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Lack of Complete Correlation between Emetic and T-Cell-Stimulatory Activities of Staphylococcal Enterotoxins†

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This study examined the emetic activity of several staphylococcal enterotoxin type A and B (SEA and SEB, respectively) mutants that had either one or two amino acid residue substitutions. New *sea* gene mutations were constructed by site-directed mutagenesis; gene products were obtained with glycine residues at position 25, 47, 48, 81, 85, or 86 of mature SEA. Culture supernatants from *Staphylococcus aureus* RN4220, or derivatives containing either *sea* or a *sea* mutation, were analyzed for the ability to stimulate proliferation of murine splenocytes, as determined by incorporation of [^{3}H]thymidine. Culture supernatants containing SEA-N25G (a SEA mutant with a substitution of glycine for the asparagine residue at position 25), SEA-F47G, or SEA-L48G did not stimulate T-cell proliferation, unlike supernatants containing the other substitution mutants. Purified preparations of SEA-N25G had weak activity and those of SEA-F47G and SEA-L48G had essentially no activity in the T-cell proliferation assay. All mutants except SEA-V85G, which was degraded by monkey stomach lavage fluid in vitro, were tested for emetic activity. SEA-C106A and two SEB mutants, SEB-D9N/N23D and SEB-F44S (previously referred to as BR-257 and BR-358, respectively), whose construction and altered immunological properties have been reported previously, were also tested in the emetic assay. Each mutant was initially administered intragastrically at doses of 75 to 100 μg per animal; if none of the animals responded, the dose was increased four- to fivefold. SEA-F47G, SEA-C106A, and SEB-D9N/N23D were the only mutants that did not induce vomiting at either dose tested; these three mutants had reduced immunological activity. However, there was not a perfect correlation between immunological and emetic activities; SEA-L48G and SEB-F44S retained emetic activity, although they had essentially no T-cell-stimulatory activity. These studies suggest that these two activities can be dissociated.

The staphylococcal enterotoxins (SEs) are emetic toxins that cause staphylococcal food poisoning syndrome (4). Five major serological types of SEs, referred to as A through E (SEA through SEE), have been characterized. The genes encoding each SE type have been cloned, and their nucleotide and derived amino acid sequences have been determined (3, 7, 8, 11, 12, 17, 18, 26, 34, 48). On the basis of nucleotide sequence comparisons, the genes can be separated into two main groups: one is composed of *sea*, *sed*, and *see*, and the other includes *seb* and *sec*. *sea* and *seb* can be considered representatives of each group. The molecular weights of SEA and SEB are 27,100 and 28,336, respectively. SEA and SEB share 34% amino acid sequence identity (6). The three-dimensional structure of SEB has been reported recently (59).

The SEs share a number of other activities; they are pyrogenic, suppress immunoglobulin secretion, enhance endotoxic shock, and induce cytokine production (e.g., interleukin-1, tumor necrosis factor, and gamma interferon) and T-cell proliferation (13, 15, 16, 19, 30, 31, 33, 38, 45, 55, 58). On the basis of their unusually strong yet specific reactivity with T cells, the SEs belong to a family of substances referred to as superantigens (reviewed in references 25, 32, 40, and 42).

Little is known about the relationship between the structure of the SEs and their emetic activity. Analysis of SEC1 fragments derived from trypsin hydrolysis has demonstrated that intravenous administration of the 22-kDa C-terminal tryptic fragment of SEC1 induces diarrhea, but not vomiting, in rhesus and cynomolgus monkeys (56). It is not known whether vomiting and diarrhea are manifestations of the same SE action. Carboxymethylation of histidine residues of SEA or SEB renders them nonemetic by the intragastric route of challenge (49, 53, 57). In addition, it has been demonstrated that the native conformation of SEA is required for full emetic activity when SEA is administered intragastrically (27).

A great deal of work has been directed towards understanding how the structure of the SEs relates to superantigenic activity. Two groups of investigators have analyzed SEC1 fragments derived from trypsin hydrolysis, with different results; one group found that the 6.5-kDa N-terminal tryptic fragment weakly induces T-cell proliferation, whereas a different group of investigators demonstrated that the 22-kDa C-terminal tryptic fragment induces T-cell proliferation at levels essentially comparable to those induced by native SEC1 (10, 56). Carboxymethylation of SEB apparently does not affect its T-cell-proliferative activities (1). The role of the SE's disulfide loop has been examined by both

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TABLE 1. Bacterial strains, plasmids, and phage used

Strain, phage, or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
Strain		
JF626	Ap^s Sea^-	8
XL-1	Sea^- Seb^-	Stratagene, La Jolla, Calif.
Plasmid		
pGEM-7Zf(+)	Ap^r	Promega Corp., Madison, Wis.
pMJB3	pBR322 derivative with *sea* insert	7
pMJB305	pGEM-7Zf(+) derivative lacking *SacI-NsiI* sites in multiple cloning region	24
pMJB263	pMJB305 with 1.4-kb *BglI-Hin*dIII containing *sea-1364*	This work
pMV2	pBluescript SK^- (Stratagene) derivative encoding SEA	23
pDG13	Derivative of pMV2 encoding SEA-C106A	23
pSEB2	pTZ18R (Pharmacia Fine Chemicals, Piscataway, N.J.) containing *seb*	35
pBR-257	pTZ18R derivative containing *seb* with mutations (which encodes SEB-D9N/N23D)	35
pBR-358	pTZ18R derivative containing *seb* with mutations (which encodes SEB-F44S)	35
Phage		
M13mp11		41
S. aureus		
Strain		
RN4220	Sea^-	36
ISP2073	Sea^- Spa^-	46
Plasmid		
pC194	Cm^r	28
pMJB273	pMV2 ligated to pC194	22
pMJB275	pDG13 ligated to pC194	22
Phage		
80α	Generalized transducing phage	43

[a] Ap, ampicillin; Sea^- and Seb^-, staphylococcal enterotoxin type A and B nonproducers, respectively; Cm, chloramphenicol; Spa^-, staphylococcal protein A-deficient strain.

chemical modification and site-directed mutagenesis (21–23). Site-directed mutagenesis of the cysteine residue at position 96 or 106 decreases the ability of SEA to induce T-cell proliferation by 100-fold without significantly affecting its binding to major histocompatibility complex (MHC) class II molecules (23). Analysis of SEA-SEE hybrid proteins has demonstrated that at least some of the residues involved in discrimination of Vβ specificity are located in the C-terminal region (29).

A number of studies with either synthetic peptides or recombinant polypeptides have indicated the importance of the N-terminal region of the SEs for T-cell proliferation (14, 20, 47). Random mutagenesis of *seb* has identified three N-terminal regions of SEB which are important for induction of T-cell stimulation (35).

This study is part of our long-term effort to identify residues of the SEs that are required for their emetic activity. Previously we have observed that mutant SEAs with deletions in either the C terminus or the N-terminal third are not as stable as native SEA (24, 27). This could explain the lack of detectable emetic activity seen in the mutants containing deletions in the C terminus (27). In an effort to obtain mutant SEs with stabilities similar to that of native SE, we constructed mutants with single-amino-acid substitutions. For this work, we concentrated on residues which are conserved among all SEs. These SEA mutants were examined in the monkey emetic assay and a murine T-cell proliferation assay. In addition, SEA-C106A (a mutant which has the cysteine residue at position 96 changed to alanine [23]) and SEB-F44S and SEB-D9N/N23D (previously referred to as BR-358 and BR-257, respectively [35]), whose construction and altered immunological properties have been reported, were tested for the ability to induce emesis. The finding that SEA-L48G and SEB-F44S were emetic despite having little (if any) superantigenic activity demonstrates that these two activities can be separated.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophage, and media. The bacterial strains, plasmids, and phage used in this study are listed in Table 1. Descriptions of the *sea* and *seb* mutations used in this study are given in Table 2. *Escherichia coli* and *Staphylococcus aureus* were propagated as described elsewhere (7). Culture conditions and media used for the transformation of *E. coli* and *S. aureus* have been described previously (27). *S. aureus* cultures were grown in 3% (wt/vol) N-Z-amine type A (Kraft, Inc., Norwich, N.Y.)–1% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) for 18 h at 37°C with aeration, and bacterial cells were pelleted by centrifugation at 7,000 rpm (Ivan Sorvall, Inc., Norwalk, Conn.) for 30 min at 4°C.

TABLE 2. Nomenclature and description of *sea* and *seb* mutations used

Allele	Description	Gene product designation	Reference or source
sea-1364	Encodes wild-type SEA	SEA	24
sea-1358	Encodes SEA with D60G[a] and codons 61–73 deleted	SEA-D60G/Δ61–73	24
sea-1365	Encodes SEA with N25G substitution	SEA-N25G	This work
sea-1370	Encodes SEA with F47G substitution	SEA-F47G	This work
sea-1371	Encodes SEA with L48G substitution	SEA-L48G	This work
sea-1367	Encodes SEA with K81G substitution	SEA-K81G	This work
sea-1368	Encodes SEA with V85G substitution	SEA-V85G	This work
sea-1369	Encodes SEA with D86G substitution	SEA-D86G	This work
ND[b]	Encodes SEA with C106A substitution	SEA-C106A	23
ND	Encodes SEB with D9N and N23D substitutions	SEB-D9N/N23D	35
ND	Encodes SEB with F44S substitution	SEB-F44S	35

[a] D60G denotes that the aspartic acid at position 60 of mature SEA has been replaced with glycine.
[b] ND, not designated.

Chemicals and enzymes. Chemicals and enzymes were purchased from the following sources: restriction enzymes and T4 DNA ligase from New England BioLabs, Inc. (Beverly, Mass.); Klenow fragment from Promega Corp. (Madison, Wis.); alkaline phosphatase from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); Geneclean kit from Bio 101, Inc. (La Jolla, Calif.); Sequenase kit from United States Biochemical (Cleveland, Ohio); Amberlite CG-50 resin from Sigma Chemical Co. (St. Louis, Mo.); CNBr-activated Sepharose 4B from Pharmacia LKB Biotechnology (Piscataway, N.J.).

Construction of mutations. *E. coli* plasmid and replicative forms of phage DNA were obtained by an alkaline lysis method (2). Staphylococcal plasmid DNA was prepared by $CsCl_2$-ethidium bromide dye-buoyant density centrifugation (44). Nucleotide sequences were determined by the dideoxy-chain termination method (51).

sea mutations were constructed with pMJB263, a pGEM-7Zf(+) derivative that contains *sea-1364* (24). This allele contains a number of engineered restriction enzyme sites which are unique to pMJB263 (Fig. 1A). The derived amino acid sequence of *sea-1364* is identical to that of wild-type *sea*. Therefore, SEA-1364 was used as the positive control and will be subsequently referred to as SEA.

Oligonucleotide-directed mutagenesis (37) was performed on an M13mp11 derivative that contained the 1-kb *Hin*cII-*Hin*dIII fragment of *sea-1364* in order to construct *sea-1369*, *sea-1370*, and *sea-1371*. The sequences of the oligonucleotides used were $5'_{321}$ aaaaaagtagGcttgtat 3' (*sea-1369*), $5'_{205}$ acgatcaaGGtttGcagca 3' (*sea-1370*), and $5'_{209}$ tcaatttGGG cagcatac 3' (*sea-1371*). The subscript indicates the 5' nucleotide in *sea* to which the oligonucleotide anneals, and the allele the oligonucleotide was used to create is indicated in parentheses. Lowercase letters are identical to the nucleotides in *sea*. Uppercase letters in italic type differ from those in *sea*; these mutations were chosen to result in the desired substitution and/or to facilitate identification of the desired mutation by destroying a restriction enzyme site. Replicative-form DNA isolated from plaques obtained from each type of mutagenesis reaction was digested with *Hin*cII and *Acc*I. The 300-bp *Hin*cII-*Acc*I fragments were gel isolated and ligated with *Hin*cII-*Acc*I-digested pMJB263 in order to form full-length mutant SEAs.

The other *sea* mutations were constructed by cassette mutagenesis, as described previously (27). The sequences of the oligonucleotide cassettes are depicted in Fig. 1B. The ligation mixtures described above were separately transformed into *E. coli* JF626 (39), and a desired clone of each type was identified by DNA sequence analysis (51). Each plasmid was then digested with *Hin*dIII and ligated with *Hin*dIII-linearized pC194. The resultant shuttle plasmids

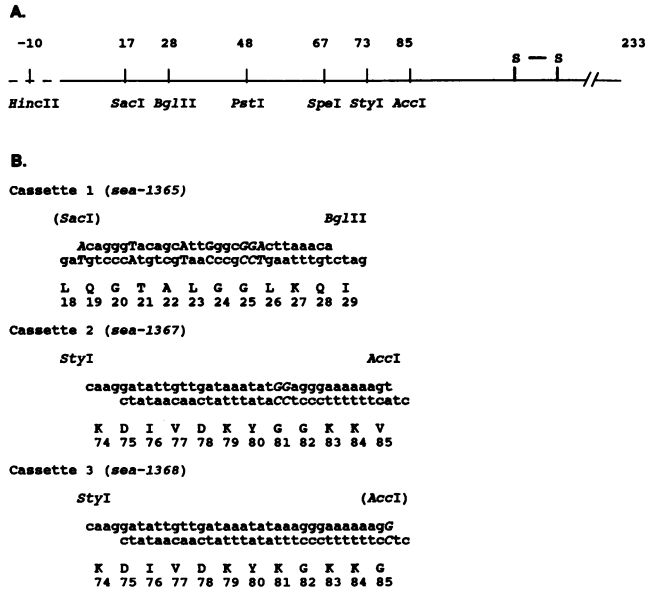

FIG. 1. (A) Diagram of *sea-1364* (not drawn to scale) indicating restriction enzyme sites unique to pMJB263, the plasmid used to obtain the mutant SEAs with substitutions in the N-terminal third. The numbers above the restriction enzyme sites correspond to the codons at the 5' end of the restriction site. The codons are numbered with the translation initiation codon as −24, and the codons corresponding to the N- and C-terminal residues of mature SEA are 1 and 233, respectively. The location of the disulfide loop formed by the cysteine residues at positions 96 and 106 is indicated. (B) Double-stranded oligonucleotide cassettes used for construction of the indicated *sea* mutations. The lowercase letters are identical to the nucleotides in *sea-1364*. Uppercase letters in italic type are mutations that resulted in an amino acid substitution and/or caused the loss of a restriction enzyme site which aided identification of the desired mutation; the loss of a restriction enzyme site is indicated by parentheses. Uppercase letters are silent mutations which were included to minimize stem-and-loop structure formation. Below the cassettes are the amino acid sequences; residue numbers correspond to those of mature SEA.

were then introduced into *S. aureus* RN4220 by electroporation, as described previously (27).

Serological assays. Western blot (immunoblot) analysis and gel double-diffusion assays were performed as described previously (27, 50). Gel double-diffusion-grade SEA and rabbit polyclonal anti-SEA and anti-SEB antisera were obtained from Merlin Bergdoll. The anti-SEA monoclonal antibodies (MAbs) C1 and E8 have been described previously (24). Normal rabbit serum was purchased from Sigma and was used for identifying non-SEA-related signals in Western blots. To quantify the amount of SEA or mutant SEAs in the culture supernatants, a twofold dilution series of each supernatant was prepared and loaded onto a polyacrylamide gel alongside known concentrations of SEA. The gels were analyzed by Western blotting, with MAb C1 as the primary antibody. The blots were then analyzed with a GS 300 densitometer (Hoefer Scientific Instruments, San Francisco, Calif.).

Stomach lavage fluid degradation assay. Monkey stomach lavage fluid was obtained from rhesus monkeys (*Macaca mulatta*) by performing a lavage with 15 ml of 0.9% (wt/vol) NaCl (Abbott Laboratories, North Chicago, Ill.) as described previously (27). Test samples (either culture supernatants or purified toxin preparations) were incubated with either phosphate-buffered saline (PBS; 0.02 M sodium phosphate containing 0.15 M NaCl, pH 7.4) or stomach lavage fluid. The samples were then analyzed by Western blotting.

Emetic assay. Staphylococcal culture supernatants were filter sterilized by passage through a 0.45-μm-pore-size filter (Nalge Co., Rochester, N.Y.) and stored on ice. When 500-μg amounts were to be administered, the supernatants were concentrated in Centriprep-10 concentrators (Amicon Division, W. R. Grace & Co., Beverly, Mass.). A concentrated culture supernatant of RN4220 (Sea^-) served as the negative control for these experiments. Purified preparations of SEs were diluted in PBS. Purified SE preparations and culture supernatants containing SEs were initially administered at doses of 75 and 100 μg per animal, respectively. When required, the dosage was increased four- to fivefold for a second group of two or three animals. Since quantification of purified preparations by absorbance determinations is more accurate than quantification of unpurified samples by Western analysis, a slightly smaller dosage of purified toxin was administered.

Rhesus monkeys (2 to 3 kg) were given samples via nasogastric intubation with a size 5 French infant feeding tube (American Pharmaseal Co., Valencia, Calif.) and were observed for 5 h. The emetic assays were performed in collaboration with the Regional Primate Research Center, Madison, Wis.

T-cell proliferation assay. T-cell proliferation assays were performed as described previously (27), using splenocytes from 6- to 8-week-old female BALB/c mice. Proliferation was determined by the incorporation of [^{3}H]thymidine. Culture supernatants containing SEA or mutant SEAs and their corresponding 10^{-1}, 10^{-2}, and 10^{-3} dilutions were tested; only the data from the last two dilutions were analyzed because the more concentrated samples were toxic to the splenocytes. Supernatant from *S. aureus* RN4220 (Sea^-) served as the negative control. Purified preparations of SEA or mutant SEAs were tested at 0.5, 0.05, and 0.005 μg per well. Statistical analysis of the data was by the *t* test, using a Minitab Release computer package (Minitab, Inc.). The level of significance is $P \leq 0.01$. Each sample was tested in triplicate in at least two separate assays. The data from one representative experiment are given.

Purification of SEs. Purification of SEA-N25G, SEA-F47G, SEA-L48G, and SEA (positive control) was accomplished first by removal of the SE from the culture supernatant with Amberlite CG-50, a carboxylic acid resin (52), and then by immunoaffinity chromatography. To avoid contamination with protein A, the plasmids containing *sea-1364*, *sea-1365*, *sea-1370*, and *sea-1371* were first introduced into the protein A-deficient strain ISP2073 (46) by generalized transduction with phage 80α, as described previously (27). In each purification, a 1-liter culture of the ISP2073 derivative producing the toxin of interest was grown overnight, and the bacteria were removed by centrifugation. The culture supernatant was adjusted to pH 5.6 and diluted to 5 liters with distilled water. CG-50 (20 g, dry weight) equilibrated previously in 5 mM sodium phosphate, pH 5.6, was then added to the diluted culture supernatant. The CG-50 was slowly stirred for about 2 h at room temperature and then allowed to settle out. The supernatant was decanted, and the slurry was packed into a column (2.7 by 23 cm; Bio-Rad Laboratories, Hercules, Calif.) to a bed height of 15 cm. The column was washed extensively with distilled water, connected to a UA-5 absorbance/fluorescence detector (Isco, Inc., Lincoln, Nebr.), and the toxin was eluted with 0.5 M sodium phosphate (pH 6.2) containing 0.5 M NaCl. The fractions containing SEA were pooled, dialyzed in PBS, and loaded onto a second column packed with CNBr-activated Sepharose 4B which had been coupled previously to MAb C1. To make this column, MAb C1 had been purified from ascites fluid with the MAbTrap G kit (Pharmacia). Then 18 mg of purified MAb C1 was coupled to 6 g of CNBr-activated Sepharose 4B as described by the manufacturer. The MAb C1-coupled Sepharose 4B resin was then packed into a column (2.7 by 23 cm; Bio-Rad Laboratories) to a bed height of 5 cm and washed with PBS. The column was stripped with 0.1 M glycine (pH 2.7) before each use, equilibrated with PBS, and connected to the absorbance/fluorescence detector.

The loaded column was washed extensively with PBS. The SEs were then eluted in 0.1 M glycine (pH 2.7) and neutralized with 0.5 M sodium bicarbonate. The SEA-containing fractions were dialyzed in PBS and concentrated with Centriprep-10 concentrators (Amicon). The amount of SEA or mutant SEA was quantified by determination of the A_{277}, using an extinction coefficient ($E_1^{1\%}{}_{cm}$) of 12.76 (54). Each toxin preparation was at least 90% pure, as determined by Coomassie brilliant blue staining of sodium dodecyl sulfate-polyacrylamide gels (data not shown), and each preparation was stored frozen.

SEA and SEA-C106A production by *E. coli* XL-1 derivatives containing pDG13 and pMV2, respectively, and by *S. aureus* RN4220 derivatives that contained pMJB273 and pMJB275, respectively, as well as their purification, has been described elsewhere (22, 23). Purified material was lyophilized and stored frozen.

Purification of SEB, SEB-F44S, and SEB-D9N/N23D produced by *E. coli* XL-1 derivatives was as described before (35). SEB and the mutant SEBs were quantified by absorbance determinations, and purified materials were stored frozen (35).

RESULTS

Examination of mutant SEAs by serological assays. For this study, several SEA mutants that had single amino acid substitutions at positions 25, 47, 48, 81, 85, and 86 were constructed. Evidence that the mutant SEAs were produced by *S. aureus* RN4220 was obtained by Western blot analysis

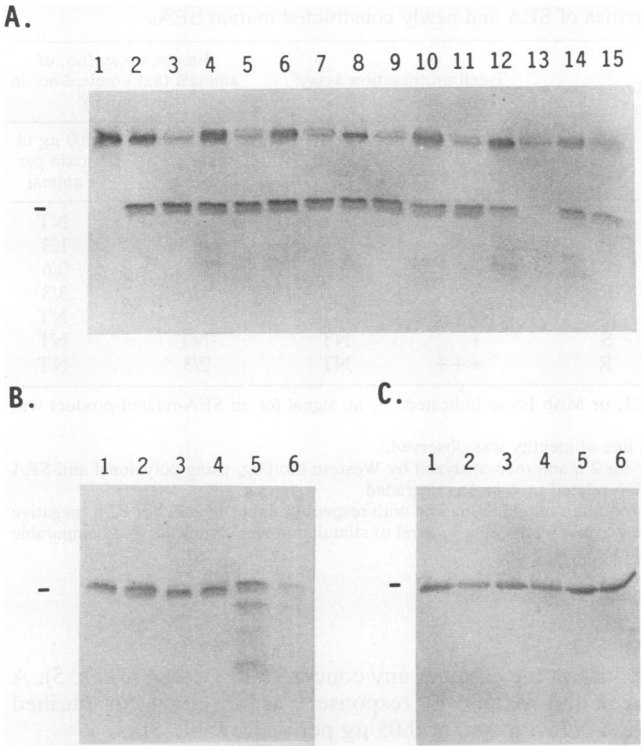

FIG. 2. Western blot analyses for detection of mutant SEs and comparison to their homologous wild-type SEs for susceptibility to degradation by monkey stomach lavage fluid in vitro. Blots A and B were reacted with polyclonal anti-SEA antiserum. Blot C was reacted with polyclonal anti-SEB antiserum. Prior to electrophoresis, all samples were incubated with either PBS (lanes 2, 4, 6, 8, 10, 12, and 14 on blot A and lanes 1, 3, and 5 on blots B and C) or monkey stomach lavage fluid (lanes 1, 3, 5, 7, 9, 11, 13, and 15 on blot A and lanes 2, 4, and 6 on blots B and C). The samples analyzed on blot A were from RN4220 (Sea$^-$; lane 1) or RN4220 derivatives producing SEA (lanes 2 and 3), SEA-N25G (lanes 4 and 5), SEA-F47G (lanes 6 and 7), SEA-L48G (lanes 8 and 9), SEA-K81G (lanes 10 and 11), SEA-V85G (lanes 12 and 13), and SEA-D86G (lanes 14 and 15). The samples analyzed on blot B were purified SEA (lanes 1 and 2), purified SEA-C106A (lanes 3 and 4), and culture supernatant of the RN4220 derivative producing SEA-D60G/Δ61-73 (lanes 5 and 6). The samples analyzed on blot C were purified SEB (lanes 1 and 2), purified SEB-F44S (lanes 3 and 4), and purified SEB-D9N/N23D (lanes 5 and 6). Samples on blots B and C were prepared and analyzed at the same time; therefore, degradation of SEA-D60G/Δ61-73 by the stomach lavage fluid served as the control for both blots. A mutant SEB that is degraded by monkey stomach lavage fluid has not been identified. The position of the signal corresponding to SEA or SEB is indicated by the dash on the left. The signals due to proteins with molecular weights larger than that of SEA were probably protein A; these signals, unlike those identified as SEA related, were seen in blots reacted only with normal rabbit serum (data not shown).

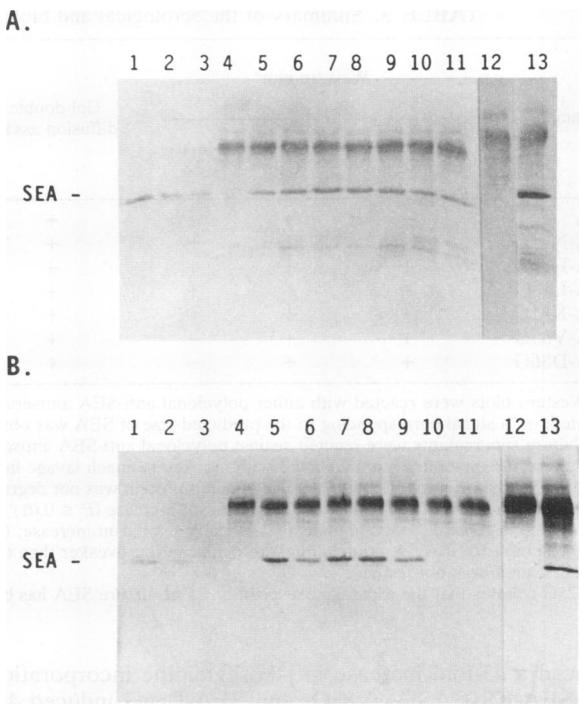

FIG. 3. Western blot analysis of purified SEA and culture supernatants from *S. aureus* RN4220 or RN4220 derivatives containing *sea* or a *sea* mutation. Blot A was reacted with MAb C1, and blot B was reacted with MAb E8. Lanes 1, 2, and 3 contained 4, 2, and 1 μg of purified SEA per ml, respectively. Samples were from RN4220 (lanes 4 and 12, the negative control) or RN4220 derivatives producing SEA (lane 5), SEA-N25G (lane 6), SEA-F47G (lane 7), SEA-L48G (lane 8), SEA-K81G (lane 9), SEA-V85G (lane 10), SEA-D86G (lane 11), or SEA-C106A (lane 13). The samples in lanes 12 and 13 were run on a separate blot. The position of the signal corresponding to wild-type SEA is indicated. The signals corresponding to proteins with molecular weights greater than that of SEA were probably protein A, as they were visible in blots reacted only with normal rabbit serum, unlike those which were SEA related (data not shown).

of culture supernatants, using polyclonal anti-SEA antiserum (Fig. 2). Samples from each RN4220 derivative producing a mutant SEA yielded a signal corresponding in size to SEA which was not present in samples of RN4220, the negative control (Fig. 2).

To determine whether any of the glycine substitutions dramatically altered the conformation of a SEA mutant compared with wild-type SEA, the SEA mutants were examined by the gel double-diffusion assay with polyclonal anti-SEA antiserum and by Western blotting with two anti-SEA MAbs. The epitopes for the two MAbs have been partially characterized previously (24). MAb C1's epitope is contained within residues 108 through 230. MAb E8 has a conformational epitope, but the residues making up its epitope have not been localized (24). All of the mutant SEAs reacted with MAb C1 (Fig. 3; Table 3). In contrast to the other mutant SEAs, SEA-V85G and SEA-D86G did not react detectably with MAb E8 (Fig. 3; Table 3). All SEA mutants formed a line of identity against polyclonal anti-SEA antiserum in the gel double-diffusion assay (data not shown and Table 3).

Induction of T-cell proliferation by mutant SEAs. The culture supernatants from the panel of RN4220 derivatives were tested for the ability to induce proliferation of murine splenocytes. The supernatants used were quantified by Western blotting with MAb C1, as described previously (24). Each mutant SEA (and wild-type SEA) was in a concentration of at least 8 μg/ml, as determined by densitometric analysis. Compared with an equivalent dilution from a culture supernatant of RN4220 (Sea$^-$), the culture supernatants containing SEA, SEA-K81G, SEA-V85G, and SEA-D86G induced a reproducible, statistically significant increase in incorporation of [^{3}H]thymidine (Table 4). SEA

TABLE 3. Summary of the serological and biological activities of SEA and newly constructed mutant SEAs

Sample	Western blot[a]			Gel double-diffusion assay[b]	Resistance to stomach lavage fluid[c]	T-cell proliferation assay[d]		Emetic assay (no. of animals that vomited/no. in test group)[e]	
	Polyclonal antiserum	MAb C1	MAb E8			Culture supernatant	Purified sample	100 μg of toxin per animal	500 μg of toxin per animal
SEA	+	+	+	+	R	+++	+++	2/3	NT
SEA-N25G[f]	+	+	+	+	R	−	+	0/3	1/3
SEA-F47G	+	+	+	+	R	−	−	0/3	0/6
SEA-L48G	+	+	+	+	R	−	−	1/6	3/3
SEA-K81G	+	+	+	+	R	+++	NT	2/3	NT
SEA-V85G	+	+	−	+	S	+	NT	NT	NT
SEA-D86G	+	+	−	+	R	+++	NT	2/3	NT

[a] Western blots were reacted with either polyclonal anti-SEA antiserum, MAb C1, or MAb E8 as indicated. −, no signal for an SEA-related product was detected; +, a signal corresponding to the predicted size of SEA was observed.

[b] Culture supernatants were reacted against polyclonal anti-SEA antiserum. +, a line of identity was observed.

[c] Culture supernatants were incubated with monkey stomach lavage fluid at 37°C for 2 h and then analyzed by Western blotting, using polyclonal anti-SEA antiserum as the primary antibody. R, SEA-related protein was not degraded; S, SEA-related protein was degraded.

[d] Samples that stimulated a statistically significant increase ($P \leq 0.01$) in the incorporation of [^{3}H]thymidine with respect to either RN4220 or PBS (negative controls). −, sample did not stimulate a statistically significant increase; NT, sample was not tested; +++, level of stimulation was significant and comparable to the response to SEA; +, stimulation was significant but weaker than the response to SEA.

[e] NT, sample was not tested.

[f] N25G denotes that the asparagine at position 25 of mature SEA has been replaced with a glycine residue.

induced a 45-fold increase in [^{3}H]thymidine incorporation, and SEA-K81G, SEA-V85G, and SEA-D86G induced 47-, 13-, and 50-fold increases in [^{3}H]thymidine uptake, respectively. Culture supernatants containing SEA-N25G, SEA-F47G, and SEA-L48G did not induce a statistically significant increase in the incorporation of [^{3}H]thymidine (Table 4).

Mutant SEAs that did not induce a significant T-cell response when tested with culture supernatants were purified by immunoaffinity chromatography, using MAb C1 coupled to Sepharose 4B. These purified preparations were tested for the ability to induce T-cell proliferation at 0.5, 0.05, and 0.005 μg per well. SEA purified in the same manner (the positive control) induced significant proliferation at all three concentrations (Table 5). Purified preparations of SEA-F47G and SEA-L48G did not cause a significant response in the assay at any concentration tested (Table 5). A weak but significant response was observed for purified SEA-N25G tested at 0.05 μg per well (Table 5).

TABLE 4. T-cell proliferation assay with culture supernatants from *S. aureus* RN4220 derivatives that express SEA or a mutant SEA[a]

Sample	Mean cpm ± SD (stimulation index)[b] for sample dilutions[c]	
	10^{-2}	10^{-3}
RN4220	549 ± 81 (1.0)	874 ± 462 (1.0)
SEA	**24,710**[d] ± 2,128 (45)	7,958 ± 4,006 (9.1)
SEA-N25G[e]	1,719 ± 691 (3.1)	890 ± 574 (1.0)
SEA-F47G	775 ± 347 (1.4)	1,614 ± 1,355 (1.8)
SEA-L48G	393 ± 130 (0.7)	489 ± 437 (0.5)
SEA-K81G	**25,848** ± 3,860 (47)	7,446 ± 3,602 (8.5)
SEA-V85G	**6,914** ± 522 (13)	1,020 ± 223 (1.2)
SEA-D86G	**27,486** ± 8,672 (50)	15,681 ± 8,277 (18)

[a] Results from one representative assay are given.

[b] The stimulation index of each test sample was determined by dividing the counts per minute for the test sample by those obtained with the corresponding dilution of the RN4220 sample.

[c] Samples were assayed in triplicate.

[d] Data from samples that induced a statistically significant difference ($P \leq 0.01$) in the uptake of [^{3}H]thymidine with respect to the negative control (RN4220) are in boldface type.

[e] N25G denotes that the asparagine at position 25 of mature SEA has been replaced with a glycine residue.

Examination of emetic activity of newly constructed mutant SEAs. Before a mutant SEA was tested for emesis, it was screened for resistance to degradation by monkey stomach lavage fluid in vitro (Fig. 2). Of the mutant SEAs, only SEA-V85G was degraded by monkey stomach lavage fluid. Therefore, SEA-V85G was not tested in the emetic assay.

For the emetic assays, the amount of SEA or a mutant SEA in the culture supernatant was quantified by Western blot analysis with MAb C1 (data not shown). When SEA, SEA-K81G, or SEA-D86G was administered at a dose of 100 μg per animal, two of the three test animals in each group vomited (Table 3). In the initial assay, only one animal that received 100 μg of SEA-L48G vomited; the other two animals did not appear ill. None of three additional animals given 100 μg of SEA-L48G showed any signs of illness. Therefore, the dose of SEA-L48G was increased to 500 μg per animal; at the higher dose, all three animals experienced emesis (Table 3). SEA-N25G did not cause emesis at a concentration of 100 μg per animal. However, one of three animals that received 500 μg of SEA-N25G did vomit, and the other two animals looked ill; both had diarrhea and one experienced retching (Table 3). None of the animals that received SEA-F47G at a dose of 100 or 500 μg per animal exhibited any signs of discomfort. As a negative control, three animals each received a concentrated RN4220 culture supernatant equivalent to the amount that would contain 500 μg of a mutant SEA. All three animals that received the negative control sample appeared healthy throughout the test period (Table 3).

Examination of emetic activity of SEA-C106A, SEB-D9N/N23D, and SEB-F44S. The emetic assays testing mutant SEBs and the initial experiment with SEA-C106A used material produced from *E. coli*. The Primate Research Center requires that SEs from *E. coli* be purified for the emetic assay. Positive controls of the homologous SE isolated by the same procedure used to purify the mutant SE were included in the assays. SEA-C106A has been shown previously to be resistant to digestion with trypsin (23).

TABLE 5. T-cell proliferation assay with purified wild-type and mutant SEAs[a]

Sample	Mean cpm ± SD (stimulation index)[b] for samples containing indicated amt of SEA or mutant SEA[c]		
	0.5 μg per well	0.05 μg per well	0.005 μg per well
SEA	**28,635**[d] ± 6,657 (50)	**65,433** ± 2,658 (117)	**49,522** ± 2,242 (114)
SEA-N25G[e]	1,993 ± 2,236 (3.5)	**9,487** ± 3,543 (17)	1,332 ± 601 (3.0)
SEA-F47G	851 ± 621 (1.5)	1,661 ± 472 (2.9)	785 ± 899 (1.8)
SEA-L48G	702 ± 955 (1.2)	2,928 ± 1,828 (5.0)	609 ± 389 (1.4)

[a] Results from one representative assay are given.
[b] The stimulation index of each sample was determined by dividing the counts per minute for the test sample by the mean counts obtained with the corresponding dilution of PBS alone (which were 563, 556, and 434 cpm, left to right columns).
[c] Samples were tested in triplicate.
[d] Data from samples that induced a statistically significant difference ($P \leq 0.01$) in the incorporation of [^{3}H]thymidine with respect to the corresponding dilution of the negative control (PBS alone) are in boldface type.
[e] N25G denotes that the asparagine at position 25 of mature SEA has been replaced with a glycine residue.

SEA-C106A, SEB-D9N/N23D, and SEB-F44S were all resistant to degradation by monkey stomach lavage fluid (Fig. 2) and were therefore examined for emetic activity.

SEA-C106A did not induce emesis at a dose of either 75 or 375 μg per animal (Table 6). Unlike native SEB, neither mutant SEB induced emesis at a dose of 75 μg per animal (Table 6). At 300 μg per animal, SEB-F44S caused emesis. At 375 μg per animal, SEB-D9N/N23D did not induce vomiting; however, both animals appeared distressed and experienced severe body tremors (appeared to be chills), behavior we have not observed before.

DISCUSSION

This is the first study examining mutant SEs with substitutions of one or two residues for emetic activity. If a mutant SE did not cause emesis, then the original amino acid residue may be required because it either is part of the active site or is important for maintaining an active conformation. The native residues at positions 81 and 86 are not required for emesis because SEA-K81G and SEA-D86G have full emetic activity. SEB-F44S did not cause emesis at 75 μg per animal but did at 300 μg per animal (Table 6). SEA-N25G did not induce emesis at 100 μg per animal, but at 500 μg per animal it did induce emesis (Table 3). SEB-D9N/N23D, SEA-F47G, and SEA-C106A did not induce emesis in any of the animals tested. It is not clear for SEB-D9N/N23D whether one or both substitutions were responsible for the loss of emetic activity. SEA-V85G was not tested in the emetic assay, because it was degraded in vitro by monkey stomach lavage fluid. This result indicated that valine at position 85 was important for maintaining a native conformation and implied that SEA-V85G would be nonemetic because of degradation in vivo.

The SEA mutants fell into several classes based on T-cell-proliferative activity. Neither purified preparations nor culture supernatants containing SEA-F47G or SEA-L48G induced a significant T-cell response (Tables 4 and 5). Analysis of purified SEA-N25G in the T-cell proliferation assay demonstrated that, compared with wild-type SEA, this mutant had a reduced but significant ability to stimulate T cells (Table 5). Culture supernatants containing SEA-V85G caused a significant increase in the incorporation of [^{3}H]thymidine in the murine splenocyte assay, although it was less than the values obtained with culture supernatants containing wild-type SEA. The lower activity of SEA-V85G in culture supernatants may be due to the lower concentration of SEA-V85G compared with SEA or to valine at position 85 having a role in T-cell-proliferative activity. This difference in concentration was apparent in Western blots with either polyclonal anti-SEA antiserum or MAb C1. We chose not to purify and further characterize SEA-V85G because SEA-V85G was degraded by monkey stomach lavage fluid in vitro, which is suggestive of altered conformation.

Examination of the emetic and T-cell-stimulatory abilities of the mutant SEs should provide data about whether these two activities are related. If the emetic activity of SEs is not dependent on their ability to stimulate T-cell proliferation, then it should be possible to dissociate these two activities. If the emetic activity of SE is a direct consequence of its ability to stimulate T-cell proliferation, then all mutants defective in T-cell stimulation should be nonemetic. However, a mutant that is fully active in the T-cell proliferation assay in vitro could be nonemetic in vivo because of degradation.

Published data are consistent with the emetic and T-cell-proliferative activities of SEs being separate. Independent laboratories have demonstrated that carboxymethylated SEA or SEB is not emetic by an intragastric route of administration (49, 53, 57). In a separate report, carboxymethylated SEB was shown to induce proliferation of monkey peripheral blood cells (1). Another line of evidence suggesting that emesis is not due to superantigenic abilities is that careful examination of toxic shock syndrome toxin 1, another bacterial superantigen, has failed to show that it is an emetic toxin by either the intravenous or the intragastric route of administration (5). It is possible, however, that the inability of toxic shock syndrome toxin 1 to induce vomiting by the intragastric route is due to its sensitivity to pepsin degradation (5).

TABLE 6. Emetic activity of SEA-C106A, SEB-D9N/N23D, and SEB-F44S

Sample	Host[a]	No. of test animals experiencing emesis/no. tested		
		75 μg of SE	300 μg of SE	375 μg of SE
SEA	*E. coli*	2/3		
SEA-C106A[b]	*E. coli*	0/3		
SEA	*S. aureus*	2/3		
SEA-C106A	*S. aureus*			0/3
SEB	*E. coli*	2/2		
SEB-D9N/N23D	*E. coli*	0/2		0/2
SEB-F44S	*E. coli*	0/2	2/2	

[a] Bacterial species that produced the recombinant SE.
[b] C106A indicates that the cysteine residue at position 106 of mature SEA has been replaced with an alanine.

SEA-L48G and SEB-F44S are the first mutant SEs to be described that retained significant emetic activity but had essentially no immunological activity (Tables 3 to 6) (35). The emetic activity of SEA-L48G was not grossly different from that of wild-type SEA (Table 3). However, SEA-L48G did not induce T-cell proliferation of murine splenocytes, even at a concentration 100-fold greater than that required to see stimulation by wild-type SEA (Tables 4 and 5). Compared with wild-type SEB, the ability of the mutant SEB-F44S to bind to human DR-1-bearing cells and to stimulate interleukin-2 production by several Vβ-bearing T-cell lines is reduced at least 1,000- and 100-fold, respectively (35). These decreases did not correlate with the decreased emetic activity of SEB-F44S; although SEB-F44S did not induce emesis at 75 μg per animal, it did so when administered at a fourfold-higher dose. These results demonstrate that these immunological assays cannot be used to identify nonemetic SEs.

Several mutant SEs had reduced abilities to induce emesis and either murine T-cell proliferation or interleukin-2 production by T-cell hybridomas. In particular, SEB-D9N/N23D, SEA-C106A, and SEA-F47G did not induce emesis in any of the animals tested. SEB-D9N/N23D does not induce interleukin-2 expression (35), and SEA-C106A and SEA-F47G do not induce murine T-cell proliferation (23) (Tables 4 and 5). If emesis does not depend on T-cell proliferation, mutant SEs defective in both activation of T-cell proliferation and emetic activity could arise as a result of a residue being critical for two different activities.

If there is a correlation between human and monkey systems, our data are consistent with the conclusion that MHC class II binding is not sufficient for emesis. SEA-C106A and SEB-D9N/N23D bind well to human HLA-DR1, but neither induced emesis at the doses tested. Alternatively, SEB-F44S, which has a 1,000-fold reduction in MHC class II binding, was emetic at 300 μg per animal, implying that binding to MHC class II by itself does not invoke the emetic response.

There were two pairs of SEA-SEB mutants that had substitutions at corresponding residues. For one pair, there was a difference in emetic activities. SEB-F44S caused emesis at 300 μg per animal, in contrast to SEA-F47G (position 47 of SEA corresponds to position 44 of SEB [9]), which did not induce a response at 500 μg per animal. Perhaps the difference in emetic activities of these two mutants was due to the differences in their substitutions. For the other pair (SEB-D9N/N23D and SEA-N25G; position 25 of SEA corresponds to position 23 of SEB [9]), it is not clear whether there is a difference in their emetic activities. SEB-D9N/N23D did not cause emesis at 375 μg per animal, whereas SEA-N25G did cause emesis at 500 μg per animal. SEB-D9N/N23D may have a lower emetic activity than SEA-N25G because of its additional mutation at position 9. Alternatively, SEB-D9N/N23D might be emetic at a dose of 500 μg per animal. We did not administer a higher dose of SEB-D9N/N23D, as the three test animals that received doses of 300 μg appeared very ill, even though there was no evidence of gastrointestinal upset (e.g., vomiting, diarrhea, or retching). It was not clear whether these nongastrointestinal symptoms were due to SEB-D9N/N23D or to a contaminating substance from *E. coli* that copurified with the mutant SEB.

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