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# Biological Activities of Staphylococcal Enterotoxin Type A Mutants with N-Terminal Substitutions†‡

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**The purpose of this study was to examine the importance of certain N-terminal amino acid residues of staphylococcal enterotoxin type A (SEA) for biological activity. The results confirm our previous observation that Asn-25, Phe-47, and Leu-48 are important for SEA's emetic and superantigen activities. Substitutions at six other sites (Leu-12, Lys-14, Ser-16, Asp-45, Gln-46, and Thr-51) did not reveal any additional residues required for biological activity. Mutant SEAs with substitutions at 25, 47, or 48 all had decreased T-cell stimulatory activity, with the mutants at position 47 being the most defective. Results of a competition assay for binding to the major histocompatibility complex (MHC) class II-expressing cell line Raji suggested that the decreased superantigen activities of the mutants with substitutions at positions 47 and 48 are due to poor interactions with MHC class II molecules, whereas the defects of the mutants at position 25 are a consequence of faulty interactions with T-cell receptors. With respect to emetic activity in rhesus monkeys, the mutants at position 25 or 48 exhibited decreased but significant activity. Interestingly, the two mutants at position 47 had different emetic activities; SEA-F47G was nonemetic when administered intragastrically at 500 μg per animal, whereas SEA-F47S was emetic at this dosage. Since the mutants at position 47 were equally defective for superantigen activity, this further supports our previous suggestion of an incomplete correlation between SEA's emetic and superantigen activities.**

The staphylococcal enterotoxins (SEs) are extracellular proteins that cause staphylococcal food poisoning syndrome (2). Five major serological types of SEs, referred to as A through E (SEA through SEE), have been characterized to date. The predicted amino acid sequence identity of the SEs ranges from 29 to 81%; SEA and SEE are the most similar, followed by SEB and SEC (for a review, see reference 4). In addition to their role in food poisoning syndrome, the SEs have other biological activities; they are pyrogenic and immunosuppressive, enhance endotoxic shock, induce production of various cytokines, and induce T-cell proliferation (for a review, see reference 7). The SEs are considered prototypical bacterial superantigens. Superantigens stimulate essentially all T cells bearing certain Vβs in their T-cell receptor (TCR), with little involvement from the other TCR variable components. Like conventional antigens, superantigens require presentation by major histocompatibility complex (MHC) class II-bearing accessory cells, but the interaction occurs outside of the conventional antigen binding groove and does not require processing (for a review, see reference 30).

Little is known about how the structure of the SEs relates to their emetic activity. It has been suggested that the toxicity of the SEs is a consequence of the large amounts of cytokines released following SE-induced T-cell activation (22, 23). In support of this model, cancer patients receiving high doses of interleukin-2 (IL-2) often experience side effects mimicking staphylococcal food poisoning, such as vomiting, diarrhea, and nausea (42). Other studies suggest that the emetic and superantigen activities of the SEs are separable. Carboxymethylation of the histidine residues of SEA or SEB abrogates emetic activity when administered intragastrically (40, 44, 50), yet carboxymethylated SEB induces proliferation of monkey peripheral blood cells (1). The intravenous administration of the 22-kDa C-terminal tryptic fragment of SEC1 induces diarrhea, but not vomiting, in rhesus and cynomolgus monkeys (49). However, there are conflicting reports regarding the superantigen capabilities of the SEC fragments obtained from limited tryptic hydrolysis (8, 49). Toxic shock syndrome toxin-1 (TSST-1) is also a superantigen, but in contrast to the SEs, TSST-1 is not emetic by either the intravenous or intragastric route of administration (3). It is possible that TSST-1's inability to induce vomiting by the intragastric route is due to its sensitivity to pepsin degradation (3).

A number of reports suggest several regions of the SEs important for superantigen activity. Deletions in the N- and C-terminal portions of SEA, SEB, and SEC result in loss of T-cell stimulatory activity and in decreased resistance to proteases (17, 19, 21, 34). Site-directed mutagenesis of SEA's disulfide loop indicates that the disulfide loop is important for interactions with the TCR (15). SEA/SEE hybrid proteins indicate that specific C-terminal residues influence Vβ specificity (20, 25, 35). A chimeric fusion of SEB residues 1 to 138 to protein A induces T-cell proliferation (9). Synthetic peptides corresponding to N-terminal and central regions of SEA and SEB inhibit SE-induced T-cell proliferation and binding to MHC class II-bearing target cells (14, 27, 38, 39). Two synthetic peptides corresponding to central regions of SEC1 stimulate human T cells (19). Random mutagenesis of *seb* demonstrates that three N-terminal regions of SEB are important for induction of T-cell stimulation (28).

The SEB three-dimensional crystal structure (51) clarifies some of the contradictions in the literature. Residues from both the N- and C-terminal portions of SEB are involved in the TCR and MHC class II binding sites (51). The crystal structure of SEB complexed to HLA-DR1 further elucidates how SEB functions as a superantigen (26). No major conformational changes occur in either MHC class II or SEB upon complex formation. A model involving unconventional contacts be-

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TABLE 1. Bacterial strains, plasmids, and phages used in this work

| Strain, phage, or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| *E. coli* | | |
| Strain JF626 | $Ap^s$ Sea | 6 |
| Plasmids | | |
| pGEM-7Zf (+) | $Ap^r$ | Promega Corp. |
| pMJB305 | pGEM-7Zf (+) derivative lacking *Sac*I/*Nsi*I sites in multiple cloning region | 17 |
| pMJB214 | pMJB305 with 1.4-kb *Bgl*I-*Hin*dIII *sea-1351* fragment inserted | 17 |
| pMJB263 | pMJB305 with 1.4-kb *Bgl*I-*Hin*dIII *sea-1364* fragment inserted | 16 |
| pMJB342 | pMJB305 with 1.4-kb *Bgl*I-*Hin*dIII *sea-1379* fragment inserted | This work |
| pMJB343 | pMJB305 with 1.4-kb *Bgl*I-*Hin*dIII *sea-1380* fragment inserted | This work |
| Phage M13mp11 | | 33 |
| *S. aureus* | | |
| Strains | | |
| RN4220 | $Sea^-$ | 31 |
| ISP2073 | $Sea^-$ $Spa^-$ | 37 |
| Plasmid pC194 | $Cm^r$ | 24 |
| Phage 80α | Generalized transducing phage | 36 |

[a] Ap, ampicillin; Cm, chloramphenicol; $Spa^-$, staphylococcal protein A-deficient strain.

tween MHC class II and TCR explains ternary complex formation (26).

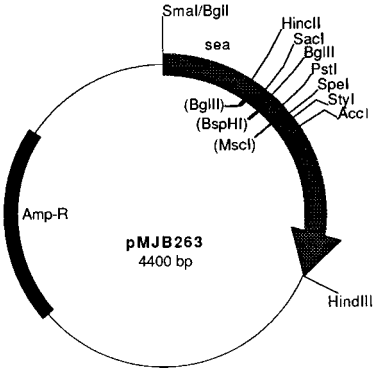
We previously identified residues 25, 47, and 48 of SEA as being important for SEA's emetic and T-cell stimulatory activities (16). In this work, different substitutions were made at these positions and at additional residues which are either completely or highly conserved among the SEs. The mutant SEAs were examined for emetic activity in rhesus monkeys and for the ability to induce proliferation and cytokine production in murine splenocytes. The mutant SEAs defective in T-cell activation were tested for the ability to compete with biotinylated SEA for binding to the human MHC class II-bearing cell line Raji to see if their defects were a consequence of impaired interactions with MHC class II molecules.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, bacteriophage, and media.** The bacterial strains, plasmids, and phages used in this study are listed in Table 1. Descriptions of the *sea* mutations used in this study are listed in Fig. 1. *Escherichia coli* and *Staphylococcus aureus* were grown as described in reference 5. Culture conditions and media used for the transformation of *E. coli* and *S. aureus* have been described elsewhere (21). *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 16 to 18 h at 37°C with aeration (200 rpm), and bacterial cells were pelleted by centrifugation at 3,820 × *g* (Ivan Sorvall, Inc., Norwalk, Conn.) for 30 min at 4°C. The supernatants were filter sterilized by passage through Nalgene filters (0.45-μm pore size; Nalge Co., Rochester, N.Y.).

**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.); alkaline phosphatase from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); Sequenase kit from United States Biochemical (Cleveland, Ohio); CNBr-activated Sepharose 4B from Pharmacia LKB Biotechnology (Piscataway, N.J.); fluorescein isothiocyanate (FITC)-avidin from Sigma Chemical Co. (St. Louis, Mo.).

**Construction of mutations.** We have previously reported the construction of *sea-1351* and *sea-1364* by oligonucleotide-directed mutagenesis (16, 17). *sea-1351* and *sea-1364* are altered *sea* genes containing a number of mutations designed to




B.

*sea-1381* (encodes SEA-L12G) made using pMJB342
(*Bgl*II) *Sac*I
gat **GGA** cga aaa aag tct gag ct
cta **CCT** gct ttt ttc aga c
11D 12**G** 13R 14K 15K 16S 17E 18L

*sea-1382* (encodes SEA-K14G) made using pMJB342
*Bgl*II *Sac*I
gat Ctg cga **GG**a aag tct gag ct
ac gct **CC**t ttc aga c
11D 12L 13R 14**G** 15K 16S 17E 18L

*sea-1383* (encodes SEA-S16G) made using pMJB342
*Bgl*II *Sac*I
gat Ctg cga aaa aag **GGA** gag ct
ac gct ttt ttc **CCT** c
11D 12L 13R 14K 15K 16**G** 17E 18L

*sea-1390* (encodes SEA-N25A) made using pMJB263
(*Sac*I) *Bgl*II
**a** cag ggt aca gc**a** tt**g** ggc **GC**t ctt aaa ca
tc ga**t** gtc cc**a** tgt cg**t** aac ccg **CG**a gaa ttt gtc tag
17E 18L 19Q 20G 21T 22A 23L 24G 25**A** 26L 27K 28Q 29I

*sea-1394* (encodes SEA-K27A) made using pMJB263
(*Sac*I) *Bgl*II
**a** cag gga aca gct tta ggc aat ctt **GCT** ca
tc ga**t** gtc cct tgt cga aat ccg tta gaa **CGA** gtc tag
17E 18L 19Q 20G 21T 22A 23L 24G 25N 26L 27**A** 28Q 29I

*sea-1384* (encodes SEA-D45G) made using pMJB343
(*Bsp*HI) (*Pst*I)
caT g**GA** caa ttt Ttg ca
**CT** gtt aaa A
44H 45**G** 46Q 47F 48L 49Q

*sea-1386* (encodes SEA-Q46G) made using pMJB343
*Bsp*H1 (*Pst*I)
caT gat **GG**a ttt Ttg ca
ta **CC**t aaa A
44H 45D 46**G** 47F 48L 49Q

*sea-1385* (encodes SEA-F47S) made using pMJB343
*Bsp*H1 (*Pst*I)
caT gat caa **AG**t Ttg ca
ta gtt **TC**a A
44H 45D 46Q 47**S** 48L 49Q

*sea-1391* (encodes SEA-L48A) made using pMJB343
*Bsp*H1 (*Pst*I)
caT gat caa ttt **GCT** ca
ta gtt aaa **CGA** gt
44H 45D 46Q 47F 48**A** 49Q

*sea-1395* (encodes SEA-T51A) made using pMJB214
(*Pst*I) (*Msc*I)
A cat **GC**A ata ttg ttt aaa ggc ttt ttt ac**T** ga
ac gt**T** gta **CG**T tat aac aaa ttt ccg aaa aaa tg**A** ct
48L 49Q 50H 51**A** 52I 53L 54F 55K 56G 57F 58F 59T 60D

FIG. 1. (A) Map of pMJB263 (encoding *sea-1364*) indicating the unique restriction enzyme sites used to construct the mutant SEAs in this study. The restriction enzyme sites in parentheses indicate the approximate position of the restriction enzyme sites unique to *sea-1379*, *sea-1380*, and *sea-1351*. *sea-1379* lacks the *Bgl*II site contained on *sea-1364*. (B) Double-stranded oligonucleotide cassettes and plasmids used in the construction of the indicated *sea* alleles. The lowercase letters are identical to the nucleotides in *sea-1364*. Uppercase letters in bold type indicate the mutations resulting in the desired amino acid substitution. Uppercase letters represent the silent mutations which were introduced to generate *sea-1351*, *sea-1379*, or *sea-1380*. Lowercase letters in bold type represent silent mutations designed to minimize stem-loop formation or to facilitate screening; in the latter case, the loss of a restriction enzyme site is indicated by parentheses. The relevant amino acid sequence is shown below each cassette; residue numbers correspond to those of mature wild-type SEA.

incorporate unique restriction enzyme sites into *sea* in order to facilitate construction of additional *sea* mutations (Fig. 1A). For the construction of some of the mutant SEAs, we used two variants of *sea-1364*, *sea-1379* and *sea-1380*. *sea-1379* was constructed by performing site-directed mutagenesis on the same altered *sea* originally used to create *sea-1364*, using the mutagenic oligonucleotide 5′-gaaaaagatCtgcgaaaaaagtc-3′ (*Bgl*II:99). *sea-1380* was constructed by performing an additional round of site-directed mutagenesis on *sea-1364*, using the mutagenic oligonucleotide 5′-gagagtcaTgatcaatttctg-3′ (*Bsp*HI:198). For each oligonucleotide, the lowercase letters indicate nucleotides identical to those in *sea-1364*, the uppercase letter is the mutation resulting in the incorporation of the indicated restriction enzyme site, and the number indicates the 5′ nucleotide in *sea* that the oligonucleotide annealed to. The resultant *Bgl*II site in *sea-1379* is unique; the altered *sea* used in its construction does not contain *sea-1364*'s *Bgl*II site (Fig. 1A).

All of the mutations in *sea-1364*, *sea-1379*, and *sea-1380* are silent mutations. One of the mutations in *sea-1351* (used to construct SEA-T51A) results in the substitution of Asp-60 with a glycine residue (17). However, the cassette used to create SEA-T51A restored residue 60 to aspartic acid (Fig. 1B). Cassette mutagenesis was performed on pMJB214, pMJB263, pMJB342, and pMJB343 (containing *sea-1351*, *sea-1364*, *sea-1379*, and *sea-1380*, respectively) in order to create the *sea* mutations encoding the various substitution mutant SEAs, as described previously (21). The sequences of the oligonucleotide cassettes and the plasmid used in each mutagenesis reaction are indicated in Fig. 1B. Each ligation mixture was separately transformed into *E. coli* JF626, and the desired constructs were identified by DNA sequence analysis (43). Each *E. coli* plasmid containing *sea* or a *sea* mutation was then digested with *Hin*dIII and ligated with *Hin*dIII linearized pC194. The resultant shuttle plasmids were introduced into *S. aureus* RN4220 by electroporation. The plasmids were subsequently introduced into the staphylococcal protein A-deficient *S. aureus* strain ISP2073 by generalized transduction (21).

**Serological assays.** Western blot (immunoblot) analysis and gel double-diffusion assays were performed as described previously (21, 41). Gel double-diffusion-grade SEA and rabbit polyclonal anti-SEA serum were obtained from Merlin Bergdoll (Madison, Wis.). Monoclonal antibodies C1 and E8 (MAb C1 and MAb E8) have been described previously (17). To quantify the amount of SEA or mutant SEAs in the culture supernatants used in the biological assays, a twofold dilution series of each supernatant was prepared and loaded on a polyacrylamide gel alongside known concentrations of SEA. The gels were analyzed by Western blotting using MAb C1 as the primary antibody. The blots were then analyzed with a GS 300 densitometer (Hoeffer Scientific Instruments, San Francisco, Calif.).

MAb C1's epitope is contained within residues 108 through 230 of SEA (17). Therefore, MAb C1's affinity for mutant SEAs with substitutions in the N-terminal region should be comparable to its affinity for wild-type SEA.

**Stomach lavage fluid degradation assay.** Monkey stomach lavage fluid was obtained from rhesus monkeys (*Macaca mulatta*) by performing a lavage using 15 ml of a 0.9% (wt/vol) NaCl solution (Abbott Laboratories, North Chicago, Ill.) as described previously (21). Test samples of culture supernatants 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 for 1 to 2 h at 37°C. 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. Each mutant SEA was initially administered at 100 μg per animal; wild-type SEA reproducibly induces a positive response in the emetic assay at this dose. Rhesus monkeys (between 2 to 3 kg) were given samples via nasogastric intubation and were observed for 5 h. The response was considered positive if emesis was observed in any of the animals. If a negative response was obtained at 100 μg per animal, the dosage was increased fivefold. When 500 μg amounts were to be administered, the supernatants were first 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. We did not increase the dosage further in order to avoid potential problems with toxicity. The emetic assays were performed in collaboration with the Regional Primate Research Center, University of Wisconsin—Madison.

**T-cell proliferation assay.** T-cell proliferation assays were performed as described in reference 21 with splenocytes from 6- to 8-week-old female BALB/c mice. Culture supernatants containing SEA or mutant SEAs and their corresponding $10^{-1}$ and $10^{-2}$ dilutions were tested; only the data from the dilutions were analyzed because the undiluted supernatants were toxic to the splenocytes. ISP2073 ($Sea^-$) culture supernatant diluted $10^{-1}$ and $10^{-2}$ served as the negative controls. Purified preparations of SEA or mutant SEAs were added to the plates at 5, 0.5, 0.05, and 0.005 μg per well. Plates were incubated at 37°C with 5% $CO_2$ for 72 h. Proliferation was determined by the incorporation of ethanol-precipitable [$^3$H]thymidine.

Statistical analysis was by the *t* test, using a Minitab Release Computer Package (Minitab, Inc.). The level of significance was $P \leq 0.01$. Each sample was tested in triplicate in at least three separate assays, and the data from one representative experiment are given.

**Detection of cytokine production by murine splenocytes stimulated with SEA or mutant SEAs.** Murine splenocyte cultures were stimulated with either SEA or the panel of mutant SEAs, under conditions similar to those used for proliferation assays. After 48 to 52 h of stimulation, the culture supernatants were collected, centrifuged, and analyzed for the presence of IL-2, IL-4, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) by enzyme-linked immunosorbent assays (ELISAs). ELISA plates specific for each murine cytokine were prepared and processed as described in reference 11. The cytokine standards and the MAb pairs specific for each murine cytokine were purchased from PharMingen (San Diego, Calif.). The lower limit of detection in each ELISA was approximately 100 pg/ml.

**Purification of the SEs.** Purification of SEA-N25G, SEA-N25A, SEA-F47G, SEA-F47S, SEA-L48G, SEA-L48A, and SEA (positive control) was performed by immunoaffinity chromatography using MAb C1 coupled to CNBr-activated Sepharose 4B (Pharmacia) (16). The amount of SEA or mutant SEA was quantified by determination of the $A_{277}$ using an extinction coefficient of 12.76 (48). 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 at −80°C until further use.

**MHC class II receptor binding assay.** The class II binding ability of the mutant SEAs was examined in a competition assay using the human Burkitt lymphoma cell line Raji (expressing HLA-DR3, HLA-DRw10, HLA-DQw1, HLA-DQw2, and HLA-DP7) (32). The Raji cells were resuspended to a concentration of $10^6$ cells per ml in FC buffer (PBS containing 1% bovine serum albumin and 0.1% sodium azide). Fifty-microliter amounts were aliquoted into Falcon 2054 culture tubes (Becton Dickinson Labware, Franklin Lakes, N.J.) and incubated with PBS, SEA, or mutant SEAs (50 μl) for 40 min on ice. The cells were washed in FC buffer and incubated with 50 μl of biotinylated SEA (2 μg/ml; Toxin Technologies, Sarasota, Fla.). As a negative control, one of the PBS-treated samples was not incubated with biotinylated SEA ("FITC only"). The cells were washed again in FC buffer and incubated with 50 μl of FITC-avidin (10 μg/ml) for 20 min. FC buffer (250 μl) was added, and the cells were analyzed for surface fluorescence with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.).

## RESULTS

**Examination of the mutant SEAs by serological assays.** Culture supernatants from each ISP2073 derivative containing *sea* or a *sea* mutation were tested in the gel double-diffusion assay to confirm expression of each gene product. Each mutant SEA formed a line of identity against polyclonal anti-SEA antiserum in the gel double-diffusion assay (data not shown). The culture supernatants of each ISP2073 derivative were also analyzed by Western blotting for reactivity with two MAbs (MAb C1 and MAb E8) directed against wild-type SEA. All of the mutant SEAs reacted strongly with MAb C1 (data not shown). Each mutant SEA also reacted with MAb E8, although the signals obtained with SEA-L12G and SEA-K14G were very weak (data not shown). These findings suggest that the substitutions did not result in dramatic conformational changes in the mutant SEAs sufficient to destroy immunological reactivity with polyclonal antibodies or MAbs.

To further investigate the possibility that the mutant SEAs had altered conformations, each mutant SEA was tested for susceptibility to degradation by monkey stomach lavage fluid. SEA-V85G was included in the assay as the positive control for degradation (16). Each of the mutant SEAs was resistant to digestion by monkey stomach lavage fluid in vitro, like wild-type SEA (Fig. 2). This is consistent with the interpretation that the mutant SEAs have conformations similar to that of wild-type SEA.

**Determination of the emetic activity of the mutant SEAs.** SEA, SEA-L12G, SEA-K14G, SEA-S16G, SEA-K27A, SEA-D45G, SEA-Q46G, SEA-L48G, and SEA-T51A induced a positive emetic response when administered intragastrically at a dose of 100 μg per animal (Tables 2 and 3) (16). SEA-N25G and SEA-N25A did not cause emesis at a concentration of 100 μg per animal. However, both SEA-N25G and SEA-N25A were emetic at 500 μg per animal (Table 2) (16). We previously reported that only one of six animals receiving 100 μg of SEA-L48G vomited; the other animals did not appear ill. However, when the dose of SEA-L48G was increased to 500 μg per animal, all three animals experienced emesis (Table 2) (16).

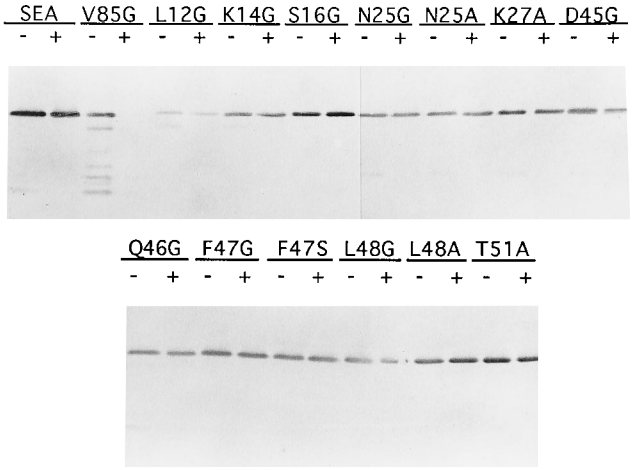



FIG. 2. Western blot analyses comparing the mutant SEAs to wild-type SEA for susceptibility to degradation by monkey stomach lavage fluid in vitro. The blots were reacted with polyclonal anti-SEA serum. Prior to electrophoresis, each sample was incubated with either PBS (−) or monkey stomach lavage fluid (+). The samples were from *S. aureus* ISP2073 derivatives producing either SEA or the indicated mutant SEAs. SEA-V85G was included as the positive control for digestion with monkey stomach lavage fluid (16).

SEA-L48A did not induce emesis in any of three animals when tested at 100 μg per animal but was emetic at 500 μg per animal (like SEA-L48G). None of the animals that received SEA-F47G at a dose of 100 or 500 μg per animal exhibited any signs of discomfort (Table 2) (16). SEA-F47S was not emetic at 100 μg per animal (like SEA-F47G). However, SEA-F47S induced emesis in all three animals when administered at a dose of 500 μg per animal (Table 2). As a negative control, three animals each received a concentrated RN4220 ($Sea^-$) 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 during the entire test period.

**Determination of the superantigen activity of the mutant SEAs.** The culture supernatants from the panel of ISP2073 derivatives were tested for the ability to induce proliferation and cytokine secretion in murine splenocytes. Splenocytes were stimulated with dilutions of each culture supernatant in triplicate on two sets of plates; one set of plates was tested in a standard proliferation assay using [$^3$H]thymidine, while the supernatants from the other set of plates were tested for cytokine production by ELISA.

The concentration of toxin in the bacterial culture supernatants ranged from 6 μg/ml (for SEA-L12G, SEA-K14G, and SEA-S16G) to 8 μg/ml (for SEA and the other mutant SEAs) as determined by Western blot analysis. We previously showed that a statistically significant T-cell proliferative response is induced with dilutions of culture supernatants containing 2 μg of wild-type SEA per ml (21). Compared to the equivalent dilutions from culture supernatants of ISP2073 ($Sea^-$), dilutions of culture supernatants containing SEA-L12G, SEA-K14G, SEA-S16G, SEA-K27A, SEA-D45G, SEA-Q46G, or SEA-T51A induced significant increases in the incorporation of [$^3$H]thymidine. These responses were strong and comparable to those obtained with wild-type SEA at the $10^{-1}$ dilutions (Fig. 3). Dilutions of culture supernatants containing SEA-N25G, SEA-N25A, SEA-L48G, or SEA-L48A induced significant responses that were much weaker than those obtained with wild-type SEA. Dilutions of culture supernatants containing SEA-F47G or SEA-F47S did not induce significant increases in the incorporation of [$^3$H]thymidine (Fig. 3 and Table 3).

TABLE 2. Emetic activity of SEA and the mutant SEAs in rhesus monkeys

| Sample | Result[a] for dose administered intragastrically (μg of toxin per animal) | | Reference |
|---|---|---|---|
| | 100 | 500 | |
| SEA | 2/3 | NT | 16 |
| SEA-L12G[b] | 1/3 | NT | This work |
| SEA-K14G | 2/3 | NT | This work |
| SEA-S16G | 3/3 | NT | This work |
| SEA-N25G | 0/3 | 1/3 | 16 |
| SEA-N25A | 0/3 | 2/3 | This work |
| SEA-K27A | 2/3 | NT | This work |
| SEA-D45G | 2/3 | NT | This work |
| SEA-Q46G | 2/3 | NT | This work |
| SEA-F47G | 0/3 | 0/6 | 16 |
| SEA-F47S | 0/3 | 3/3 | This work |
| SEA-L48G | 1/6 | 3/3 | 16 |
| SEA-L48A | 0/3 | 2/3 | This work |
| SEA-T51A | 2/3 | NT | This work |

[a] Number of animals that experienced emesis/number of animals tested. NT, not tested.
[b] L12G indicates that the leucine at position 12 has been replaced with a glycine residue.

TABLE 3. Summary of the biological activities of SEA and mutant SEAs with N-terminal substitutions

| Sample | T-cell proliferation assay result[a] | | Induction of cytokine production[b] | MHC class II molecule binding assay result[c] | Emetic assay result[d] |
|---|---|---|---|---|---|
| | Culture supernatant | Purified sample | | | |
| SEA | +++ | +++ | +++ | + | ++ |
| SEA-L12G[e] | +++ | NT | +++ | NT | ++ |
| SEA-K14G | +++ | NT | +++ | NT | ++ |
| SEA-S16G | +++ | NT | +++ | NT | ++ |
| SEA-N25G | + | + | + | + | + |
| SEA-N25A | + | + | + | + | + |
| SEA-K27A | +++ | NT | +++ | NT | ++ |
| SEA-D45G | +++ | NT | +++ | NT | ++ |
| SEA-Q46G | +++ | NT | +++ | NT | ++ |
| SEA-F47G | − | − | − | − | − |
| SEA-F47S | − | − | − | − | + |
| SEA-L48G | + | + | − | − | ++ |
| SEA-L48A | + | + | − | − | + |
| SEA-T51A | +++ | NT | +++ | NT | ++ |

[a] Samples that stimulated a statistically significant increase ($P \leq 0.01$) in the incorporation of [$^3$H]thymidine with respect to either ISP2073 or PBS (negative controls). −, sample did not stimulate a significant increase; NT, sample was not tested; +++, level of stimulation was significant and similar to the response to SEA; +, stimulation was significant but weaker than the response to SEA.
[b] Samples that stimulated a statistically significant increase ($P \leq 0.01$) in the production of IL-2 or IFN-γ with respect to ISP2073 (negative control). −, sample did not stimulate a significant increase; +++, level of stimulation was significant and comparable to that of SEA; +, level of stimulation was significant but weaker than the response to SEA.
[c] Symbols: +, sample competed with biotinylated SEA for binding to Raji cells similarly to SEA; −, sample did not compete with biotinylated SEA for binding to Raji cells like SEA; NT, sample was not tested.
[d] Symbols: ++, sample induced a positive emetic response in monkeys when administered at 100 μg per animal; +, sample induced a positive response when administered at 500 μg per animal; −, sample did not induce a positive response when administered at 100 or 500 μg per animal.
[e] L12G indicates that the leucine at position 12 has been substituted with a glycine residue.

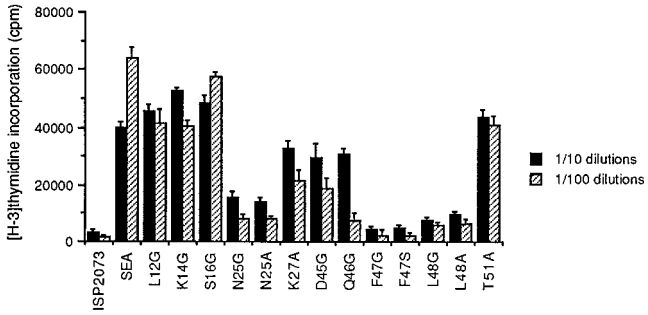



FIG. 3. Induction of murine splenocyte proliferation by SEA and the mutant SEAs. Dilutions of culture supernatants from *S. aureus* ISP2073 (Sea$^-$; negative control) or its derivatives expressing either SEA (positive control) or the indicated mutant SEAs were incubated with murine splenocytes for 72 h followed by an 18-h pulse with [$^3$H]thymidine. Each sample was tested in triplicate, and the results are reported as the mean counts per minute of a representative experiment. Each mutant SEA was tested at least three times in this assay. The standard deviation is indicated as a line above the bars.

IL-4 and TNF-α were not detected in splenocyte cultures stimulated with either wild-type or any of the mutant SEAs (data not shown). IL-2 and IFN-γ were detected in cultures stimulated with either wild-type or mutant SEAs (Fig. 4). The amount of IL-2 induced by a given toxin (and its statistical significance) corresponded well with the degree of proliferation induced by that toxin (Fig. 3 and 4). All of the mutant SEAs induced less IFN-γ than wild-type SEA (Fig. 4). However, the pattern of IFN-γ production mirrored the pattern of proliferation induced by the panel of mutant SEAs (Fig. 3 and 4).

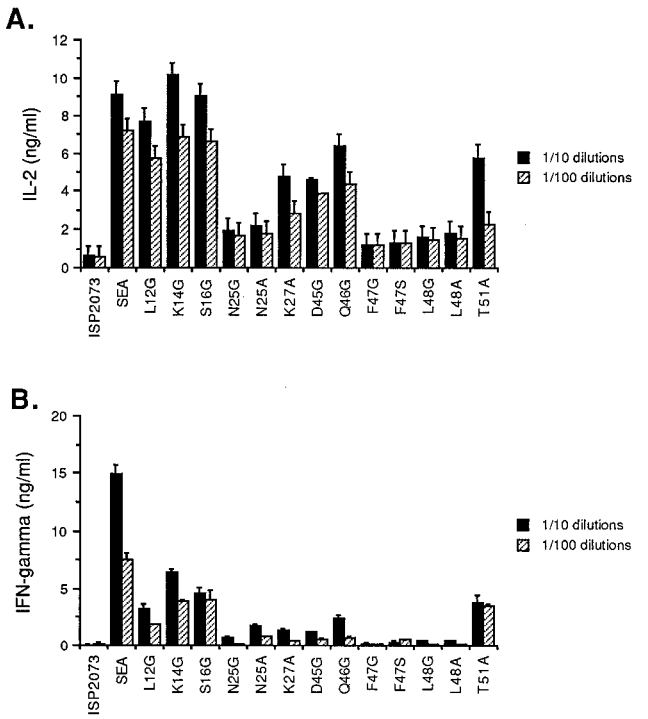



FIG. 4. Induction of cytokine production by SEA and the mutant SEAs. Dilutions of culture supernatants from *S. aureus* ISP2073 (Sea$^-$; negative control) or its derivatives expressing either SEA or the indicated mutant SEAs were incubated with murine splenocytes for 48 h. The splenocyte culture supernatants were clarified by centrifugation and analyzed for IL-2 and IFN-γ production by ELISA. This particular assay was set up at the same time as the proliferation assay whose results are shown in Fig. 3; the bacterial culture supernatant dilutions and splenocyte suspension used were the same in both experiments. Results are expressed in nanograms per milliliter as compared to recombinant cytokine standards. (A) Amount of IL-2 detected by an ELISA specific for murine IL-2. (B) Amount of IFN-γ detected by an ELISA specific for murine IFN-γ.

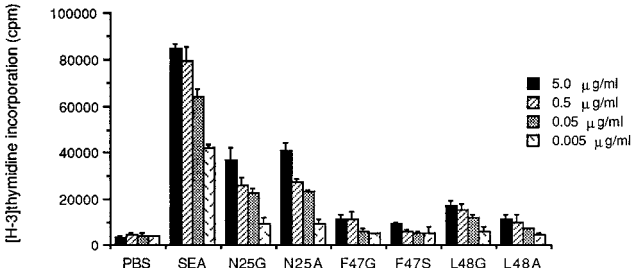



FIG. 5. Induction of murine T-cell proliferation by purified SEA or mutant SEAs. Purified preparations of SEA and the indicated mutant SEAs were incubated with murine splenocytes for 72 h followed by an 18-h pulse with [$^3$H]thymidine. Each sample was tested in triplicate, and the results are given as the mean counts per minute of a representative experiment. The standard deviation is indicated as a line above the bars. Each purified toxin was tested in at least three separate assays.

SEA-N25G, SEA-N25A, SEA-F47G, SEA-F47S, SEA-L48G, and SEA-L48A were subsequently purified and tested for the ability to induce T-cell proliferation at 5, 0.5, 0.05, and 0.005 μg per well. SEA purified in the same manner induced a strong response at all four concentrations (Fig. 5). Purified preparations of SEA-N25G, SEA-N25A, SEA-L48G, and SEA-L48A induced significant responses that were much weaker than those observed with wild-type SEA. SEA-F47G and SEA-F47S did not reproducibly induce significant responses at any concentration tested (Fig. 5). We previously reported that SEA-L48G does not induce a significant response in the proliferation assay when tested at 0.5, 0.05, or 0.005 μg per well (16). In this study we observed a weak yet significant response to SEA-L48G when the mutant was added at 5 μg per well.

**MHC class II binding abilities of mutant SEAs defective for T-cell activation.** Since binding to MHC class II molecules is prerequisite for the SEs' interaction with TCR, mutant SEAs with reduced superantigen activity could arise from faulty interactions with either MHC class II molecules or TCR. To further investigate these possibilities, SEA-N25G, SEA-N25A, SEA-F47G, SEA-F47S, SEA-L48G, SEA-L48A, and wild-type SEA were tested for the ability to bind to Raji cells in a competition assay against biotinylated SEA (Fig. 6). The

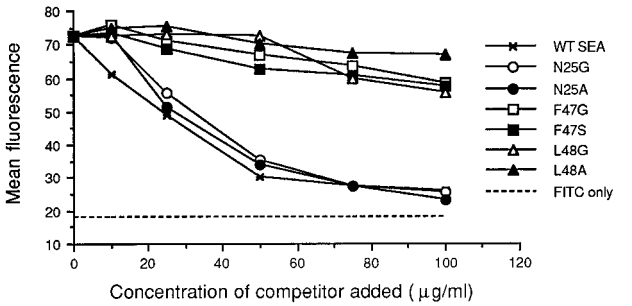



FIG. 6. MHC class II binding activities of the mutant SEAs. SEA or the indicated mutant SEAs were used as competitors for the binding of biotinylated SEA to human MHC class II-bearing Raji cells. The results are plotted as the mean fluorescence of the cells versus the concentration of competitor added. WT, wild type.

curves obtained with SEA-N25G and SEA-N25A were superimposible on the curve obtained with wild-type SEA. In contrast, the curves obtained with SEA-F47G, SEA-F47S, SEA-L48G, and SEA-L48A were different from the curve obtained with wild-type SEA (Fig. 6 and Table 3). Although slight decreases in fluorescence were observed when the Phe-47 and Leu-48 mutants were tested at the higher concentrations, the Phe-47 and Leu-48 mutants do not appear to compete effectively with biotinylated SEA for binding to Raji cells bearing MHC class II molecules.

## DISCUSSION

We are interested in how the structure of the SEs relates to their biological activities. We previously showed that substitution of Asn-25, Phe-47, or Leu-48 of SEA with glycine (yielding SEA-N25G, SEA-F47G, and SEA-L48G, respectively) greatly decreases T-cell stimulatory activity and either decreases or abolishes emetic activity (16). In this work we have more closely examined the biological activities of SEA-N25G, SEA-F47G, and SEA-L48G. We have also analyzed the biological activities of mutant SEAs with different substitutions at these positions (SEA-N25A, SEA-F47S, and SEA-L48A) and at other conserved residues in the N-terminal region of SEA.

The finding that both Asn-25 mutants competed with biotinylated SEA for binding to Raji cells similarly to wild-type SEA indicates that their T-cell activation defect is not due to poor binding to MHC class II molecules. Presumably, the Asn-25 mutants are instead impaired for interactions with the TCR. Since the Asn-25 mutants retain some stimulatory activity, they may weakly interact with all SEA-reactive TCRs or with just a particular subset. Our findings are in accordance with previous studies of SEB (26, 28, 51). Five mutant SEBs at position 23 (corresponding to SEA's position 25) are defective for T-cell activation; four of the five Asn-23 mutants bind to MHC class II like wild-type SEB, suggesting the importance of this residue in TCR recognition. Interestingly, the other mutant at position 23 exhibits decreased MHC class II binding activity, lending support for the suggestion that this residue may also be involved in MHC class II binding (28). On the basis of the three-dimensional structure of SEB, Asn-23 is in an α-helix (α2) with its side chain pointing towards the solvent (51). Asn-23 is part of a shallow cavity formed between the two domains of SEB. Mutational and topological data for SEB are consistent with this cavity constituting the TCR binding site (26, 51).

The demonstration that the Phe-47 and Leu-48 mutants compete poorly for binding to Raji cells is consistent with the interpretation that these residues are required for effective interactions with MHC class II molecules. Mutant SEBs with substitutions at positions 44 and 45 (corresponding to Phe-47 and Leu-48 in SEA) are defective for binding to MHC class II molecules (28). Phe-44 and Leu-45 are located in a turn between β1 and β2 in the SEB structure (51); Phe-44 is positioned with its side chain exposed to solvent such that hydrophobic contacts with MHC class II would be favorable (51). The three-dimensional structure of the SEB/MHC class II complex indicates that key SEB residues at the interface with HLA-DR lie in a turn between β1 (residues 33 to 39) and β2 (residues 48 to 52) and in β3 (residues 63 to 68; 26). The SEB/HLA-DR1 interface is comprised of two distinct regions: one predominately hydrophobic (including Phe-44 and Leu-45) and the other mainly hydrophilic (26).

SEA-D45G, SEA-Q46G, and SEA-T51A all retained significant amounts of T-cell stimulatory and emetic activity. This indicates that Asp-45, Gln-46, and Thr-51 are not required for SEA's biological activities, despite the presumed proximity of these residues to Phe-47 and Leu-48 by analogy to the loop in SEB between β1 and β2. However, SEA-D45G and SEA-Q46G do exhibit somewhat reduced superantigen activity with respect to SEA. Perhaps these residues have a minor role in SEA's interaction with MHC class II molecules. The corresponding glutamine residue in SEB (Gln-43) is part of the SEB/MHC class II interface, potentially engaging in hydrogen bonding with HLA-DR1 residues (26).

A segment of SEB spanning residues 9 through 23 is implicated by mutational analysis to be important for SEB's interactions with TCR and MHC class II molecules (28). Specifically, mutant SEBs with substitutions at positions 9, 14, 17, or 23 are defective for T-cell activation. The three-dimensional structure of SEB places these residues on the molecular surface with locations favorable for making contacts with MHC class II molecules or the TCR (51). In contrast to the importance of SEB residues 9 to 23, we found that deletion of SEA residues 3 through 17 does not abolish T-cell stimulatory activity (17). In this study the substitution of the three conserved residues in this region (Leu-12, Lys-14, and Ser-16) with glycine residues had no significant effect on superantigen activity. This finding further supports our conclusion that residues 3 through 17 are not required for SEA's superantigen activity (17). It has been reported recently that SEC1 residues 6 through 18 are not required for the binding of SEC1 to either MHC class II molecules or the TCR (19).

Several lines of evidence suggest that the mechanisms whereby SEA and SEB function as superantigens differ in important respects. Results of competition studies are consistent with SEA and SEB binding to overlapping but distinct regions on HLA-DR. SEB and TSST-1 bind to distinct sites on HLA-DR and HLA-DQ (45). SEA cross-competes with both TSST-1 (46, 47) and SEB (12) for MHC class II binding. However, SEB and TSST-1 do not inhibit SEA binding to HLA-DR (10). Mutational analysis has indicated that His-81 of HLA-DR's β chain is required for the binding of SEA, but not for the binding of SEB or TSST-1, to HLA-DR (18, 29). In addition, the binding of SEA to HLA-DR is dependent on the presence of zinc, whereas a zinc requirement for SEB/HLA-DR binding has not been established (13). It has been suggested that SEA may crosslink two MHC class II molecules; SEA's N-terminal domain could interact with the α1 domain of one MHC class II receptor (similarly to SEB), and its C-terminal domain could associate with the β chain of a second MHC class II receptor (26). Our studies have identified N-terminal SEA residues (Phe-47 and Leu-48) that indeed function in MHC class II recognition similarly to the corresponding residues in SEB.

We reported previously that SEB-F44S induces emesis when tested at 300 μg per animal, in contrast to SEA-F47G, which does not induce a response at 500 μg per animal (16). In this study we attempted to resolve this discrepancy by substituting Phe-47 of SEA with a serine residue. The finding that SEA-F47S is emetic at 500 μg per animal indicates that the nature of the substitution at position 47 influences SEA's emetic activity. Serine may be a more permissible substitution than glycine for the role phenylalanine has in emesis. Alternatively, SEA-F47G may be degraded in vivo despite its in vitro resistance to degradation. Although SEA-F47G and SEA-F47S had different emetic activities, they were equally defective for induction of T-cell proliferation and cytokine production. Both mutant SEAs also competed poorly in the MHC class II binding assay. Although we did not test SEB-F44S in this study,

SEB-F44S is similarly defective for T-cell activation and MHC class II recognition (28).

The relationship between SEA's T-cell stimulatory and emetic activities remains unresolved. SEA-L48G and SEA-L48A induced significant increases in the incorporation of [$^3$H] thymidine. However, the levels never approached those induced by wild-type SEA, even when the mutants were present at concentrations 1,000-fold greater than those required to see strong stimulation by wild-type SEA. SEA-F47S did not induce a significant response in the proliferation assay at any of the concentrations tested (Fig. 3). All three mutants induced very little IL-2 and IFN-γ in the splenocyte cultures (Fig. 4). However, SEA-F47S, SEA-L48G, and SEA-L48A all retained significant emetic activity (Table 2). These findings corroborate our previous conclusion that activity in the murine splenocyte assay does not predict emetic activity in rhesus monkeys (16). Despite the imperfect correlation, activity in the two assays does appear to be somehow related. Mutant SEAs with substitutions as Asn-25, Phe-47, Leu-48, or Cys-106 all have decreased T-cell stimulatory activity and have either decreased or abrogated emetic activity (Table 2) (16).

In summary, we examined the emetic and superantigen activities of a number of mutant SEAs. Although there is not a strict correlation between activity in the murine T-cell stimulation assays and in the simian emetic assay, activity in these assays appears to be somehow related. Our studies have better defined the role of Asn-25, Phe-47, and Leu-48 in SEA's superantigen activity. Our results are consistent with Phe-47 and Leu-48 of SEA being required for effective interactions with MHC class II molecules and Asn-25 being important for TCR recognition.

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