unlike conventional antigens, superantigens do not require processing and are displayed outside of the peptide binding groove (9). The three-dimensional structure of SEB has been determined, which, in conjunction with mutagenesis data, allows prediction of sites in SEB which make contact with the TCR (24, 42). Further, the three-dimensional structure of SEB complexed with the human MHC class II molecule HLA-DR1 allows identification of SEB residues which make direct contact with the α chain of the MHC class II molecule (22).

SE binding to MHC class II is sufficient to stimulate macrophages to produce the inflammatory cytokines interleukin-1 and tumor necrosis factor alpha; this stimulation is enhanced by gamma interferon (13). Superantigens bind to different sites on the MHC class II molecule. For example, SEA can compete with SEB (10) and toxic shock syndrome toxin 1 (TSST-1), another staphylococcal superantigen, for binding to the MHC class II molecule, but SEB and TSST-1 cannot compete with SEA for binding (8). The structure of TSST-1 complexed with HLA-DR1 shows that TSST-1 binds to a region of the MHC class II α chain similar to that bound by SEB, but with significant differences (25). SEA and SEE require zinc to bind the MHC class II molecule, while SEB binding was not affected by EDTA (12). Mutations at positions 81, 137, and 152 of the β chain of HLA-DR1 affect binding of SEA but not SEB, suggesting that SEA binds the α and β chains of the MHC class II molecule (3, 19).

The relationship between the emetic and the superantigen activities of the SEs is unclear. Some investigators suggest that the high levels of interleukin-2 production provoked by superantigen exposure lead to emesis, similar to the emetic effect of chemotherapeutic interleukin-2 treatment (35). A growing body of data indicates, however, that the two activities are separable. One group showed that carboxymethylated SEB (CM-SEB) is unable to induce emesis and can block the emetic activity of SEB (32), while a separate study showed that CM-SEB is active in a primate T-cell proliferation assay (2). Mutational analysis of SEA and SEB has also shown that the two activities can be separated (14, 15). Residues 25, 47, and 48 of SEA are required for the superantigen activity of SEA, with residue 25 interacting with the TCR and residues 47 and 48 playing a role in MHC class II molecule binding (14). However, some mutations at these positions which result in loss of superantigen activity do not cause loss of emetic activity.

Earlier chemical modification of the histidine residues of SEA showed that CM-SEA is unable to provoke an emetic response in monkeys (40); CM-SEA was not tested for concomitant superantigen activity. In the present study, CM-SEA

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TABLE 1. Mutant *sea* alleles

Mutant allele	Template	Site-directed mutation(s)[a]	Effect[b]
sea-1325	*sea*	A483G	*Kpn*I
		T489C	*Sty*I
		G537A	*Bgl*II
		G615A, A618C	*Stu*I
		T619C	
		T637A, C638G	*Spe*I
sea-1401	*sea-1364*	C202G, A203T	H44A, *Pst*I −
		C204T	
sea-1402	*sea-1364*	C202G	H44D, *Pst*I −
sea-1403	*sea-1364*	C220G, A221C	H50A, *Pst*I −
sea-1404	*sea-1364*	C220G	H50D, *Pst*I −
sea-1405	*sea-1364*	C253G, A254C	H61A, *Spe*I −
sea-1406	*sea-1364*	C253G	H61D, *Spe*I −
sea-1407	*sea-1325*	C412G, A413C	H114A, *Cla*I −
		T423C	
sea-1408	*sea-1325*	C412G, A426T	H114D, *Cla*I −
sea-1409	*sea-1325*	C631G, A632C	H187A, *Spe*I −
sea-1410	*sea-1325*	C631G	H187D, *Spe*I −
sea-1411	*sea-1325*	C734G, A735C	H225A, *Nsi*I −
sea-1412	*sea-1325*	C734G	H225D, *Nsi*I −

[a] The nucleotide indicated at the left of the indicated position was changed to the nucleotide indicated at the right.

[b] Amino acid substitution or restriction site created or lost (−) by substitution.

and SEA with the individual histidine residues at positions 44, 50, 61, 114, 187, and 225 substituted by site-directed mutagenesis were tested for the ability to stimulate murine T-cell proliferation and for susceptibility to monkey stomach fluid degradation. Further, mutants which were defective in the ability to stimulate T-cell proliferation were tested by flow cytometry for the ability to compete with SEA for MHC class II binding to Raji cells, which constitutively express HLA-DR.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* and *Staphylococcus aureus* were grown under established conditions (20). *S. aureus* was grown in 3% (wt/vol) N-Z amine type A (Kraft, Inc., Norwich, N.Y.)–1% yeast extract (Difco Laboratories, Detroit, Mich.) at 37°C with aeration (200 rpm) for 16 to 18 h and then harvested by centrifugation. Nalgene filters (0.45-μm pore size; Nalge Co., Rochester, N.Y.) were used to sterilize culture supernatants before testing.

Chemicals and enzymes. Chemical and enzymatic reagents were obtained from the following sources: restriction enzymes and T4 ligase were from New England Biolabs, Inc. (Beverly, Mass.); bromoacetic acid and fluorescein isothiocyanate-avidin were from Sigma Chemical Co. (St. Louis, Mo.); the Sequenase kit was from United States Biochemical (Cleveland, Ohio); and the Unique Site Elimination kit and CNBr-activated Sepharose 4B were from Pharmacia Biotec (Milwaukee, Wis.).

Chemical modification. Samples containing 0.3 mg of SEA were incubated with bromoacetic acid (0.5 M, pH 6.8) or potassium phosphate buffer (0.5 M, pH 6.8) as a negative control for 14 days in the dark at room temperature. The samples subsequently were dialyzed extensively against phosphate-buffered saline (PBS; 0.02 M sodium phosphate, 0.15 M NaCl [pH 7.4]) to remove the bromoacetic acid and were concentrated with Centricon-10 units (Amicon Division, W. R. Grace & Co., Beverly, Mass.). Amino acid analysis of the carboxymethylated samples was performed at the Medical College of Wisconsin's Protein/Nucleic Acid Shared Facility of the Cancer Center (Milwaukee).

Site-directed mutagenesis. The *sea* allele used as a template for mutagenesis was *sea-1364* (15) or *sea-1325* with unique restriction sites in the coding regions for the N terminus or the C terminus of SEA, respectively. Sequential rounds of mutagenesis (27) were used to construct *sea-1325* (Table 1).

The Unique Site Elimination kit (Pharmacia) was used to construct most of the altered *sea* alleles, with the exception of mutations resulting in *sea-1410* and *sea-1412*; PCR was performed to make *sea-1410*, and modified Kunkel mutagenesis (27) was used to construct *sea-1412*. All mutations were confirmed by DNA sequence analysis (36). Table 1 describes the specific nucleotide substitutions made for each mutant *sea* as well as the resulting amino acid substitutions and changes in unique restriction sites which resulted from these substitutions.

Following confirmation of the mutation, the plasmid with the mutant *sea* was digested with *Hin*dIII and ligated to *Hin*dIII-cut pC194 (20, 21). The resulting plasmids were then electroporated into *S. aureus* RN4220 (20, 26). Generalized transduction with phage 80∝ (30) was used to introduce the plasmids into the protein A-negative *S. aureus* strain ISP2073 (31).

Serological assays. Western blots (immunoblots) and gel double-diffusion assays were performed by using established protocols (20, 34). The polyclonal antisera used were the gift of Merlin Bergdoll (University of Wisconsin—Madison); the monoclonal antibodies (MAbs) C1 and E8 have been characterized previously (16).

Murine T-cell proliferation assay. Splenocytes from 6- to 8-week-old BALB/c female mice (Harlan, Sprague, Dawley, Madison, Wis.) were used in T-cell proliferation assays (20). Dilutions of culture supernatants containing SEA, mutant SEAs, or purified samples (including chemically modified SEA) were incubated at 5.0, 0.5, or 0.05 μg per well. The extent of proliferation was determined by measuring the incorporation of [^{3}H]thymidine. The degree of statistical significance ($P < 0.01$) was determined by Student's t test, using Minitab (Minitab, Inc.).

Purification of SEs. SEA, SEA-H225A, and SEA-H225D were purified from protein A-negative strains by immunoaffinity chromatography with MAb C1 as described previously (15). Purity of samples was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The concentration of the purified samples was determined by UV adsorption at 277 nm, using an extinction coefficient of 12.76 (39).

MHC class II receptor binding assay. Samples containing various amounts of purified SEA or mutant SEA were coincubated with 5 μg of biotinylated SEA (Toxin Technologies, Sarasota, Fla.) per ml and Raji cells (5×10^6 cells per ml), washed with FC buffer (PBS [pH 6.8], 1% bovine serum albumin, 0.1% NaN_3), and incubated with fluorescein isothiocyanate-avidin (10 μg/ml). Binding was determined by flow cytometry using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) flow cytometer (14).

Stomach fluid degradation assay. Samples with culture supernatants containing SEA, CM-SEA, and control SEA in PBS or supernatants containing mutant SEAs were incubated with monkey stomach lavage fluid or PBS for 2 h at 37°C. The stomach fluid was obtained by sterile lavage with 15 ml of 0.9% (wt/vol) NaCl solution (Abbott Laboratories, Chicago, Ill.) from rhesus monkeys (*Macaca mulatta*). The samples were then tested by Western blot (immunoblot) analysis. SEA-V85G, previously shown to degrade in stomach fluid, was used as a positive degradation control (15).

Emetic assays. SEA is able to stimulate a strong emetic response in monkeys at a dose of 100 μg per animal. Sterilized culture supernatants from RN4220 derivatives containing 100 μg of mutant SEA were administered by nasogastric intubation to rhesus monkeys weighing 2 to 3 kg. The amount of SEA (or mutant SEA) in RN4220 culture supernatants was determined by serial dilution and comparison with quantitated purified SEA (15). The monkeys were observed for 5 h. Samples which did not induce an emetic response at the dose were tested at 500 μg per animal. The samples containing higher amounts were prepared by concentrating culture supernatants with Amicon Centriprep units. RN4220 supernatants served as a negative control for these experiments. Emetic assays were done in collaboration with the Wisconsin Regional Primate Research Center, Madison.

RESULTS

Carboxymethylation of SEA. An earlier study using CM-SEA did not test the modified toxin for its ability to stimulate T-cell proliferation and did not determine whether the loss of emetic activity was due to degradation by stomach fluid (40). To address these questions in the present study, SEA was carboxymethylated to further characterize CM-SEA. The modification was successful in that an average of 5.5 of the 6 histidine residues were modified as determined by amino acid analysis. CM-SEA was able to bind polyclonal antisera against SEA (Fig. 1A) and MAbs C1 and E8 (data not shown), consistent with the earlier report that CM-SEA is not significantly altered in conformation (40). CM-SEA was tested for its ability to stimulate murine T-cell proliferation and did not produce a statistically significant response compared with SEA (Fig. 1B). CM-SEB, in contrast, was able to stimulate primate T cells to proliferate (2). CM-SEA was tested for susceptibility to degradation by monkey stomach fluid in an in vitro assay. Compared with SEA-V85G, a mutant SEA susceptible to degradation in monkey stomach fluid (15), CM-SEA was found to be resistant to degradation under these conditions, suggesting that the loss of emetic activity was probably not due to degradation (Fig. 1A). These findings suggested that one or more histidine residues play a role in the superantigen and emetic activities of SEA.

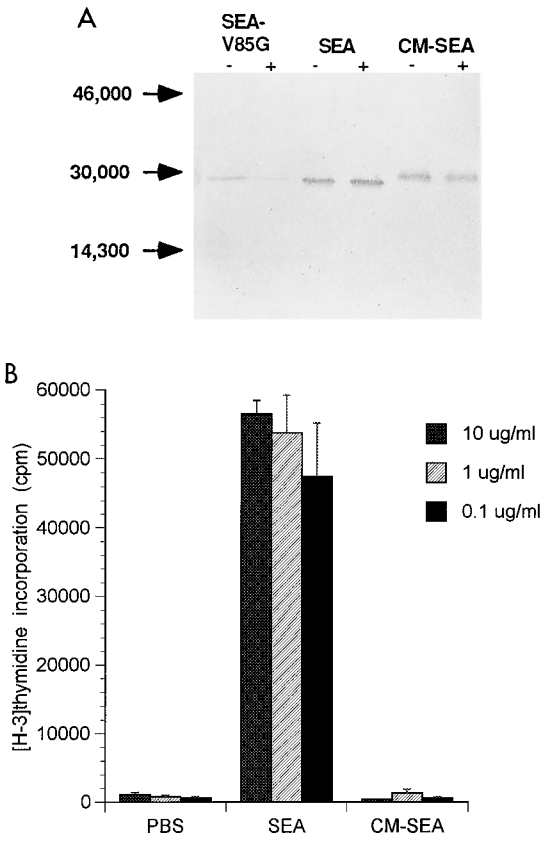

FIG. 1. Characterization of CM-SEA. (A) Western blot comparing SEA-V85G (culture supernatant in ISP2073 background), SEA (5 μg/ml, initial concentration), and CM-SEA (5 μg/ml, initial concentration) for susceptibility to stomach fluid degradation and ability to bind polyclonal antiserum against SEA. Lanes: −, samples incubated with PBS; +, samples incubated with monkey stomach lavage fluid. (B) Induction of T-cell proliferation by SEA and CM-SEA. Samples were incubated with murine splenocytes for 72 h and then pulsed with [^{3}H]thymidine for 18 h. Results are means of a representative experiment in which each sample was tested in triplicate. Data shown are representative of at least three separate proliferation assays.

Serological characterization of mutant SEAs. To identify the specific histidine residues involved in the superantigen and emetic activities of SEA, mutant SEAs were constructed by site-directed mutagenesis. Aspartic acid substitutions were made in order to mimic the results obtained by acidic modification. Alanine substitutions were made to address the possibility that the aspartic acid substitutions do not affect metal binding and because aspartic acid is found at some analogous positions in other SEs (4). The production of immunoreactive material by these mutant alleles was confirmed by gel double-diffusion assays and Western blot analysis of *S. aureus* RN4220 supernatants, using polyclonal antisera raised against SEA (data not shown). The plasmids were then transduced into the protein A-negative *S. aureus* strain ISP2073, and these strains were used for further analysis of the mutants.

All of the mutant SEAs formed a band of identity with polyclonal antisera raised against SEA in both gel double-diffusion assays and Western blots. All mutants were also able to form a band of identity with MAb C1, which recognizes an epitope in residues 108 to 230 of SEA, and with MAb E8 (data not shown), which recognizes a conformational epitope (16). None of the mutants were degraded by incubation with monkey stomach lavage fluid (Fig. 2), which was a further indication that the substitutions did not result in significant conformational change in the proteins that would expose buried protease cleavage sites.

T-cell stimulation by mutant SEAs. Culture supernatants from ISP2073 derivatives producing SEA or mutant SEAs were tested for the ability to stimulate murine splenocyte proliferation. Dilutions of each supernatant were tested in triplicate wells. Substitution of the histidine at position 44, 61, 114, or 187 with either alanine or aspartic acid did not affect the ability of SEA to stimulate murine T-cell proliferation (Fig. 3A). SEA-H50D and SEA-H114A stimulated a lower level of proliferation than did SEA, but the proliferative response was statistically significant ($P < 0.01$) compared with the negative control. SEA-H225A and SEA-H225D were inactive in the murine T-cell proliferation assay.

SEA-H225A and SEA-H225D were purified by immunoaffinity chromatography to determine if they could stimulate T-cell proliferation at concentrations higher than those found in culture supernatants. When tested at 5, 0.5, and 0.05 μg per well, these mutant SEAs were unable to stimulate T-cell proliferation (Fig. 3B), indicating that the histidine at position 225 plays an important role in the superantigen activity of SEA.

Binding of SEA-H225A and SEA-H225D to MHC class II molecules. Two types of mutations would potentially affect the superantigen activity of SEA: a defect in MHC class II binding or a defect in the ability to bind the TCR. Because MHC class II binding is prerequisite for optimal interaction with the TCR and because MHC class II binding alone is sufficient to stimulate inflammatory cytokine production, we tested these mutants for the ability to bind Raji cells, which constitutively express HLA-DR1. Neither SEA-H225A nor SEA-H225D was able to compete effectively with biotinylated SEA for binding to Raji cells (Fig. 4). This finding provides evidence that histidine 225 plays a role in the MHC class II binding of SEA.

Emetic activity of mutant SEAs. All of the mutants resistant to degradation by monkey stomach fluid in an in vitro assay (Fig. 2), with the exception of SEA-H114A and SEA-H114D, were tested for emetic activity. SEA-H114A and SEA-H114D were not tested because SEA with a deletion of this histidine residue and SEA-H114R were emetic when fed at 100 μg per animal (19a). SEA-H44A, SEA-H44D, SEA-H50A, SEA-H50D, SEA-H187A, and SEA-H187D were emetic at 100 μg per animal (Table 2). A mutant SEA which induces a response in at least one animal is considered emetic, but comparisons

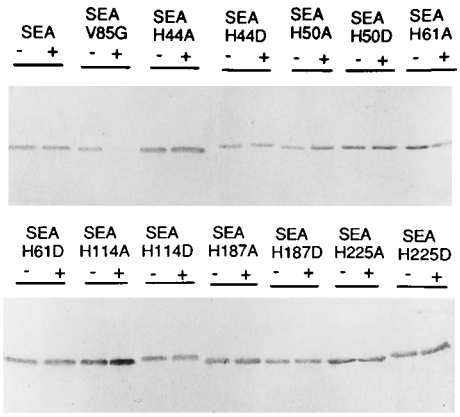

FIG. 2. Incubation of mutant SEAs with monkey stomach lavage fluid. Culture supernatants containing SEA or mutant SEAs, including SEA-V85G as a control, were incubated with PBS (−) or stomach fluid (+) and then subjected to Western blotting. The blots were detected with polyclonal antisera raised against SEA.

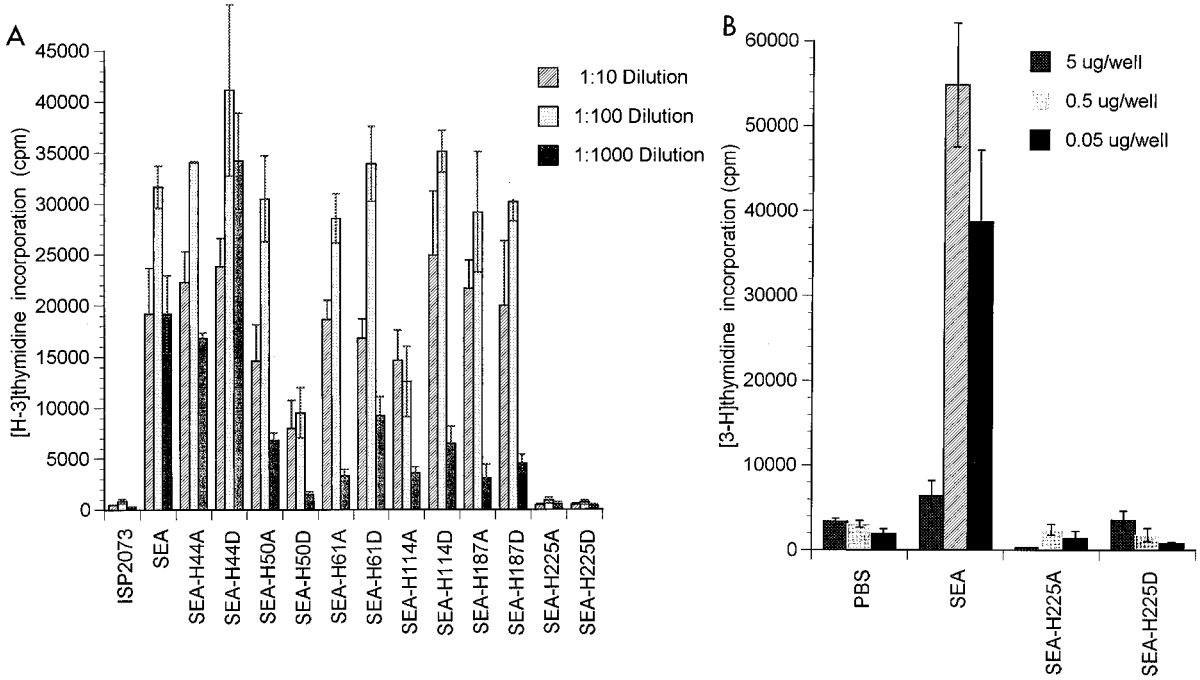

FIG. 3. Induction of T-cell proliferation by mutant SEAs in culture supernatants from ISP2073 (Sea$^-$; negative control) or derivatives expressing either SEA or a mutant SEA (A) or purified SEA-H225A and SEA-H225D (B). Samples were incubated with murine splenocytes for 72 h and then pulsed with [^{3}H]thymidine for 18 h. Results are means of a representative experiment in which each sample was tested in triplicate. Data shown are representative of at least three separate proliferation assays.

between emetic samples cannot be made because of limited availability of monkeys. SEA-H61A and SEA-H61D did not provoke an emetic response when fed at 100 μg per animal. When tested at 500 μg per animal, neither SEA-H61A nor SEA-H61D provoked an emetic response in any of the six animals tested (Table 2). SEA-H225D did not provoke an emetic response at 100 μg per animal but did cause emesis in two of six monkeys at 500 μg per animal (Table 2). SEA-H225A was not tested at 100 μg per animal because SEA-H225D was emetic only at the higher dose and was not emetic at 500 μg per monkey (Table 2).

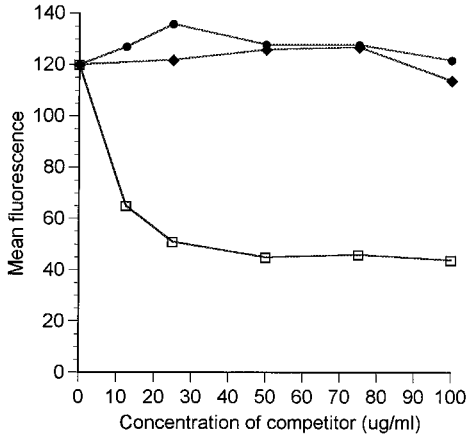

FIG. 4. MHC class II binding assay with SEA-H225A and SEA-H225D. Various concentrations of SEA (□), SEA-H225A (●), and SEA-H225D (◆) were tested for the ability to compete with biotinylated SEA for binding to Raji cells. Results are mean fluorescence of the cells following incubation with fluorescein isothiocyanate-avidin and detection by flow cytometry.

DISCUSSION

This study identifies two histidine residues of SEA which are important for the biological activities of this enterotoxin, confirming and extending an earlier finding which suggested that histidine residues play a role in the emetic activity of SEA (40). Other data also indicate that the histidine residues of SEA may be important for biological activity. Sequence analysis of the SEs shows that the histidine at position 114 is conserved among all SEs characterized. The zinc requirement for MHC class II binding implicates amino acids which are able to bind

TABLE 2. Emetic activities of SEAs

Sample[a]	Stomach fluid[b]	No. of monkeys exhibiting emesis	
		100 μg/animal (3 animals/group)	500 μg/animal (6 animals/group)
RN-4220	NT	0	NT
SEA	R	3	NT
SEA-V85G	S	NT	NT
SEA-H44A	R	3	NT
SEA-H44D	R	3	NT
SEA-H50A	R	1	NT
SEA-50D	R	1	NT
SEA-H61A	R	0	0
SEA-H61D	R	0	0
SEA-H187A	R	1	NT
SEA-H187D	R	1	NT
SEA-H225A	R	NT	0
SEA-H225D	R	0	2

[a] Samples were in RN4220 culture supernatants which had been filter sterilized.

[b] NT, not tested; R, sample is resistant to degradation by monkey stomach lavage fluid; S, sample is sensitive to degradation by monkey stomach lavage fluid.

zinc; cysteine and histidine residues are commonly found in zinc coordination sites, with aspartic acid and glutamic acid also common. The two cysteines of SEA form a stable disulfide bond and are unlikely to be directly involved in zinc coordination. The histidines at positions 187 and 225 are found in both SEA and SEE, which require zinc for the ability to bind the MHC class II molecule.

CM-SEA is unable to stimulate murine T-cell proliferation, demonstrating that one or more histidine residues of SEA are required for superantigen activity. This contrast with CM-SEB is not surprising since only the histidine at position 114 is conserved between the two. CM-SEA was not susceptible to degradation by stomach fluid (Fig. 1), suggesting that the lack of emetic activity reported in the earlier study was probably not due to degradation.

To identify the specific histidine residues which are required for the emetic and superantigen activities of SEA, site-directed mutagenesis was performed to create 12 SEAs in which single histidine residues were replaced with aspartic acid or alanine. None of the substitutions resulted in significant conformational changes that affected binding of polyclonal antisera to the mutant proteins or resulted in susceptibility to degradation by gastric fluid. The binding of MAb C1, which recognizes an epitope in the C terminus of SEA (16), is not affected by any of the mutations, including those in the C terminus; MAb E8 is also able to bind all of the mutants. All of the mutant proteins were resistant to degradation by stomach fluid, indicating that the overall conformation of the mutant proteins was not altered to expose a buried protease recognition site.

The substitutions at amino-terminal positions 44, 50, and 61 did not affect the ability of SEA to stimulate murine T cells. Histidines 44 and 50 are near residues 47 and 48, which had previously shown to be important for the MHC class II binding activity of SEA (14). In SEB, the five residues at positions 43 to 47, which align with residues 46 to 50 of SEA, make contact with the α chain of the HLA-DR (22). However, mutations at positions 46 (14) and 50 in SEA did not dramatically affect superantigen activity. Also, the histidines at positions 44 and 50 are not required for emetic activity.

Histidine 61 is near a site identified in SEB as being important in determining TCR specificity (17, 24). In SEA, a residue adjacent to histidine 61, aspartic acid 60, may play a role in interaction with the murine TCR (15, 28). Histidine 61 is important for the emetic activity of SEA but is not required for the ability to stimulate murine T-cell proliferation. One possible explanation for the observed change in emetic activity but not in murine T-cell stimulation is that this residue is important for primate but not murine TCR binding. It is also possible that the loss of emetic activity is due to in vivo degradation, in spite of the in vitro resistance of these mutants to stomach fluid. Another possible explanation is that the two activities are indeed distinct and that this residue is required for emesis but not T-cell activation.

Some mutations in the carboxy-terminal region affected both activities. Histidine 114 is the only histidine that is conserved among all of the enterotoxins characterized. Deletion of this histidine, or substitution of the histidine at this position with arginine, does not affect the emetic or superantigen activity of SEA (19a). Both SEA-H114A and SEA-H114D are active in murine T-cell proliferation assays. Thus, this conserved histidine is not required for emetic or superantigen activity.

Substitution of histidine 187 with an aspartic acid or alanine did not affect T-cell stimulation or emetic activity, although this histidine plays a role in the flexibility of the zinc binding site (36). The activity of SEA-H187A in a murine T-cell proliferation assay is in apparent contrast to the report that this substitution affects MHC class II binding (1). Our finding that SEA-H187A and SEA-H187D have superantigen activity and the report that SED has an aspartic acid at this position (4) indicate that residues other than histidine can be functional at this position. SEA-H225A and SEA-H225D were unable to stimulate T-cell proliferation, even at high amounts. These mutants were also unable to compete with biotinylated SEA for binding to cell surface-expressed MHC class II molecules. The histidine at position 225 is also important for emetic activity because SEA-H225D was emetic only when fed at 500 μg per animal and SEA-H225A was not emetic at 500 μg per animal.

SEA-H61A and SEA-H61D are the first mutant SEAs to be described with activity in the murine splenocyte proliferation assay that are altered in their emetic activities. The finding that SEA-H225D is emetic at the higher dose but does not stimulate T-cell proliferation is also consistent with earlier findings that the two activities do not perfectly correlate. However, it is worth noting that SEAs with mutations at sites in both the amino- and carboxy-terminal regions which affect MHC class II binding (residues 47, 48, and 225) have altered emetic activity. The nature of the substitution influences the degree of the effect on emetic activity; for example, SEA-F47S (14) and SEA-H225D are emetic at 500 μg per animal, while SEA-F47G (15) and SEA-H225A are not.

The identification of a specific amino acid in the C terminus of SEA which is important for MHC class II binding is consistent with the finding that a recombinant peptide consisting of residues 107 to 233 (18) is able bind the MHC class II molecule. Inspection of the SEA three-dimensional structure suggests that this residue is on the opposite face of the molecule relative to the predicted N-terminal MHC class II binding site. This finding indicates that SEA has two distinct MHC class II binding sites, consistent with the cross-linking model proposed by Jardtzky et al. (22). The two sites seem to be cooperative because substitutions at either site affect MHC class II molecule binding (this work and references 1 and 14).

The three-dimensional structure of SEA indicates that the amino acids which bind a metal atom are serine 1, histidines 187 and 225, and the aspartic acid at position 227 (37). The inability of mutants at position 225 to compete with biotinylated SEA for binding to the MHC class II molecule can be attributed to inability to bind zinc. However, the T-cell-stimulatory activity of substitutions at histidine 187 suggest that this residue is not vital for metal binding, consistent with the report of Abrahmsen et al. that SEA with an alanine at this position is able to bind the MHC class II molecule, though more weakly than SEA (1). The different emetic activities of SEA-H225A and SEA-H225D suggest that zinc binding could play a role in the emetic activity of SEA. While we were unable to show that SEA-H225D is able to compete for binding to the MHC class II receptor, it is possible that in the gastrointestinal tract, this mutant is able to bind zinc whereas SEA-H225A is not.

In summary, this study identifies histidine residues in SEA which play a role in the emetic and superantigen activities of this molecule. Histidine 61 was shown to be important for emetic activity but not for the ability to stimulate murine T-cell proliferation. A carboxy-terminal residue in the metal binding site of SEA important for MHC class II binding and emetic activity was identified. This is the first examination of the role of the metal binding site in the emetic activity of SEA and the first identification of a specific carboxy-terminal residue which plays a role in the emetic activity of SEA. The results reported in this paper are consistent with earlier findings that activity in a murine T-cell proliferation assay is not predictive of emetic

activity in primates and add to previous evidence indicating that the two activities are not clearly linked.

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REFERENCES

1. **Abrahmsen, L., M. Dohlsten, S. Segren, P. Bjork, E. Jonsson, and T. Kalland.** 1995. Characterization of two distinct MHC class II binding sites in the superantigen staphylococcal enterotoxin A. EMBO J. **14:**2978–2986.
2. **Alber, G., D. K. Hammer, and B. Fleischer.** 1990. Relationship between enterotoxic and T lymphocyte-stimulating activity of staphylococcal enterotoxin B. J. Immunol. **144:**4501–4506.
3. **Bavari, S., and R. G. Ulrich.** 1995. Staphylococcal enterotoxin A and toxic shock syndrome toxin compete with CD4 for human major histocompatibility complex class II binding. Infect. Immun. **63:**423.
4. **Bayles, K. W., and J. J. Iandolo.** 1989. Genetic and molecular analysis of the gene encoding staphylococcal enterotoxin D. J. Bacteriol. **171:**4799–4806.
5. **Bergdoll, M. S.** 1983. Enterotoxins, p. 559–598. *In* C. S. F. Easmon and C. Adlam (ed.)., Staphylococci and staphylococcal infections. Academic Press, New York.
6. **Betley, M. J., D. W. Borst, and L. B. Regassa.** 1992. Staphylococcal enterotoxins, toxic shock syndrome toxin and streptococcal pyrogenic exotoxins: a comparative study of their molecular biology. Chem. Immunol. **55:**1–35.
7. **Betley, M. J., and J. J. Mekalanos.** 1988. Nucleotide sequence of the type A staphylococcal enterotoxin gene. J. Bacteriol. **170:**34–41.
8. **Chintagumpala, M. M., J. A. Mollick, and R. R. Rich.** 1991. Staphylococcal toxins bind to different sites on the HLA-DR. J. Immunol. **147:**3876–3881.
9. **Dellabonna, P., J. Peccoud, J. Kappler, P. Marrack, C. Benoist, and D. Mathis.** 1990. Superantigens interact with MHC class II molecules outside of the antigen groove. Cell **62:**1115–1121.
10. **Fraser, J. D.** 1989. High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR. Nature (London) **339:**221–223.
11. **Fraser, J. D., S. Lowe, M. J. Irwin, N. R. J. Gascoigne, and K. R. Hudson.** 1993. Structural model of staphylococcal enterotoxin A interaction with MHC class II antigens, p. 7–29. *In* B. Huber and E. Palmer (ed.), Superantigens: a pathogen's view of the immune system. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
12. **Fraser, J. D., R. G. Urban, J. L. Strominger, and H. Robinson.** 1992. Zinc regulates the function of two superantigens. Proc. Natl. Acad. Sci. USA **89:** 5507–5511.
13. **Grossman, D., J. G. Lamphear, J. A. Mollick, M. J. Betley, and R. R. Rich.** 1992. Dual roles for class II major histocompatibility complex molecules in staphylococcal enterotoxin-induced cytokine production and in vivo toxicity. Infect. Immun. **60:**5190–5196.
14. **Harris, T. O., and M. J. Betley.** 1995. Biological activities of staphylococcal enterotoxin type A mutants with N-terminal substitutions. Infect. Immun. **63:** 2133–2140.
15. **Harris, T. O., D. Grossman, J. W. Kappler, P. Marrack, R. R. Rich, and M. J. Betley.** 1993. Lack of complete correlation between emetic and T-cell stimulatory activities of staphylococcal enterotoxins. Infect. Immun. **61:** 3175–3183.
16. **Harris, T. O., W. O. Hufnagle, and M. J. Betley.** 1993. Staphylococcal enterotoxin A internal deletion mutants examined for serological activity and induction of T-cell proliferation. Infect. Immun. **61:**2059–2068.
17. **Hayball, J. D., J. H. Robinson, R. E. O'Hehir, A. Verhoef, J. R. Lame, and R. A. Lake.** 1993. Identification of two binding sites in staphylococcal enterotoxin B that confer specificity for TCR V β gene products. Int. Immunol. **6:**199–211.
18. **Hedlund, G., M. Dohlsten, T. Herrmann, G. Buell, P. A. Lando, S. Segren, J. Schrimsher, H. R. MacDonald, H. O. Sjogren, and T. Kalland.** 1991. A recombinant C-terminal fragment of staphylococcal enterotoxin A binds to human MHC class II products but does not activate T cells. J. Immunol. **147:**4082–4085.
19. **Herman, A., N. Labrecque, J. Thibodeau, P. Marrack, J. Kappler, and R. Sekaly.** 1991. Identification of the staphylococcal enterotoxin A superantigen binding site in the β1 domain of the human histocompatibility antigen HLA-DR. Proc. Natl. Acad. Sci. USA **88:**9954–9958.

19a. **Hufnagle, W., and M. Betley.** Unpublished results.

20. **Hufnagle, W. O., M. T. Tremaine, and M. J. Betley.** 1991. The carboxyl-terminal region of staphylococcal enterotoxin A is required for a fully active molecule. Infect. Immun. **59:**2126–2134.
21. **Iorandescu, S.** 1975. Recombinant plasmid obtained from two different compatible staphylococcal plasmids. J. Bacteriol. **24:**597–601.
22. **Jardetzky, T. S., J. H. Brown, J. C. Gorga, L. J. Stern, R. G. Urban, Y. Chi, Cl. Stauffacher, J. L. Strominger, and D. C. Wiley.** 1994. Three dimensional structure of a human class II histocompatibility molecule complexed with superantigen. Nature (London) **368:**711–718.
23. **Jones, C. L., and S. A. Khan.** 1986. Nucleotide sequence of the enterotoxin B gene from *Staphylococcus aureus*. J. Bacteriol. **166:**29–33.
24. **Kappler, J. W., A. Herman, J. Clements, and P. Marrack.** 1992. Mutations defining functional regions of the superantigen staphylococcal enterotoxin B. J. Exp. Med. **175:**387–396.
25. **Kim, J., R. G. Urgan, J. L. Strominger, and D. C. Wiley.** 1994. Toxic shock syndrome toxin-1 complexed with a class II major histocompatibility molecule HLA-DR1. Science **266:**1870–1874.
26. **Kreisworth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome toxin exotoin structural gene is not detectably transmitted by a prophage. Nature (London) **305:**709–712.
27. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-directed mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
28. **Mahanam, W., R. Al-Daccak, C. Leveille, J. Valet, J. Hebert, M. Ouellette, and W. Mourad.** 1995. A natural mutation of the amino acid residue at position 60 destroys staphylococcal enterotoxin A murine T-cell mitogenicity. Infect. Immun. **63:**2826–2832.
29. **Marrack, P., and J. Kappler.** 1990. The staphylococcal enterotoxins and their relatives. Science **248:**705–717.
30. **Novick, R. P.** 1963. Analysis of by transduction of mutations affecting penicillinase formation in Staphylococcus aureus. J. Gen. Microbiol. **33:**121–136.
31. **Patel, A. H., T. J. Foster, and P. A. Pattee.** 1989. Physical and genetic mapping of the protein A gene in the chromosome of *Staphylococcus aureus* 3825-4. J. Gen. Microbiol. **135:**1799–1807.
32. **Reck, B., P. H. Scheuber, W. Londong, B. Sailer-Kramer, K. Bartsch, and D. K. Hammer.** 1988. Protection against the staphylococcal enterotoxin-induced intestinal disorder in the monkey by anti-idiotypic antibodies. Proc. Natl. Acad. Sci. USA **85:**3170–3174.
33. **Ren, K., J. Bannan, V. Pancholi, A. Cheung, J. Robbins, V. Fischetti, and J. Zabriskie.** 1994. Characterization and biological properties of new staphylococcal exotoxin. J. Exp. Med. **180:**1675–1683.
34. **Robbins, R., S. Gould, and M. S. Bergdoll.** 1974. Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. Appl. Microbiol. **28:**946–950.
35. **Rosenberg, S. A., M. T. Lotze, L. M. Muul, A. E. Chang, F. P. Avis, S. Leitman, W. M. Linehan, C. N. Robertson, R. E. Lee, J. T. Rubin, C. A. Sepp, C. G. Simpson, and D. E. White.** 1987. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. N. Engl. J. Med. **316:** 889–897.
36. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **12:**5436–5467.
37. **Schad, E. M., I. Zaitseva, V. N., M. Dohlsten, T. Kalland, P. M. Schlievert, D. H. Ohlendorf, and L. A. Svensson.** 1995. Crystal structure of the superantigen staphylococcal enterotoxin type A. EMBO J. **14:**3292–3301.
38. **Scheuber, P. H., J. R. Golecki, B. Kickhofen, D. Scheel, G. Beck, and D. K. Hammer.** 1985. Skin reactivity of unsensitized monkeys upon challenge with staphylococcal enterotoxin B: a new approach for investigating the site of toxin action. Infect. Immun. **50:**869–876.
39. **Singh, B. R., and M. J. Betley.** 1989. Comparative structural analysis of staphylococcal enterotoxins A and E. J. Biol. Chem. **264:**4404–4411.
40. **Stelma, G. N., Jr., and M. S. Bergdoll.** 1982. Inactivation of staphylococcal enterotoxin A by chemical modification. Biochem. Biophys. Res. Commun. **105:**121–126.
41. **Su, Y., and A. C. Wong.** 1995. Identification and purification of a new staphylococcal enterotoxin, H. Appl. Environ. Microbiol. **61:**1438.
42. **Swaminathan, S., W. Furey, J. Pletcher, and M. Sax.** 1992. Crystal structure of staphylococcal enterotoxin B, a superantigen. Nature (London) **359:**801–805.

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