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Staphylococcal Enterotoxin Type A Internal Deletion Mutants: Serological Activity and Induction of T-Cell Proliferation†

THERESA O. HARRIS, WENDY O. HUFNAGLE,‡ AND MARSHA J. BETLEY*
University of Wisconsin-Madison, Madison, Wisconsin 53706

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Previous findings indicate that the N-terminal region of staphylococcal enterotoxin type A (SEA) is required for its ability to induce T-cell proliferation. To better localize internal peptides of SEA that are important for induction of murine T-cell proliferation, SEA mutants that had internal deletions in their N-terminal third were constructed. A series of unique restriction enzyme sites were first engineered into *sea*; only one of these changes resulted in an amino acid substitution (the aspartic acid residue at position 60 of mature SEA was changed to a glycine [D60G]). Because the D60G substitution had no discernible effect on serological or biological activity, the *sea* allele encoding this mutant SEA was used to construct a panel of mutant SEAs lacking residues 3 to 17, 19 to 23, 24 to 28, 29 to 49, 50 to 55, 56 to 59, 61 to 73, 68 to 74, or 74 to 85. Recombinant plasmids with the desired mutations were constructed in *Escherichia coli* and transferred to *Staphylococcus aureus*. Staphylococcal culture supernatants containing the mutant SEAs were examined. Western immunoblot analysis with polyclonal anti-SEA antiserum revealed that each of the recombinant *S. aureus* strains produced a mutant SEA of the predicted size. All the mutant SEAs exhibited increased sensitivity to monkey stomach lavage fluid in vitro, which is consistent with these mutants having conformations unlike that of wild-type SEA or the SEA D60G mutant. In general, deletion of internal peptides had a deleterious effect on the ability to induce T-cell proliferation; only SEA mutants lacking either residues 3 to 17 or 56 to 59 consistently produced a statistically significant increase in the incorporation of [^{3}H]thymidine. In the course of this work, two monoclonal antibodies that had different requirements for binding to SEA in Western blots were identified. The epitope for one monoclonal antibody was contained within residues 108 to 230 of mature SEA. Binding of the other monoclonal antibody to SEA appeared to be dependent on the conformation of SEA.

The staphylococcal enterotoxins (SEs) are extracellular proteins produced by *Staphylococcus aureus* that cause staphylococcal food poisoning syndrome (3). At present, five major serological types have been characterized, A through E (designated SEA through SEE). The genes (*sea* through *see*) have been cloned, and their nucleotide and derived amino acid sequences have been determined (2, 5, 6, 10, 11, 18, 19, 32, 39, 56). The SEs are all simple single-chain molecules, with molecular weights ranging from 26,360 to 28,336 (2, 5, 6, 10, 11, 18, 19, 32, 39, 56). The percentage of amino acid sequence identity ranges from 29 to 81%; SEA and SEE are the most closely related pair, followed by SEB and SEC, which have 66% amino acid sequence identity (4).

The SEs share a number of other biological activities; they are pyrogenic, induce the production of interleukin-1, gamma interferon, and tumor necrosis factor and induce T-cell proliferation (13, 16, 17, 22, 38, 45, 54, 59). As determined from some common requirements for T-cell proliferation, the SEs belong to a group of substances referred to as superantigens. Other members of this group include toxic shock syndrome toxin type 1, the streptococcal pyrogenic exotoxins and M proteins, the *Clostridium perfringens* enterotoxin, the *Mycoplasma arthritidis*-derived mitogen, the *Yersinia enterocolitica*-derived antigen, and endogenous self-superantigens, such as the mammary tumor virus-encoded Mls antigens (12, 62, 64; reviewed in references 31, 48, and 50). Superantigens have the ability to stimulate T cells expressing particular Vβ elements of the T-cell receptor regardless of what other variable elements are present (65). Like conventional antigens, the superantigens require major histocompatibility (MHC) class II-bearing antigen-presenting cells to stimulate T cells. However, superantigens do not require processing for presentation (15, 23), and superantigens bind outside of the conventional antigen-binding groove (20; for review, see references 31 and 50). The bacterial superantigens have been implicated as virulence factors in infections characterized by severe shock symptoms (8, 47). With respect to this type of disease, toxic shock syndrome toxin 1 is the best studied of the superantigens. It has been suggested that the severe shock symptoms are due to release of cytokines caused by the interaction of the superantigens with the immune system (34, 35, 54).

A variety of techniques have been used to identify the regions involved in SEA's various immunological activities. SEA's ability to induce T-cell proliferation is destroyed by treatment with either chymotrypsin, pronase, proteinase K, or cyanogen bromide (24). This is consistent with the interpretation that SEA's superantigenic domain spans more than one polypeptide generated by a given reagent. Chemical modifications and site-directed mutagenesis of SEA's disulfide loop indicate that the disulfide loop is critical for interaction with the T-cell receptor (28, 29). A synthetic peptide corresponding to the N-terminal 27 residues of SEA blocks SEA's ability to induce T-cell proliferation and gamma interferon production (55). Furthermore, the binding of SEA to MHC class II-bearing Raji cells is blocked by

* Corresponding author.
† Primate Center publication no. 32-024.
‡ Present address: Virginia Mason Research Center, Seattle, Wash.

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

Strain, phage, or plasmid	Relevant characteristics[a]	Reference or source
E. coli JF626	Ap^s Sea^-	J. J. Mekalanos, Harvard Medical School, Boston, Mass. (6)
E. coli plasmids		
pGEM-7Zf(+)	Ap^r	Promega Corp.
pMJB3	pBR322 derivative with an insert fragment containing *sea*	5
pMJB305	pGEM-7Zf(+) derivative lacking *Sac*I and *Nsi*I sites in multiple cloning region [pGEM-7Zf(+) digested with *Nsi*I and *Sac*I, blunted with T4 DNA polymerase, and ligated]	This work
pMJB198	pMJB305 with 1.4-kb *Bgl*I-*Hin*dIII *sea* fragment from pMJB3 inserted	This work
pMJB214	pMJB305 with 1.4-kb *Bgl*I-*Hin*dIII *sea-1351* fragment inserted	This work
E. coli phage M13mp11		49
S. aureus		
RN4220	Sea^-	R. P. Novick, Public Health Research Institute, New York, N.Y. (42)
RN450	Sea^-	R. P. Novick (52)
SA20	Sea^-	S. Iordanescu, Public Health Research Institute, New York, N.Y. (41)
S. aureus plasmid pC194	Cm^r	R. P. Novick (36)
S. aureus phage 80α	Generalized transducing phage	P. A. Pattee, Iowa State University, Ames (51)

[a] Ap, ampicillin; Cm, chloramphenicol.

synthetic peptides corresponding to residues 1 to 45, 39 to 66, 62 to 86, and 121 to 149 of SEA (26). This latter finding suggests that the binding of SEA to MHC class II molecules involves multiple domains in both the N-terminal and central regions of the molecule. Another group of investigators have reported that a recombinant SEA fragment containing residues 107 to 233 binds to HLA-DR and HLA-DP, although it does not activate T cells (30). These authors suggested that the C-terminal portion of SEA is sufficient for binding MHC class II molecules but that additional regions of the protein are required for T-cell activation (30). The analysis of SEA-SEE hybrid proteins has demonstrated that C-terminal residues are involved in discrimination of Vβ specificity (37).

Limited tryptic hydrolysis of SEC1 has yielded different results; one group of investigators reported that the small N-terminal tryptic fragment causes T-cell proliferation, whereas another group found that the C-terminal tryptic fragment is pyrogenic and induces T-cell proliferation (9, 60).

The analysis of truncated SEB-protein A fusion proteins indicates that residues 1 to 138 of SEB contain a region required for the stimulation and induction of anergy in T cells bearing Vβ3.1 gene elements (14). A collection of SEB mutants containing one to three amino acid substitutions that are defective in stimulation of cells bearing Vβ7 or Vβ8.3 have been generated (40). Most of the substitutions fall within three N-terminal regions corresponding to residues 9 to 23, 41 to 53, and 60 to 61 of SEB. The genetic analysis, in conjunction with the three-dimensional structure analysis of SEB (63), has revealed that the T-cell receptor site is a shallow cavity and that the MHC class II binds to an adjacent site.

The purpose of this study was to better localize internal peptides in the N-terminal third of SEA (corresponding to residues 1 to 85) that are important for SEA's ability to induce T-cell proliferation. A series of *sea* mutations with internal in-frame deletions were constructed. Each mutant SEA was examined for the ability to react, in either a gel double-diffusion assay or Western immunoblot, with polyclonal anti-SEA antiserum; for sensitivity to degradation by monkey stomach lavage fluid; and for the ability to induce T-cell proliferation of murine splenocytes. In addition, to aid in quantification of the mutant SEAs, a monoclonal antibody (MAb) prepared against SEA with a binding epitope outside of the N-terminal third of SEA was identified. A second MAb against SEA that apparently recognizes a conformational epitope was also identified.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and media. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. Descriptions of the *sea* mutations used are in Table 2. *Escherichia coli* and *S. aureus* were propagated as described elsewhere (5). The culture conditions and media used for the transformation of *E. coli* and *S. aureus* and transduction of *S. aureus* have been described previously (33). For biological and serological assays, *S. aureus* strains were grown in 3% (wt/vol) NZ-amine type A (Kraft Inc., Norwich, N.Y.) and 1% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) for 18 h at 37°C with aeration, and bacterial cells were removed by centrifugation at 7,000 rpm for 30 min at 4°C.

Chemicals and enzymes. Purified SEA was obtained from Toxin Technologies (Sarasota, Fla.); restriction enzymes, T4 DNA polymerase, and T4 DNA ligase were from New England Biolabs Inc. (Beverly, Mass.); Klenow fragment was from Promega Corp. (Madison, Wis.); alkaline phosphatase was from Boehringer Mannheim (Indianapolis, Ind.); the Geneclean kit was from Bio101 Inc. (La Jolla, Calif.); the Sequenase kit was from United States Biochemical (Cleveland, Ohio); and cyanogen bromide (CNBr) was

TABLE 2. Nomenclature for *sea* mutations used in this study

Allele	Codon(s) deleted[a] or restriction sites added	Mature protein product		Reference or source
		Designation	Calculated mol wt	
sea	None (wild-type allele)	SEA	27,093	6
sea-1309	169–233 of *sea*	SEA-1309	19,574	33
sea-1310	195–233 of *sea*	SEA-1310	22,465	33
sea-1315	227–233 of *sea*	SEA-1315	26,237	33
sea-1316	231–233 of *sea*	SEA-1316	26,742	33
sea-1317	233 of *sea*	SEA-1317	27,006	33
sea-1364	*Sac*I, *Bgl*II, *Pst*I, *Spe*I, and *Sty*I sites added (all silent changes)	SEA-1364	27,093	This work
sea-1351	*Sac*I, *Bgl*II, *Pst*I, *Msc*I, *Spe*I, and *Sty*I sites added (*Msc*I addition resulted in D60G substitution)	SEA-1351	27,035	This work
sea-1352	3–17 of *sea-1351*	SEA-1352	25,220	This work
sea-1372	19–23 of *sea-1351*	SEA-1372	26,564	This work
sea-1373	24–28 of *sea-1351*	SEA-1373	26,494	This work
sea-1355	29–49 of *sea-1351*	SEA-1355	24,404	This work
sea-1374	50–55 of *sea-1351*	SEA-1374	26,295	This work
sea-1375	56–59 of *sea-1351*	SEA-1375	26,582	This work
sea-1358	61–73 of *sea-1351*	SEA-1358	25,442	This work
sea-1359	68–74 of *sea-1351*	SEA-1359	26,230	This work
sea-1361	74–85 of *sea-1351*	SEA-1361	25,632	This work

[a] The mature form of SEA is 233 amino acid residues in length (6). The codons have been numbered starting with the amino-terminal residue of mature SEA.

from Eastman Kodak (Rochester, N.Y.). All chemicals and enzymes were used according to the manufacturers' recommendations.

Construction of mutations. *E. coli* plasmid DNA and replicative forms of phage DNA were prepared by an alkaline lysis method (1). Staphylococcal plasmid DNA was obtained by CsCl-ethidium bromide dye-buoyant density centrifugation (53). Nucleotide sequences were determined by the dideoxy chain termination method (58).

To facilitate the construction of in-frame deletions in *sea*, unique restriction enzyme sites were introduced into *sea* by oligonucleotide-directed mutagenesis (Fig. 1A) (43, 44). The 1-kb *Hinc*II-*Hin*dIII fragment of *sea* from pMJB3 was ligated into *Hinc*- and *Hin*dIII-digested M13mp11 DNA. Five consecutive rounds of mutagenesis were performed with the following oligonucleotides: 5′-aagtctgaGCtCcaggaaca-3′ (*Sac*I; 117); 5′-cttaaacaGatctattat-3′ (*Bgl*II; 150); 5′-gatcaatt tCtGcagcatact-3′ (*Pst*I; 207); 5′-ttttttacTgGCcattcgtgg-3′ (*Msc*I; 243); and 5′-gatttaCtagtagattttgattcCaaggat-3′ (*Spe*I and *Sty*I; 270). The lowercase letters indicate nucleotides identical to those in *sea*, uppercase letters show mutations that result in incorporation of the restriction enzyme site(s) indicated in parentheses, and the number after the restriction site denotes the 5′ nucleotide of *sea* to which the oligonucleotide anneals. The oligonucleotides were obtained from the University of Wisconsin Biotechnology Center (Madison, Wis.).

After the five rounds of mutagenesis, the 300-bp *Hinc*II-*Acc*I *sea* fragment containing the unique restriction enzyme sites was subcloned into *Hinc*II- and *Acc*I-digested pMJB198, yielding *sea-1351*, in pMJB214. pMJB198 had been constructed by ligating the 1.4-kb *Bgl*I-*Hin*dIII fragment of pMJB3 that contains *sea* into pMJB305, a pGEM-7Zf(+) derivative (Table 1). The nucleotide sequence of the *Hinc*II-*Acc*I fragment of pMJB214 was determined; only the desired mutations were present. All unique sites incorporated into the *sea-1351* fragment resulted in silent mutations with the exception of *Msc*I, which changed the aspartic acid residue at position 60 of mature SEA to a glycine residue.

To determine whether *sea-1351* would be an appropriate template for deletion construction, SEA-1351 was analyzed serologically and tested for induction of emesis activity and of T-cell proliferation. As indicated in Table 3, SEA-1351 acted like wild-type SEA. As a control, *sea-1364*, which contains all the unique restriction sites of *sea-1351* except *Msc*I, was also constructed; its gene product (SEA-1364) has the same predicted amino acid sequence as wild-type SEA.

When the *sea* open reading frame could be retained, deletions were constructed by removing an intervening region of DNA between two unique restriction sites. For the construction of *sea-1355*, pMJB214 was digested with *Bgl*II and *Pst*I, treated with mung bean nuclease to create blunt ends, and ligated. To make *sea-1358*, pMJB214 was digested with *Msc*I and *Sty*I, reacted with Klenow fragment to blunt the *Sty*I overhang, and ligated. For the construction of *sea-1359*, pMJB214 was digested with *Spe*I and *Sty*I, treated with mung bean nuclease to blunt the ends, and ligated. To make *sea-1361*, pMJB214 was digested with *Acc*I and reacted with Klenow fragment to blunt the ends. The *Acc*I-linearized pMJB214 was then digested with *Sty*I, reacted with mung bean nuclease to blunt the resulting *Sty*I overhang, and ligated.

For the other deletions constructed in this study, cassette mutagenesis was used to maintain *sea*'s open reading frame. For each construction, two synthetic complementary oligonucleotides with ends compatible to the two restriction sites flanking the region to be deleted were synthesized. The nucleotide sequences of these oligonucleotides are shown in Fig. 1B. One microgram of each oligonucleotide, as determined by A_{260} (46), was annealed with its complement for 30 min at 37°C and ligated into appropriately digested pMJB214.

Each ligation mixture was transformed separately into *E. coli* JF626, and the desired constructs were identified by DNA sequence analysis. Each *E. coli* plasmid containing *sea* or an *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 electro-

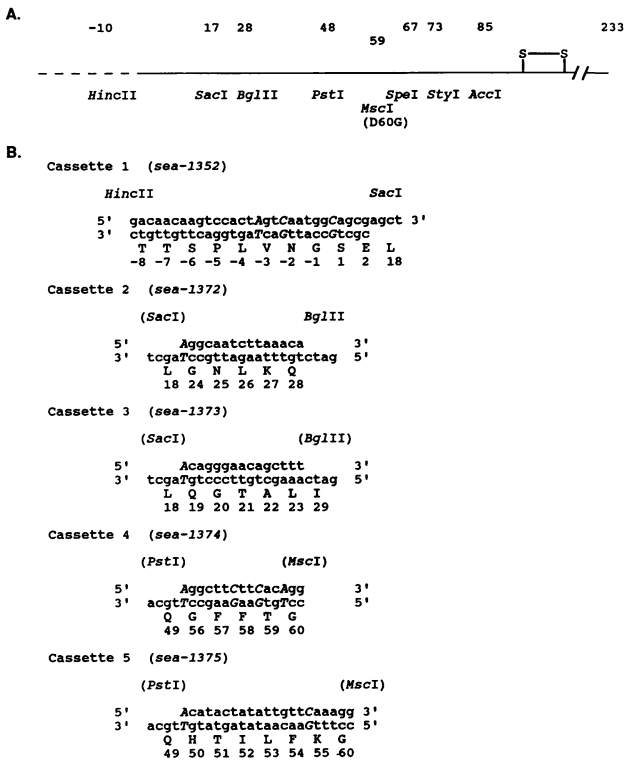

FIG. 1. (A) Diagram of *sea-1351* (not drawn to scale), indicating restriction enzyme sites that are unique to pMJB214, the plasmid substrate used to construct the *sea* deletion mutations. *Sac*I, *Bgl*II, *Pst*I, *Msc*I, *Spe*I, and *Sty*I sites were introduced into *sea* by site-directed mutagenesis. The *Hin*cII and *Acc*I sites are present in wild-type *sea*. The numbers above the restriction sites correspond to the codons located at the 5′ end of the restriction site. The codons are numbered with the translation initiation codon as −24, the first codon of mature SEA-1351 as 1, and the last codon as 233. The location of the disulfide loop formed by the cysteine residues at positions 96 and 106 is indicated (S—S). (B) Double-stranded oligonucleotide cassettes used for construction of *sea* deletion mutations. For each cassette, the ends were compatible with the indicated restriction enzyme cleavage products, and these restriction enzymes were used to substitute the cartridge for the corresponding region in *sea-1351*. Lowercase letters indicate nucleotides which are identical to those in *sea-1351*. Uppercase letters in italic type represent silent mutations which were designed either to minimize stem-loop formation or to facilitate screening; in the latter case, the loss of a restriction site is indicated by parentheses. The amino acid sequence is shown below the cassette. The amino acid residues have been numbered so that the first residue of mature wild-type SEA is 1 and the last residue of the signal peptide is −1.

poration and subsequently moved into *S. aureus* SA20 by transduction (33).

Serological assays. Rabbit polyclonal anti-SEA antiserum and gel double-diffusion-grade SEA were obtained from Merlin Bergdoll. Western blot analysis and gel double-diffusion assays were performed as previously described (33, 57). The amount of each antibody preparation used in Western blot analysis was standardized to contain 1.1 to 1.2 mg of total protein. Full-length mutant SEAs were quantitated by densitometric analysis of Western blots (GS 300 densitometer; Hoefer Scientific Instruments, San Francisco, Calif.), with known concentrations of SEA used as standards. Normal rabbit serum was purchased from Sigma Chemical Co. (St. Louis, Mo.) and used for indicating non-SEA-related signals in Western blot analysis.

Stomach lavage fluid assay. A sterile saline lavage (15 ml of 0.9% [wt/vol] NaCl; Abbott Laboratories, North Chicago, Ill.) was performed on rhesus monkeys (*Macaca mulatta*) by nasogastric intubation as described previously (33). Forty microliters of each SA20 culture supernatant was mixed with either 10 μl of monkey stomach lavage fluid or phosphate-buffered saline (0.02 M sodium phosphate containing 0.15 M NaCl [pH 7.4]) and incubated at 37°C for 2 h. An equal amount of loading buffer was added, and 5 μl of each sample was analyzed by Western blotting.

Emetic assay. The SA20 derivative producing SEA-1351 was grown in 100 ml of 3% NZ-amine type A plus 1% yeast extract in a 2-liter Erlenmeyer flask for 18 h at 37°C with aeration. The cells were harvested by centrifugation at 7,000 rpm for 30 min, and the culture supernatant was sterilized by passage through a 0.45-μm pore size filter (Gelman Sciences, Ann Arbor, Mich.). The concentration of SEA-1351, determined by visual inspection of Western blots made with polyclonal anti-SEA antiserum and known amounts of SEA as standards, was at least 8 μg/ml. Twelve milliliters of this supernatant was administered to six rhesus monkeys by nasogastric intubation. Two of six monkeys vomited during the 5-h assay. The emetic assay was performed in collaboration with the Regional Primate Center, University of Wisconsin-Madison.

CNBr treatment of SEA. Each reaction mix contained 200 μl of formic acid (70%, vol/vol) with 50 μg of purified SEA and either no CNBr (control) or CNBr in either 4,000-, 6,000-, or 8,000-fold molar excess over SEA. The reaction mixes were incubated in the dark at room temperature overnight. The samples were diluted in distilled water, lyophilized, and resuspended in 500 μl of phosphate-buffered saline. Five-microliter samples were analyzed by Western blotting.

Generation of anti-SEA MAbs. Hybridomas producing MAbs to SEA were generated at the University of Wisconsin Hybridoma Facility (Madison, Wis.) as described previously (25). Spleen cells from BALB/c mice immunized with 1 mg of SEA (61) were fused to 10^7 NS-1 myeloma cells by using polyethylene glycol 1500 (Boehringer-Mannheim) and selected in Dulbecco's modified Eagle's medium (GIBCO/BRL, Grand Island, N.Y.) supplemented with hypoxanthine, aminopterin, and thymidine (Sigma). Culture supernatants were screened by enzyme-linked immunosorbent assay (ELISA) by a modification of the method of Engvall and Perlmann (21), as adapted for the use of fluorogenic enzyme substrates (66), and by Western blot analysis with purified SEA. Hybrids producing anti-SEA antibodies were cloned by limiting dilution and expanded for ascites fluid production and for cyropreservation.

To prepare ascites fluid, mice (BALB/c) primed with pristane (2,6,10,14-tetramethylpentadecane; Aldrich, Milwaukee, Wis.) were injected intraperitoneally with 2×10^6 to 4×10^6 hybridoma cells (0.5 ml) derived from single MAb-producing clones. Ascites fluid was collected 8 to 20 days postinjection. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.), and isotypes were determined with an isotype identification kit (Binding Site Inc., San Diego, Calif.). All assays were performed with unpurified ascites fluid preparations.

T-cell proliferation assay. Stimulation of T-cell proliferation by purified SEA or by culture supernatants containing either SEA or mutant SEAs and their corresponding 10^{-1} and 10^{-2} dilutions was determined by incorporation of

TABLE 3. Activities of mutant SEAs in culture supernatants of *S. aureus* SA20 or SA20 derivatives

Sample[a]	Western blot[b]			Gel double-diffusion assay[c]	Susceptibility to stomach lavage fluid[d]	T-cell proliferation assay[e]
	Polyclonal antiserum	MAb C1	MAb E8			
SA20	−	−	−	−	−	−
SEA	+	+	+	+	R	+
SEA-1351 (D60G)	+	+	+	+	R	+
SEA-1352 (Δ3–17)	+	+	IC	−	S	+
SEA-1372 (Δ19–23)	+	+	+	+	S	−
SEA-1373 (Δ24–28)	+	+	−	−	S	−
SEA-1355 (Δ29–49)	+	+	−	−	S	−
SEA-1374 (Δ50–55)	+	+	+	+	S	−
SEA-1375 (Δ56–59)	+	+	+	+	S	+
SEA-1358 (Δ61–73)	+	+	−	−	S	−
SEA-1359 (Δ68–74)	+	+	−	−	S	−
SEA-1361 (Δ74–85)	+	+	−	−	S	−

[a] In SEA-1351, the aspartic acid residue at position 60 of wild-type SEA has been substituted with a glycine residue. For other mutants, the residues of SEA-1351 that have been deleted are shown in parentheses.

[b] Western blots were reacted with either polyclonal anti-SEA antiserum, MAb C1, or MAb E8. Symbols: +, blot revealed an SEA-related signal corresponding to the predicted size; −, no signal for an SEA-related product detected; IC, inconclusive because the concentration of SEA was below the level of detection.

[c] Culture supernatants were reacted against polyclonal anti-SEA antiserum. Symbols: +, precipitin line detected; −, no visible reaction observed. The same results were obtained when the supernatants were concentrated fivefold.

[d] Culture supernatants were incubated with stomach lavage fluid in vitro for 2 h at 37°C and then subjected to Western analysis with polyclonal anti-SEA antiserum. Symbols: −, sample contained no SEA-related proteins (negative control); R, SEA-related protein(s) resistant to degradation; S, SEA-related protein(s) susceptible to degradation.

[e] Symbols: +, culture supernatants stimulated a statistically significant ($P \leq 0.01$) increase in T-cell proliferation over that in the SA20 culture supernatant; −, results of proliferation assays were not all statistically significant.

[^{3}H]thymidine by murine splenocytes, as described previously (33). Data from the 10^{-1} and 10^{-2} dilutions of the supernatants were analyzed because these dilutions contained sufficient amounts of SEA to be in the maximal mitogenic range (10^{-2} to 10 μg/ml) (45) yet minimized the toxicity of the bacterial culture medium to the T cells (33). Data from the 10^{-1} and 10^{-2} dilutions from test and SA20 negative-control samples were analyzed with a Minitab Release 7.1 computer package (Minitab Inc.). The level of significance was set at $P < 0.01$. All mutant SEAs were tested at least twice in the T-cell proliferation assay, and the data from one representative experiment are given.

RESULTS

Anti-SEA MAbs. Five different MAb preparations which reacted strongly with intact SEA, as determined by Western blot analysis and a direct ELISA (titers, >40,000), were obtained. The concentrations ranged between 31 and 46 mg of protein per ml, and all were of the immunoglobulin G1 isotype.

Characterization of anti-SEA MAbs. A Western blot procedure was used to quantify unpurified preparations of mutant SEAs that had deletions within the first 85 amino acid residues of the molecule relative to wild-type SEA. Our strategy was to identify an MAb that could react efficiently in a Western blot with a CNBr-derived SEA fragment lacking residues 1 through 85 or more of SEA.

The preferred cleavage site of CNBr is methionine residues (27). Figure 2A is a diagram of SEA, showing the locations of its two methionine residues and the fragments predicted to be generated from complete and partial cleavage with CNBr. These fragments were designated CNBr-SEA[1–224], CNBr-SEA[108–233], CNBr-SEA[108–224], CNBr-SEA[1–107], and CNBr-SEA[225–233]; the numbers in brackets indicate the residues of mature SEA contained in the fragment. Western blot analysis of CNBr-treated SEA with polyclonal anti-SEA antiserum resulted in visualization

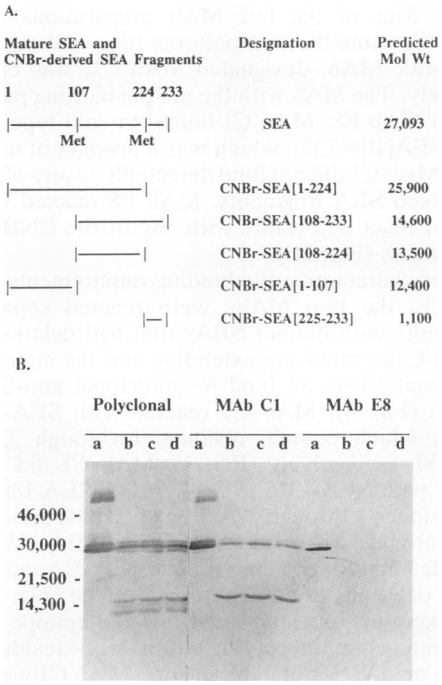

FIG. 2. (A) Diagram of mature SEA and CNBr cleavage products (not drawn to scale). The numbers correspond to the residues in mature SEA, with the N-terminal residue as 1. The locations of methionine residues are indicated. (B) Western blot analysis of purified SEA. Blots were reacted with polyclonal anti-SEA antiserum, MAb C1, or MAb E8. For each blot, purified SEA was treated with 70% formic acid containing no CNBr (lanes a), an 8,000-fold molar excess of CNBr (lanes b), a 6,000-fold molar excess of CNBr (lanes c), or a 4,000-fold molar excess of CNBr (lanes d). The positions of molecular weight standards are indicated on the left.

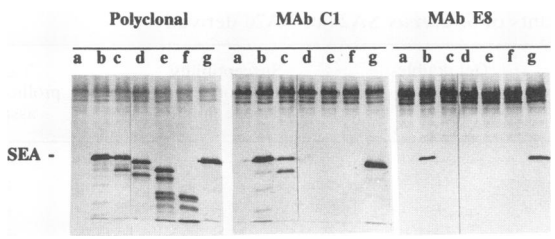

FIG. 3. Western blot analysis of culture supernatants from *S. aureus* SA20 or SA20 derivatives containing *sea* or an *sea* mutation. Blots were reacted with either polyclonal antiserum, MAb C1, or MAb E8. The samples were from SA20 producing no SEA (lane a; negative control) or SA20 derivatives producing SEA-1317 (lane b), SEA-1316 (lane c), SEA-1315 (lane d), SEA-1310 (lane e), SEA-1309 (lane f), or SEA (lane g). The position of SEA is indicated by the dash on the left. Signals corresponding to proteins with molecular weights greater than 30,000 are probably protein A; these signals are also visible in blots reacted only with normal rabbit serum (data not shown).

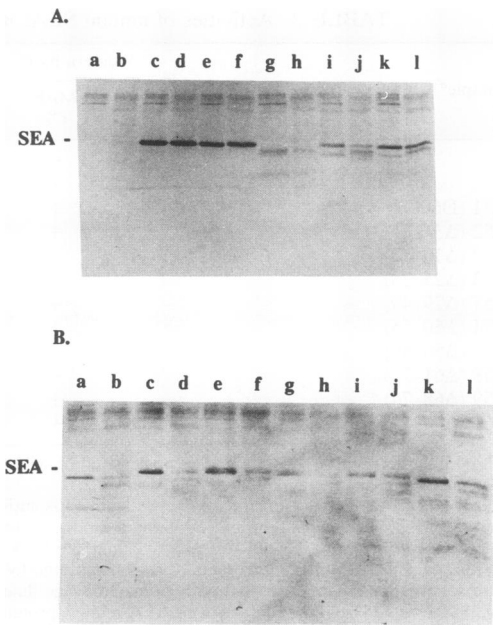

FIG. 4. Comparison of susceptibility of SEA and mutant SEAs to degradation by monkey stomach lavage fluid. Western blot analysis with polyclonal anti-SEA antiserum was performed on culture supernatants from *S. aureus* SA20 derivatives containing *sea* or an *sea* mutation that had been incubated with phosphate-buffered saline (lanes a, c, e, g, i, and k) or stomach lavage fluid (lanes b, d, f, h, j, and l) for 2 h at 37°C prior to electrophoresis. The samples analyzed on blot A were from SA20 producing no SEA (lanes a and b) or SA20 derivatives producing SEA-1364 (lanes c and d), SEA-1351 (lanes e and f), SEA-1352 (lanes g and h), SEA-1372 (lanes i and j), or SEA-1373 (lanes k and l). The samples analyzed on blot B were from SA20 derivatives producing SEA-1355 (lanes a and b), SEA-1374 (lanes c and d), SEA-1375 (lanes e and f), SEA-1358 (lanes g and h), SEA-1359 (lanes i and j), or SEA-1361 (lanes k and l). The position of the signal corresponding to wild-type SEA is indicated on the left. Signals corresponding to proteins with molecular weights greater than 30,000 are probably protein A; these signals, unlike those identified as SEA related, are visible in blots reacted only with normal rabbit serum (data not shown).

of all the expected CNBr-derived fragments except CNBr-SEA[225–233]; perhaps this fragment lacks an antigenic epitope, or the fragment migrated out of the gel. The signal corresponding to CNBr-SEA[108–224] was very light but was visible between the more intense signals which corresponded to CNBr-SEA[108–233] and CNBr-SEA[1–107] (Fig. 2B).

When tested for their ability to bind to CNBr-derived SEA fragments, four of the five MAb preparations examined produced the same blotting patterns (data not shown); one representative MAb, designated MAb C1, was chosen for further study. The MAb with the unique blotting pattern was designated MAb E8. MAb C1 bound to wild-type SEA and to CNBr-SEA[108–233], which was a product of incomplete cleavage. MAb C1 did not bind detectably to any of the other CNBr-derived SEA fragments. MAb E8 reacted with SEA but did not react detectably with any of the CNBr-derived SEA fragments (Fig. 2B).

To better characterize the binding requirements of MAbs C1 and E8, the two MAbs were reacted separately in Western blots with mutant SEAs that had deletions beginning at the C terminus and extending into the molecule (33). These mutant SEAs all bind to polyclonal anti-SEA antiserum (33) (Fig. 3). MAb C1 reacted with SEA-1317 and SEA-1316, which contain residues 1 through 232 and 1 through 230, respectively (Fig. 3). MAb C1 did not react detectably with SEA-1315, SEA-1310, or SEA-1309, which contain residues 1 through 226, 1 through 194, and 1 through 168, respectively. MAb E8 reacted with SEA-1317 but did not react detectably with any of the other SEA mutants that had larger deletions in the C-terminal region (Fig. 3).

These experiments established that the epitope for MAb C1 was contained in the peptide consisting of residues 108 to 230. In the next series of experiments, MAb C1 was used in Western blots to quantify unpurified mutant SEAs containing internal deletions in their N-terminal third.

Examination of N-terminal mutant SEAs by serological assays. The culture supernatants of the different SA20 derivatives were tested for their ability to react with polyclonal anti-SEA antiserum in a Western blot. All mutant SEAs reacted with polyclonal serum, and the signals corresponded to the predicted sizes (Fig. 4 and Table 3). These supernatants were tested for sensitivity to monkey stomach lavage fluid, a treatment that does not degrade wild-type SEA (33). SEA-1364 (with a predicted amino acid sequence identical to that of wild-type SEA) and SEA-1351 (D60G; the positive control for the SEA deletion mutants) were both resistant to this treatment (Fig. 4). Each of the SEA deletion mutants was degraded by the stomach fluid to different extents (Fig. 4).

MAb C1 and MAb E8 were used as the primary antibodies in Western analysis of culture supernatants containing the mutant SEAs (Fig. 5). All the mutant SEAs reacted with MAb C1. MAb C1 also bound efficiently to SEA-related breakdown products in each culture supernatant. This is in contrast with MAb E8, which gave a detectable reaction only with SEA-1364, SEA-1351, SEA-1372, SEA-1374, and SEA-1375 (Fig. 5).

The concentrations of the mutant SEAs in the SA20 culture supernatants were quantified by Western analysis with MAb C1. A twofold dilution series of each culture supernatant was prepared and run on an acrylamide gel alongside known concentrations of SEA. Except for SEA-1352, each undiluted supernatant that contained a mutant SEA had at least 4 μg of full-length SEA-related material per ml (data not shown). Western analysis of SEA-1352 consis-

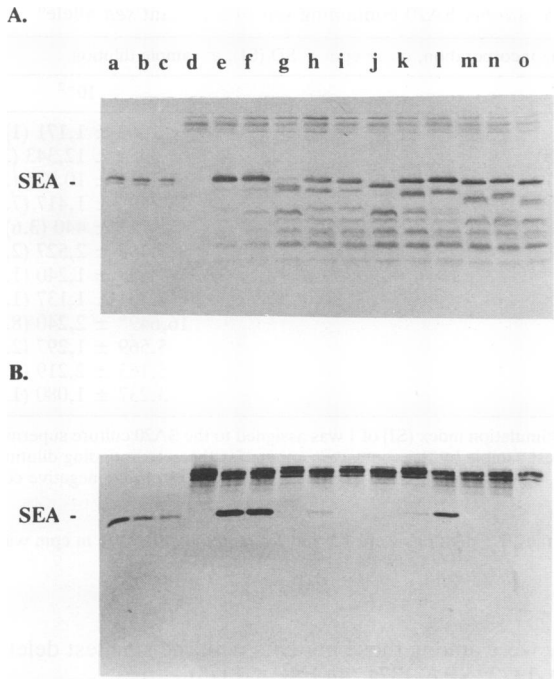

FIG. 5. Western blot analysis of purified SEA and culture supernatants from *S. aureus* SA20 or SA20 derivatives that contained *sea* or an *sea* mutation. Blot A was reacted with MAb C1, and blot B was reacted with MAb E8. Lanes a, b, and c contained 4, 2, and 1 μg of SEA per ml, respectively. Samples were from strain SA20 (lane d; produces no SEA), SEA-1364 (lane e), SEA-1351 (lane f), SEA-1352 (lane g), SEA-1372 (lane h), SEA-1373 (lane i), SEA-1355 (lane j), SEA-1374 (lane k), SEA-1375 (lane l), SEA-1358 (lane m), SEA-1359 (lane n), and SEA-1361 (lane o). The position of the signal corresponding to wild-type SEA is indicated by the dash. Signals corresponding to proteins with molecular weights greater than 30,000 are probably protein A; these signals, unlike those identified as SEA related, are visible in blots reacted only with normal rabbit serum (data not shown).

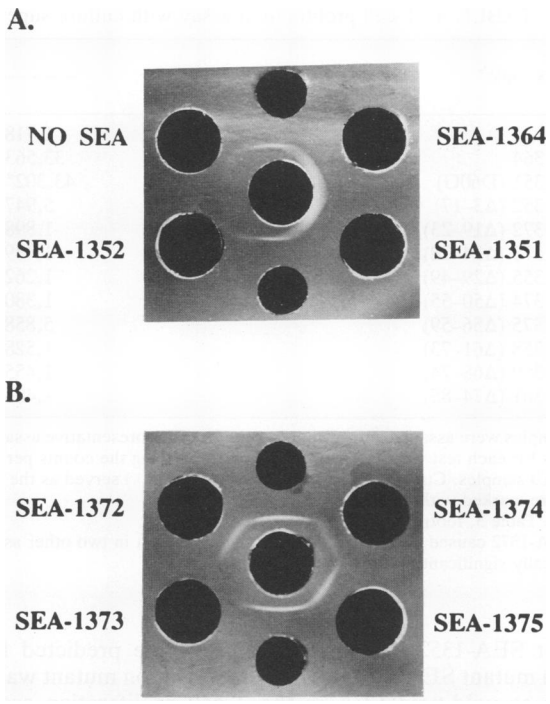

FIG. 6. Gel double-diffusion assay. The center well of each plate contained polyclonal anti-SEA antiserum. The top and bottom wells of each plate contained 4 μg of SEA per ml. Side wells contained culture supernatant from SA20 or SA20 derivatives expressing the indicated wild-type or mutant SEA.

tently revealed a faint signal corresponding to the predicted size and a more intense signal which migrated slightly faster; the concentrations of the two signals were less than 1 μg/ml and about 4 μg/ml, respectively.

The culture supernatants from each SA20 derivative were tested in a gel double-diffusion assay against anti-SEA antiserum. SEA-1364, SEA-1351, SEA-1372, SEA-1374, and SEA-1375 formed precipitin lines in the assay (Fig. 6). SEA-1352, SEA-1373, and the remaining mutant SEAs did not react in this assay, even when the culture supernatants were concentrated fivefold (Fig. 6 and data not shown).

Induction of T-cell proliferation by N-terminal mutant SEAs. Culture supernatants from the panel of SA20 derivatives and their corresponding 10^{-1} and 10^{-2} dilutions were tested for the ability to induce T-cell proliferation of murine splenocytes (Table 4). The supernatants used were the same preparations quantified by Western analysis with MAb C1, as described above, and both experiments were performed on the same day. All supernatants except that containing SEA-1352 had at least 4 μg of the full-length mutant SEA per ml. Unlike the equivalent dilutions from a culture supernatant of SA20 (Sea^-), the culture supernatants containing SEA-1364, SEA-1351, SEA-1352, and SEA-1375 induced a reproducible, statistically significant increase in the incorporation of [^{3}H]thymidine. SEA-1352 and SEA-1375 induced about a 7-fold increase in [^{3}H]thymidine incorporation, and 30- and 26-fold increases in [^{3}H]thymidine incorporation were induced by SEA-1364 and SEA-1351, respectively. SEA-1373, SEA-1355, SEA-1374, SEA-1358, SEA-1359, and SEA-1361 did not induce a statistically significant increase in the incorporation of [^{3}H]thymidine. SEA-1372 induced a weak response which was only statistically significant in one of three assays.

DISCUSSION

SEAs with internal deletions in the region corresponding to residues 1 to 85 of SEA were examined for serological reactivity and ability to induce T-cell proliferation. Our strategy was to construct a series of mutant SEAs that had deletions spanning short segments within this region. First, a number of unique restriction sites were introduced into the N-terminal region of *sea*, yielding *sea-1351*. SEA-1351 had one amino acid substitution (D60G) compared with wild-type SEA. This residue is not among those conserved in any of the other SEs (7). SEA-1351 was as resistant as wild-type SEA in the stomach lavage fluid assay, reacted the same as SEA in Western blot and gel double-diffusion assays, was emetic, and induced murine T-cell proliferation. Because SEA-1351 reacted like SEA, its gene, *sea-1351*, was an appropriate template for deletion constructions. SEA-1351 served as the positive control during characterization of the deletion mutants.

Our laboratory has previously established that a reproducible, statistically significant T-cell proliferative response is obtained with dilutions of an SA20 culture supernatant containing 2 μg of SEA per ml (33). All of the mutant SEAs

TABLE 4. T-cell proliferation assay with culture supernatants from *S. aureus* SA20 containing *sea* or a mutant *sea* allele[a]

Sample[b]	[^{3}H]thymidine incorporation, mean cpm ± SD (SI), at sample dilution:	
	10^{-1}	10^{-2}
SA20	1,718 ± 1,089 (1)	2,062 ± 1,171 (1)
SEA-1364	33,563 ± 18,687 (19.5)	65,558* ± 12,343 (30.8)
SEA-1351 (D60G)	43,302* ± 1,267 (25.2)	54,703* ± 10,975 (26.5)
SEA-1352 (Δ3–17)	5,947 ± 4,038 (3.4)	14,790* ± 1,417 (7.2)
SEA-1372 (Δ19–23)	1,898 ± 2,148 (1.1)	7,511* ± 440 (3.6)[c]
SEA-1373 (Δ24–28)	1,489 ± 1,309 (0.8)	4,486 ± 2,527 (2.2)
SEA-1355 (Δ29–49)	1,262 ± 634 (0.7)	3,625 ± 1,240 (1.8)
SEA-1374 (Δ50–55)	1,380 ± 1,322 (0.8)	2,704 ± 1,137 (1.3)
SEA-1375 (Δ56–59)	5,858 ± 2,831 (3.4)	16,649* ± 2,240 (8.0)
SEA-1358 (Δ61–73)	1,528 ± 1,044 (0.9)	5,569 ± 1,297 (2.7)
SEA-1359 (Δ68–74)	1,455 ± 820 (0.8)	5,163 ± 2,219 (2.5)
SEA-1361 (Δ74–85)	1,403 ± 818 (0.8)	3,237 ± 1,080 (1.5)

[a] Samples were assayed in triplicate; results from a representative assay are shown. A stimulation index (SI) of 1 was assigned to the SA20 culture supernatant. The SIs for each test sample were calculated by dividing the counts per minute for the test sample by the counts per minute for the corresponding dilutions of the SA20 samples. Culture supernatant from SA20 (Sea$^-$) served as the negative control. Values that differed significantly ($P < 0.01$) from the negative control value are marked with an asterisk.

[b] See Table 3, footnote *a*.

[c] SEA-1372 caused a weak response in this assay, but in two other assays, the SIs of the 10^{-2} dilutions were 1.1 and 1.4 (and the difference in cpm was not statistically significant).

except SEA-1352 had at least 4 μg of the predicted full-length mutant SEA per ml. If an SEA deletion mutant was as active as wild-type SEA in the T-cell proliferation assay, significant incorporation of [^{3}H]thymidine should have been observed. Of the deletion mutants, only SEA-1352 and SEA-1375 consistently induced a statistically significant response in the T-cell proliferation assay. These results indicate that residues 3 through 17 and 56 through 59 of SEA are not required for T-cell proliferation of murine splenocytes. The magnitude of the response induced by culture supernatants containing SEA-1352 or SEA-1375 was lower than that induced by the positive control, SEA-1351 (stimulation indices of the 10^{-2} dilution were 7, 8, and 26, respectively). The lower activity of the mutants could be due either to decreased specific activity of the mutants or to lower concentrations of the mutant SEAs than of SEA-1351. Mutants that failed to give a positive response in this assay are clearly less active than wild-type SEA. However, they may have weak activity which might be evident if tested at higher concentrations.

All of the SEA deletion mutants had conformations that differed from that of wild-type SEA. Western blot analysis of culture supernatants revealed substantially more SEA-related breakdown products for the SEA deletion mutants than for either SEA or SEA-1351. Each mutant SEA was degraded by monkey stomach lavage fluid in vitro under conditions that did not degrade wild-type SEA or SEA-1351 (Table 3). These two findings indicate that the SEA deletion mutants have conformations unlike that of wild-type SEA or SEA-1351. Because the SEA deletion mutants were degraded in vitro by stomach lavage fluid, they were not tested for emetic activity under the assumption that they would be degraded in vivo.

The lack of reaction of SEA-1373, SEA-1355, SEA-1358, SEA-1359, and SEA-1361 in the gel double-diffusion assay also indicated that these mutants had different conformations than wild-type SEA. However, it is possible that the deletions resulted in the loss of a major antigenic determinant. The lower limit of detection in the gel double-diffusion assay is 0.5 μg/ml (57), which is lower than the concentration of each of the mutant SEAs tested. The only mutant SEAs that produced precipitin lines in the gel double-diffusion assay were among those mutants with the smallest deletions (SEA-1372, SEA-1374, and SEA-1375).

From the observation that a synthetic peptide corresponding to residues 1 to 27 of SEA competes with SEA in a T-cell proliferation assay with human peripheral mononuclear cells but does not cause T-cell proliferation, Pontzer et al. suggested that this peptide contains residues important for MHC class II binding (55). We found that SEA-1352 (lacking residues 3 to 17) was effective at inducing proliferation of murine splenocytes. If the requirements for MHC class II binding are the same in the human and murine systems, then residues 1 and 2 and/or 18 to 27 may contain residues required for MHC class II binding. Alternatively, the deletion in SEA-1352 may result in the loss of one MHC class II binding site, but there may be other sites contained on SEA-1352 which are sufficient for MHC class II binding. There is evidence of multiple binding sites on SEA (26).

Kappler et al. have demonstrated the importance of residue 14 in SEB for binding to MHC class II molecules (40). SEA-1352 (lacks SEA residues 3 to 17, which include the residue corresponding to residue 14 of SEB) stimulated proliferation of murine splenocytes. Apparently the SEA peptide spanning residues 3 to 17 of SEA does not contain residues required for binding murine MHC class II molecules, in contrast to the corresponding region in SEB, which is required for binding to human MHC class II molecules.

Some of the residues conserved between SEA and SEB may serve the same function in T-cell stimulation. Four of our SEA mutants which did not induce T-cell proliferation (SEA-1372, SEA-1373, SEA-1355, and SEA-1374) lacked peptides which included residues conserved with SEB and which Kappler et al. have demonstrated as being important for SEB's stimulation of T cells. However, it is unknown whether the loss of T-cell-stimulatory activity of the individual SEA deletion mutants was due to loss of an active-site residue (e.g., required for binding to either MHC class II or Vβ) or to a change in conformation compared with wild-type SEA.

Two MAbs that have different requirements for binding to SEA were identified. CNBr-SEA[108–233] contains residues necessary for efficient binding of MAb C1. Taken together with the finding that MAb C1 reacted with SEA-1317 but not

with the other C-terminal SEA deletion mutants, it appears that the epitope for MAb C1 lies within residues 108 to 230 of mature SEA. Consistent with this interpretation is the observation that MAb C1 reacted efficiently with each mutant SEA that had a deletion within the region corresponding to residues 3 through 85.

There was no visible reaction between MAb E8 and any of the CNBr-derived SEA fragments, including the incomplete cleavage products (CNBr-SEA[1–224] and CNBr-SEA[108–233]). This finding suggested that MAb E8 does not recognize a sequential epitope. MAb E8 bound detectably with 1 μg of wild-type SEA per ml. All of the N-terminal deletion mutants of SEA except SEA-1352 were present at a concentration of at least 4 μg/ml. Therefore, if the reactions between the mutant SEAs and MAb E8 were as efficient as the reaction between wild-type SEA and MAb E8, then binding of MAb E8 to the deletion mutants should have been detected. However, MAb E8 did not react detectably with SEA-1373, SEA-1355, SEA-1358, SEA-1359, SEA-1361, or SEA-1316, which had deletions corresponding to SEA residues 24 to 28, 29 to 49, 61 to 73, 68 to 74, 74 to 85, and 231 to 233, respectively. MAb E8's lack of reaction with these six different mutant SEAs further supports the suggestion that MAb E8's epitope is conformational rather than sequential.

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