Analysis of Toxicity of Streptococcal Pyrogenic Exotoxin A Mutants

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Received 30 December 1996/Returned for modification 6 March 1997/Accepted 30 April 1997

Streptococcal pyrogenic exotoxin A (SPE A) is secreted by some strains of *Streptococcus pyogenes* and is strongly associated with streptococcal toxic shock syndrome (STSS), a severe and often fatal illness. SPE A possesses a number of biological properties, some of which are shared with a group of exotoxins of streptococcal and staphylococcal origins, the pyrogenic toxin superantigens (PTSAgs). SPE A's most extensively studied property is superantigenicity. Superantigenic activation of T cells and monocytes stimulates the release of cytokines such as tumor necrosis factors alpha and beta, interleukin 1, and gamma interferon. These endogenous mediators are considered to be the primary cause of capillary leak, hypotension, and shock, the most severe manifestations of STSS. However, several studies have suggested that other properties of SPE A, such as ability to greatly enhance host susceptibility to endotoxin and ability to interact directly with endothelial cells, may play substantial roles in the syndrome. In this work we generated single- and double-site mutations of SPE A at residues K16, N20, C87, C90, C98, K157, S195, N20/C98, and N20/K157. The mutant SPE A's were analyzed in vivo for their lethal activity and in vitro for their superantigenic ability. Our results indicate that SPE A's ability to induce lethality and endotoxin enhancement does not require superantigenicity, and conversely superantigenicity does not necessarily lead to lethality. Thus, these properties and their relative contributions to the onset of hypotension and shock may be separable. Furthermore, evidence is presented that certain mutant toxins may be suitable for use as vaccine toxoids.

Streptococcal pyrogenic exotoxin A (SPE A) (or scarlet fever toxin A) is made and secreted by many strains of *Streptococcus pyogenes* (also known as group A streptococci) isolated in cases of streptococcal toxic shock syndrome (STSS), a severe and often fatal multiorgan illness (2, 3). Several lines of evidence indicate that SPE A is a significant virulence factor in the pathogenesis of the illness: the toxin is very often present in streptococcal M types associated with STSS, and the majority of those streptococcal strains produce SPE A or carry its coding gene (11, 29); SPE A in a purified form can induce symptoms of STSS in a rabbit animal model (22) and in humans in laboratory accidents; furthermore, the purified toxin administered to rabbits confers significant protection from challenge with viable M1 and M3 streptococci (36), the two M types most often associated with STSS.

SPE A is a member of a large family of toxins of streptococcal and staphylococcal origin (2, 30, 35), the pyrogenic toxin superantigens (PTSAgs), grouped because of their functional and structural similarities. Among these toxins are SPE A, B, C, and F and streptococcal superantigen, which are associated with cases of STSS, and the staphylococcal exotoxins toxic shock syndrome toxin 1 (TSST-1) and enterotoxins A to I excluding F, associated with toxic shock syndrome (TSS). From recent reports on crystallographic studies of the latter group of molecules (14, 33, 34, 41) it is apparent that the toxins' three-dimensional structures are closely related. Computer modeling studies propose the same type of folding for SPE A (12) (Fig. 1). Major features of the kidney-shaped molecule are a 26-residue amino-terminal segment at the top of domain A, of which only the last portion is highly organized. The remainder of the amino-terminal half of the protein (up to residue 106)

folds into domain B, which is composed of a β -barrel structure, a short α -helix at the bottom, and an apparently unorganized 12-residue fragment. In the staphylococcal enterotoxin structure a disulfide bridge between two cysteine residues holds this apparently unorganized fragment in a loop. Although analog cysteine residues in the SPE A primary sequence are present, no evidence thus far has directly demonstrated the presence of a cystine loop. The carboxyl half of the toxin is folded into domain A (Fig. 1), whose major features are a wall of β -strands, the back long diagonal α -helix, and the front short α -helix.

Several of SPE A's immunobiological properties are shared with the other toxins of the family. The most extensively studied property for all the PTSAgs is superantigenicity (26, 35), which consists of their ability to interact with and activate up to 50% of the host T lymphocytes. Superantigens bind to the variable region of the β chain of the T-cell receptor and to major histocompatibility complex (MHC) class II outside the antigen binding site and promote MHC class II and T-cell receptor cross-linking independently of antigen specificity. Since the binding specificity of superantigens is limited only to the β chain of the T-cell receptor, each toxin can stimulate a large variety of T-cell clones. Superantigenic activation leads to T-cell proliferation and secretion of abnormally high levels of a number of cytokines both from lymphocytes and monocytes. Superantigenicity may be a contributing cause of shock and death in individuals affected by TSS.

Other properties of the PTSAgs may also contribute to the symptoms and the fatal outcome of this illness. All of the toxins enhance by several orders of magnitude host susceptibility to endotoxin shock (2), they can cause lethality in a rabbit model of TSS (22, 31), and they cause B-lymphocyte immunosuppression (2). TSST-1 has been shown also to bind isolated endothelial cells (20, 23). In addition to these properties, SPE A also possesses the unique capabilities to enhance cardiac dam-

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TABLE 1. Codon changes and respective amino acid changes introduced in SPE A by site-directed mutagenesis

Mutant	Codon change(s)	Amino acid change(s)
K16N	AAA→AAC	Lys→Asn
N20D	AAT→GAT	Asn→Asp
C87S	TGT→AGC	Cys→Ser
C90S	TGT→TCT	Cys→Ser
C98S	TGT→TCG	Cys→Ser
K157E	AAG→GAG	Lys→Glu
S195A	TCT→GCT	Ser→Ala
N20D/C98S	AAT→GAT, TGT→TCG	Asn→Asp, Cys→Ser
N20D/K157E	AAT→GAT, AAG→GAG	Asn→Asp, Lys→Glu

age from endotoxin or streptolysin O (39, 40) and to form a stable complex with lipopolysaccharide (LPS) that is lethal to T lymphocytes (24).

In this study we attempted to define domains of SPE A contributing to some of its immunobiological activities. The residues chosen for mutagenesis are present in organized domains of SPE A. We generated several simple site-specific mutants of SPE A. These residues are exposed to the surface and are in regions proposed in other PTSAGs to affect biological activities. We also present evidence to suggest that some of these mutants may be useful as toxoid vaccines. Finally, we present data further suggesting that SPE A, similarly to the staphylococcal enterotoxins, has a sulfhydryl linkage between two of its cysteine residues.

MATERIALS AND METHODS

Site-directed mutagenesis. Single-site mutants of SPE A were generated by PCR, by a modification of the protocol of Perrin and Gilliland (32). Briefly, for

each mutant an internal primer containing the appropriate nucleotide changes and a flanking 3' primer were used in the ratio 5:100 in a PCR. The template was a DNA fragment, previously cloned in pBR328 (17), containing the *speA* structural gene from phage T12, together with known and probable regulatory sequences. The products of these reactions were end flushed by Klenow fragment of DNA polymerase (5, 13), electrophoresed in agarose, purified by the Gene-clean method, and used as primers, together with a flanking 5' primer, in a second PCR. The products of the latter reactions were DNA fragments of 1.75 kb containing mutated *speA*. The mutant proteins generated after mutagenesis and the corresponding nucleotide changes are listed in Table 1. Their localization in the SPE A molecule is shown in Fig. 1. Reagents for PCR (Ampli-Taq DNA polymerase) were purchased from Perkin-Elmer (Norwalk, Conn.).

Cloning. Mutant DNA fragments were cloned in shuttle vector pMIN164. This vector was constructed by fusion of *Escherichia coli* plasmid pBR328 (Amp^r) and *Staphylococcus aureus* plasmid pE194 (Em^r) (27, 28). Ligated DNA was transformed in *E. coli* DH5 α . Clones were tested for SPE A expression in a double-immunodiffusion assay using rabbit polyclonal antibodies raised against wild-type SPE A. Clones that tested positive were completely sequenced, and their DNAs were transformed into *Staphylococcus aureus* RN4220 by protoplast transformation (4). This staphylococcal host does not secrete enterotoxins A to E or TSST-1 as determined by lack of reactivity of culture fluids with antisera specific for those toxins. Furthermore, culture fluids lack lymphocyte mitogenic activity. Toxin expression and secretion by erythromycin-resistant colonies were detected by a double-immunodiffusion assay.

Double-stranded DNA was sequenced by a modification of the dideoxynucleotide chain termination reaction for double-stranded DNA (16) (Sequenase T7 DNA polymerase version 2.0 kit; U.S. Biochemicals, Cleveland, Ohio).

Double-mutant construction. Double mutant N20D/K157E SPE A was constructed by PCR, as described above, using N20D SPE A DNA as template and the mutant primer for K157E. Double mutant N20D/C98S was constructed by swapping the *Bam*HI-*Bst*EII restriction fragment of C98S DNA with the corresponding restriction fragment in N20D mutant DNA. In both cases, the resulting double-mutant genes were completely sequenced.

Toxin production and purification. Toxins were produced and purified from *Staphylococcus aureus* RN4220 as previously described (38). Briefly, strains carrying wild-type or mutant *speAs* in pMIN164 were grown to stationary phase at 37°C with vigorous shaking for 18 h in pyrogen-free dialyzed beef heart medium containing 5 μ g of erythromycin per ml. Cell cultures were precipitated with ethanol, and pellets were resuspended in small volumes of pyrogen-free water and cleared by centrifugation. Supernatants were dialyzed and subjected to

FIG. 1. Ribbon diagram of the modeled three-dimensional structure of SPE A. Organized structures such as β -strands and α -helices are represented. Domains A and B are indicated. Alpha carbons of the mutated residues are represented as spheres.

successive flatbed isoelectric-focusing separations in pH gradients of 3.5 to 10 and 4 to 6. Toxin-containing fractions were extensively dialyzed in pyrogen-free water, aliquoted, and kept lyophilized until use. Purity was assessed by Coomassie staining of sodium dodecyl sulfate (SDS)–15% polyacrylamide gels (21).

Stability measurements of mutants. Indication of mutant stability was obtained from evaluating toxin reactivity to anti-SPE A polyclonal antibodies and toxin susceptibility to trypsin cleavage. To evaluate reactivity to antibodies, equal amounts of toxins, as determined from band density in Coomassie-stained gels, were serially diluted 1:2. Each dilution was incubated with polyclonal antiserum to wild-type SPE A at 37°C for 4 h in a double-immunodiffusion assay. For all the toxins the highest dilution forming a precipitation band was determined. The lowest wild-type SPE A concentration at which a precipitate can be seen was previously determined to be 5 µg/ml. Since toxin quantity, as determined by density of bands in SDS-polyacrylamide gels, and toxin amounts determined by serial-dilution double immunodiffusion agree with each other, both assays were routinely utilized to quantify both wild-type and mutant SPE A's. To determine susceptibility of the mutants to trypsin cleavage, toxins (5 µg) were incubated with trypsin (10 µg) in a 100-µl reaction mixture at 37°C. Samples were withdrawn at 0, 10, and 60 min. Reactions were stopped by addition of SDS sample buffer, proteins were resolved in SDS–15% polyacrylamide gel electrophoresis, and the toxins were Western immunoblotted.

Lethality. Lethality was assessed by subcutaneous implantation of miniosmotic pumps in American Dutch belted rabbits (22, 31). The pumps were preloaded with 200 or 500 µg of wild-type or mutant toxin in phosphate-buffered saline (0.005 M NaPO₄, 0.15 M NaCl) at pH 7.2. These devices are designed to release a constant amount of toxin over a period of 7 days. Animals were monitored for symptoms of STSS, and deaths were recorded over a 15-day period. Residual toxic effects were evaluated by necropsic examination of the surviving animals.

The actual amount of SPE A released from miniosmotic pumps was measured after both 2 and 4 days of exposure of two rabbits to SPE A (200 µg/animal). At those time points pumps were removed from the animals and the amount of SPE A remaining within each pump was quantified by serial 1:1.1 dilution double immunodiffusion beginning with a 1:16 dilution. Control for antibody reactivity was the same SPE A preparation diluted comparably except that it was not administered to rabbits in miniosmotic pumps. The results indicated that the animals were exposed to 40 µg of toxin by day 2 and 80 µg by day 4. These levels are approximately 70% of those predicted by the manufacturer but suggest that the exposure rate was constant at 20 µg/day.

Endotoxin shock enhancement. American Dutch belted rabbits were pre-treated with sublethal doses of wild-type or mutant SPE A (5 µg/kg of body weight), administered intravenously (i.v.) in the marginal ear vein (38). Four hours later, animals were challenged with sublethal doses of endotoxin from *Salmonella typhimurium* as prepared by the hot-phenol method (42) (1 or 10 µg/kg of body weight, equal to 1/500 and 1/50 50% lethal dose, respectively) via the same route. Deaths were recorded for the 48 h after the second injection. Residual toxic effects were evaluated by necropsic examination of surviving animals.

Superantigenicity. Rabbit or mouse splenocytes or human peripheral blood mononuclear cells in RPMI cell culture medium were dispensed in 96-well microtiter plates, 2×10^5 /well for rabbit and human cells and 5×10^5 /well for mouse cells, and incubated at 37°C in 7% CO₂ with serial 1:10 dilutions of wild-type or mutant SPE A for 72 h (1). Cells were pulsed for 18 h with [³H]thymidine (Amersham Corporation, Arlington Heights, Ill.), 1 µCi/well. Cells were harvested on fiberglass filters, and counts per minute due to [³H]thymidine incorporation into DNA of proliferating cells were measured in a scintillation counter. Each sample was tested in quadruplicate. Counts from cells not treated with toxin were considered background.

RESULTS

Generation of SPE A mutants. Single- and double-site mutants were generated by PCR. The residues individually changed and characteristics of the mutations are listed in Table 1. Figure 1 shows the position of those residues in the SPE A three-dimensional structure. Mutant DNA fragments were cloned in vector pMIN164 to facilitate cloning in *E. coli* and to allow expression and production of the toxin in *Staphylococcus aureus*. The choice of the bacterial host for toxin production was dictated by the necessity to obtain the toxin in an LPS-free environment, due to SPE A's ability to bind LPS, yet devoid of contaminant streptococcal virulence factors. Previous work has shown that SPE A obtained from *Staphylococcus aureus* conserves intact biological properties associated with streptococcus-derived toxin (2). In addition, the staphylococcal host used, RN4220, does not make detectable PTSAGs (data not shown).

Toxin-expressing clones were identified based on reactivity

TABLE 2. Stability measurements of SPE A and PCR-generated mutants^a

Protein	Yield ^b
SPE A.....	400
K16N.....	200
N20D.....	500
C87S.....	25
C90S.....	420
C98S.....	130
K157E.....	370
S195A.....	330
N20D/C98S.....	ND ^c
N20D/K157E.....	ND

^a All proteins were resistant to cleavage after 5 µg of wild-type or mutant SPE A was incubated with 10 µg of trypsin in 100-µl volumes, as evaluated by use of SDS-polyacrylamide gels.

^b In micrograms of purified SPE A or mutant recovered per liter of stationary-phase culture. The data are averages for several toxin preparations as determined by toxin concentrations estimated by both intensity of Coomassie blue-stained gels and serial twofold-dilution double immunodiffusion.

^c ND, not determined.

of cell culture supernatants to anti-SPE A polyclonal antibodies in double-immunodiffusion assays.

Stability of SPE A mutants. The possibility of loss of stability of the toxins due to the single or double amino acid changes introduced was investigated by several methods (Table 2). The mutants were all equally reactive to polyclonal antibodies raised against native SPE A, indicating that major epitopes were not affected by the mutations introduced. This was expected since presumed surface-exposed residues were mutagenized. Resistance to trypsin digestion was also evaluated, since in spite of having 24 potential cleavage sites, SPE A is normally resistant to trypsin cleavage. Mutant SPE A's displayed resistance to prolonged trypsin digestion identical to wild type, suggesting that trypsin cleavage sites did not become exposed as a consequence of introducing mutations. Mutants K16N, N20D, C90S, K157E, S195A, N20D/C98S, and N20D/K157E were recovered from culture fluids in amounts comparable to native SPE A. Mutants C87S and C98S were recovered in much lower amounts. Both of the latter mutant proteins copurify with a band of lower molecular weight which cross-reacts with antibodies to SPE A in Western blots and is also resistant to further trypsin cleavage (Fig. 2). Such a band is likely to be a degradation product of a less stable mutant protein.

Lymphocyte proliferative activity. The ability to stimulate lymphocyte proliferation was measured in vitro as [³H]thymidine incorporation into cellular DNA of splenocytes incubated with serial dilutions of the mutant toxins. Rabbit cells were chosen for one set of studies, in order to maintain consistency with the rabbit animal models used for in vivo toxicity studies. Results of these assays are shown in Table 3. Experiments with the mutants were carried out at different times, and therefore, wild-type control data for each experiment are presented. Mutant K16N retained the ability to induce wild-type lymphocyte proliferation. However, a mutation, N20D, only 4 residues away but localized in a more organized structure of the molecule, had a dramatic effect on the lymphocyte proliferative ability of the toxin. N20D induced lymphocyte proliferation only slightly above background at all concentrations except the highest dose tested. Individual cysteine residues changed to serines caused diverse effects on SPE A lymphoproliferative properties. Substitution of the sulfhydryl group of cysteines 87 and 98 with a hydroxyl group caused a dramatic reduction of the toxin's lymphoproliferative ability. In contrast, the same

FIG. 2. Western blot of wild-type SPE A and mutants C87S and C98S, each before incubation (T0) or after incubation with trypsin at 37°C (10 or 60 min). Proteins were resolved in SDS-15% polyacrylamide gels and blotted onto nitrocellulose (pore size, 0.45 μ m), and toxins were visualized with rabbit polyclonal antibodies to SPE A, alkaline phosphatase-conjugated anti-rabbit antibodies, and then substrate. The positions of molecular mass marker bands (in kilodaltons [kD]) are indicated.

substitution at position 90 had less significant effects. Mutant S195A was comparable to wild-type SPE A in its ability to stimulate lymphocyte proliferation. When mutants C90S and S195A were assayed, splenocytes showed peak stimulation by both control and test toxins in the 1- to 10-ng/well range, rather than at the highest dose used (1,000 ng/well). Mutant

TABLE 3. Rabbit splenocyte proliferative ability of SPE A mutants compared to the wild type

Protein	1,000 ng/well			100 ng/well			10 ng/well			1 ng/well		
	cpm (10 ³) ^a	SD ^b	% ^c	cpm (10 ³)	SD	%	cpm (10 ³)	SD	%	cpm (10 ³)	SD	%
K16N	71	17	72	92	12	120	81	4	96	72	17	86
SPE A	98	13		76	9		84	15		84	8	
N20D	57	7	16	46	6	13	12	2	5	12	2	27
N20D/C98S	0.7	1	0.2	9	1	2.5	20	2	9	17	2	39
N20D/K157E	41	5	12	39	5	11	15	2	7	11	1	25
SPE A	355	45		359	45		221	28		44	6	
C87S	0	0	0	0	0	0	7	2	23	4	2	18
C98S	0	0	0	5	2	9	11	2	37	6	2	27
SPE A	101	9		53	10		30	6		22	2	
C90S	9	1	33	21	3	60	47	7	90	58	2	130
SPE A	27	6		35	5		52	5		44	8	6
S195A	0.8	3	40	0.8	0	1.8	11	3	12	63	8	85
SPE A	2	2		45	8		91	27		74	7	
K157E	0.7	0.4	2	10	1	30	26	2	104	19	2	70
SPE A	42	8		33	5		25	5		27	3	

^a Resulting from incorporation of [³H]thymidine into DNA of proliferating splenocytes.

^b For quadruplicate samples.

^c Mutant activity divided by wild-type SPE A activity at the same dose and in the same assay $\times$ 100.

TABLE 4. Murine-splenocyte proliferative ability of SPE A mutants compared to the wild type

Protein	1,000 ng/well			100 ng/well			10 ng/well		
	cpm (10 ³) ^a	SD ^b	% ^c	cpm (10 ³)	SD	%	cpm (10 ³)	SD	%
N20D	7	3	6	19	3	13	12	5	18
C87S	0	0	0	0	0	0	0.2	2	0.3
C98S	1	2	0.9	4	2	3	2	2	3
K157E	121	5	110	33	6	23	5	3	7
SPE A	110	14		144	19		67	2	
N20D/C98S	0.1	0.2	0.6	0.2	0.1	7	2.0	0.9	87
N20D/K157E	2	2	12	0.3	0.2	10	0.7	0.8	30
SPE A	17	0.6		3	1		2.3	0.8	

^a Resulting from incorporation of [³H]thymidine into DNA of proliferating cells.

^b For quadruplicate samples.

^c Mutant activity divided by wild-type SPE A activity at the same dose and in the same assay $\times$ 100.

K157E was consistently inhibitory at 1,000 and 100 ng/well, although its control was not, indicating that this effect may be a consequence specific to the mutation introduced. At doses of 10 ng/well and lower the K157E mutant was able to stimulate lymphocyte proliferation as efficiently as wild-type SPE A.

The double mutant N20D/C98S was devoid of mitogenic activity at all toxin doses tested. Mutant N20D/K157E showed some mitogenic activity at the highest dose tested (though less than the single mutant N20D) but was nonmitogenic at all other doses.

Some mutant proteins were also evaluated for capacity to stimulate murine-splenocyte (Table 4) and human peripheral blood mononuclear cell (Table 5) proliferation as measures of cross species consistency in activity. Although some differences were seen, mutant proteins generally stimulated (or failed to stimulate) proliferation of murine and human cells similarly to what was seen with rabbit cells. Thus, the single-residue mutant N20D and both double mutants (N20D/C98S and N20D/K157E), when tested with murine and human lymphocytes, were significantly less mitogenic than the wild-type SPE A. Similarly, the two cysteine mutants C87S and C98S were not mitogenic for murine lymphocytes and were significantly less mitogenic at low doses compared to C90S when tested with

TABLE 5. Human peripheral blood mononuclear cell proliferative ability of SPE A mutants compared to the wild type

Protein	1,000 ng/well			100 ng/well			10 ng/well			1 ng/well		
	cpm (10 ³) ^a	SD ^b	% ^c	cpm (10 ³)	SD	%	cpm (10 ³)	SD	%	cpm (10 ³)	SD	%
N20D	31	5	78	8	3	21	3	0.1	4	0.3	0.4	
C87S	15	5	35	41	4	66	33	6	40	2	1.6	2.6
C90S	19	8	48	30	3.5	79	36	5	43	60	5	78
C98S	34	1.6	85	29	2.6	76	21	2	25	4	0.7	5
K157E	31	1.3	78	31	1.6	82	79	5	95	76	12	99
N20D/C98S	21	1.8	53	16	4	42	8	0.9	10	5	1.2	6
N20D/K157E	28	1.5	70	26	15	68	5	1.6	6	0.5	0.1	0.6
SPE A	40	5		38	3		83	8		77	6	

^a Resulting from incorporation of [³H]thymidine into DNA of proliferating cells.

^b For quadruplicate samples.

^c Mutant activity divided by wild-type SPE A activity at the same dose and in the same assay $\times$ 100.

TABLE 6. In vivo assays of lethality of mutant and wild-type SPE A's

Mutant	No. of rabbits that died/total no. of rabbits						<i>P</i> ^c
	Miniosmotic pumps ^a		Endotoxin enhancement ^b		Combined		
	Mutant	Wild type	Mutant	Wild type	Mutant	Wild type	
K16N	NT ^d	NT	6/7	3/3	6/7	3/3	0.7
N20D	0/4	3/4	1/6	6/6	1/10	9/10	0.0005
C87S	NT	NT	4/6	4/6	4/6	4/6	0.45
C90S	3/3	3/4	NT	NT	3/3	3/4	0.57
C98S	NT	NT	1/6	4/6	1/6	4/6	0.11
S195A	3/3	3/4	NT	NT	3/3	3/4	0.57
K157E							
200 µg	0/4	3/4	1/5	3/4	1/9	6/8	0.013
500 µg	1/5	4/5					0.10
N20D/K157E	NT	NT	0/3	3/3	0/3	3/3	0.05
N20D/C98S	NT	NT	0/3	3/3	0/3	3/3	0.05

^a 200 µg of protein.^b Rabbits were injected i.v. with sublethal doses of SPE A or mutant at 0 h and then injected i.v. at 4 h with sublethal doses of endotoxin derived from *Salmonella typhimurium*.^c Comparison of data combined from miniosmotic-pump and endotoxin enhancement experiments to the corresponding data for wild-type SPE A by Fisher's exact probability test.^d NT, not tested.

human lymphocytes. All of the cysteine mutants were comparably active at the higher doses tested. Finally, mutant K157E retained significant lymphocyte proliferative activity for both murine and human lymphocytes.

In vivo lethality studies. As a measure of lethality we tested the ability of the mutant toxins to enhance host susceptibility to endotoxin shock and/or their ability to cause shock in animals when released from subcutaneous miniosmotic pumps. These in vivo assays are typically well correlated. Endotoxin enhancement was induced by pretreating rabbits with sublethal doses of wild-type or mutant SPE A given i.v. and challenging 4 h later with sublethal doses of endotoxin from *Salmonella typhimurium* via the same route. Miniosmotic pumps, loaded with 200 or 500 µg of wild-type or mutant toxin, were implanted subcutaneously in the flank and released a constant amount of toxin over a period of 7 days.

Mutations K16N, C90S, and S195A did not reduce the lethal ability of SPE A compared to wild-type toxin (Table 6). Mutants N20D, N20D/C98S, and N20D/K157E, which were severely reduced in lymphocyte proliferative properties (Tables 3 to 5), were also significantly less lethal (Table 6) than the wild-type control, as tested by both miniosmotic-pump implantation and endotoxin susceptibility enhancement. Mutations C87S and C98S, both of which decreased superantigenicity (Tables 3 to 5), had different effects on lethality, with C87S being as lethal as the wild-type (Table 6) and C98S being less lethal, as judged by susceptibility to endotoxin enhancement. When mutant K157E, which conserved the ability to induce splenocyte proliferation in both rabbits and mice, was administered to rabbits in miniosmotic pumps at the dose of 200 µg/rabbit, all the rabbits tested survived the treatment (Table 6). None of the animals treated showed signs of distress. To assess whether this mutation caused complete loss of toxic ability of SPE A, lethality of the mutant was evaluated by using miniosmotic pumps loaded with a higher dose. At 500 µg/rabbit, one of the five rabbits tested succumbed. Similarly, this mutant was able to enhance susceptibility to endotoxin shock in one of the five rabbits tested. The rabbits surviving treat-

ment with both the high dose in miniosmotic pumps and the endotoxin shock enhancement were subjected to necropsy. Visual observation revealed abnormalities in several organs (i.e., severe congestion of lungs, spleen discoloration, and necrotic patches on both heart and liver). Together, these findings indicate that mutant K157E has much reduced lethal activity but conserves some residual multiorgan toxicity. Multiorgan abnormalities were not visually detected in surviving animals treated with either double-mutant toxin.

DISCUSSION

This study was initiated in the attempt to define domains of SPE A contributing to its immunobiological properties. Directed single amino acid changes were introduced in several regions of SPE A as defined by a model of the molecule's three-dimensional structure (Fig. 1) (12). A summary of the superantigenic and lethal properties of these mutants is presented in Table 7. Mutations of residues K16 and N20 caused profoundly different effects on the biological activities tested, in spite of their proximity in the primary structure. Although K16N conserved the ability to induce lymphocyte proliferation (Table 3) and was as able to enhance susceptibility to endotoxin shock as wild-type SPE A (Table 6), N20D had severely reduced lymphocyte proliferative activity and was not lethal in either in vivo model used. The different behaviors of the two mutant toxins are consistent with the predicted topology of residues 16 and 20 in the folded molecule. K16 is in a relatively loose part of the structure (Fig. 1), facing the front of the molecule; N20 is located in α -helix 3, which borders a deep groove on the back of the molecule. The latter residue aligns with N23 of SEB and N25 of SEC3 (6, 7, 9, 10, 14, 18, 25), both of which share 50% sequence similarity with SPE A. A mutation in this Asn residue impairs the ability of these staphylococcal enterotoxins to bind the T-cell receptor. Finally, a recent study of the three-dimensional structure of SEC3 complexed with the β chain of the T-cell receptor indicated that residue N25 is a contact residue for the β chain (7). N20 of SPE A was suggested to have the analogous role of interacting with the T-cell receptor. This possibility is in agreement with the localization of residue N20 at the border of a structural deep groove that could well be a docking place for the T-cell receptor. Several residues in the analogous groove of TSST-1 have also been suggested to be important for T-cell receptor interaction.

In a recent paper, Kline and Collins (19) noted that single-amino-acid mutants (S12A, S13A, and N17A) had mitogenic activity nearly comparable to activity of wild-type SPE A. These data are consistent with the results obtained in the present study in which K16N also retained wild-type activity.

TABLE 7. Summary of toxicity studies of mutants of SPE A

Protein	Superantigenicity	Lethality
Wild type	+	+
K16N	+	+
N20D	—	—
N20D/C98S	—	—
N20D/K157E	—	—
C87S	—	+
C90S	+	+
C98S	—	—
K157E	+	—
S195A	+	+

However, in the prior study (19) it was reported that the mutant N20A retained 65% of wild-type SPE A mitogenicity, whereas in this study N20D was essentially nonmitogenic. This difference in activity may result from the N20D charge change of that surface residue such that the T-cell receptor cannot dock properly in the presence of that negatively charged residue, whereas docking may be permitted by the relatively innocuous N-to-A change.

Residue K157 is part of the diagonal α -helix on the back of the SPE A molecule, and its side chain is likely not to be facing the back groove. The K157E mutant was much less active than wild-type SPE A in inducing lethality in both the miniosmotic-pump and the endotoxin enhancement models (Table 6). However, as seen in necropsies of surviving animals, this mutant conserved some residual toxicity. Interestingly, although toxicity was significantly reduced, lymphocyte mitogenicity was not (Tables 3 to 5). This observation can be interpreted in different ways. First, residue K157 may itself interact with or stabilize interaction of the toxin with host receptors other than MHC class II and T-cell receptor—for example, those on the liver or on endothelial cells (20, 23, 37). The change from Lys to Asp could disturb interactions with such receptors and decrease toxicity while still permitting superantigenic effects. Alternatively, interaction of mutant K157E with immune-cell receptors may be sufficient to induce proliferation and stimulate production of some cytokines but not all the mediators necessary to inevitably tip the equilibrium toward induction of lethality.

Mutant S195A did not change SPE A toxicity. The mutant stimulated lymphocyte proliferation (Table 3) and was as lethal to rabbits as wild-type SPE A (Table 6). Since residue 195 is located in α -helix 6 and probably faces the solvent, it appears as though this front portion of SPE A is not involved in toxin-host cell interaction. This is consistent also with data of Kline and Collins (19), who observed that mutants with mutations in the same region, Q194A and L198A, retained 67 and 75% of wild-type SPE A mitogenicity.

Individual Cys residues changed to Ser had diverse effects on SPE A properties. The observation that mutants C87S and C98S were severely reduced in mitogenic ability (Tables 3 to 5) was not surprising. The region corresponding to domain B where cysteines 87 and 98 are located (Fig. 1) is important for the interaction of staphylococcal enterotoxins A and C1 with the T-cell receptor (8, 15, 19). Of greater interest was the observation that Cys 87 and 98, but less so Cys 90, were important for the lymphocyte proliferative activity of SPE A. This is suggestive of the possibility that Cys 87 and 98, but not Cys 90, are linked in a disulfide bond. This possibility was suggested as a result of prior analysis of SPE A superantigenicity (19). This interpretation is supported by our observation that individual mutants C87S and C98S are recovered from culture fluids in much lower amounts relative to wild type. Furthermore, these proteins are accompanied by a possible degradation product (Fig. 2), suggesting the mutants acquired susceptibility to one or more of the several proteases secreted by *Staphylococcus aureus* in stationary phase. Such a disulfide linkage may confer conformational stability as well as protease resistance to the long loop in domain B. As shown with SEC1 (15), the structure of the cysteine loop, and neither the sulfhydryl groups themselves nor other specific residues of the loop, would be required for efficient binding to an immune-cell receptor. The Cys mutants were also tested for their lethal capability. C90S conserved full lethality in miniosmotic pumps (Table 6), suggesting that Cys 90 is not implicated in this biological activity. Interestingly, ability to enhance endotoxin shock was affected differently by the other two Cys mutants,

with C98S being only weakly toxic but C87S being strongly toxic (Table 6). This observation may be interpreted as the result of different stabilities of the C87S and C98S molecules. Although both mutations may affect the conformation of the loop, C98S may affect it to a lesser extent. Although not detectable by the measurements we used, these differences might have a great impact on the interaction of the loop area with the receptors responsible for induction of lethality. Alternatively, we can speculate that in the absence of a sulfhydryl group at position 87, C90 might interact with C98 in either a disulfide linkage or a hydrogen bond. Although not optimal, and perhaps present in a small percentage of the molecules in solution, this conformation may stabilize the structure of the loop. The fraction of stabilized molecules may not be enough to elicit a full superantigenic response but may significantly contribute to toxicity, for example by interacting with MHC class II and stimulating monocytes to secrete cytokines.

Finally, in this study two double mutants were shown to have less biological activity than any of the single-amino-acid mutants. For example, the N20D/C98S mutant lacked detectable superantigenic activity, even at the highest doses tested. Similarly, the mutant lacked ability to induce lethality. The other double mutant N20D/K157E exhibited no superantigenic activity at any dose tested except the highest dose, where the activity observed was still below that seen with single mutants. Both of these mutants have the ability to induce antibodies in rabbits capable of neutralizing biological activities (mitogenicity, capacity to enhance susceptibility to lethal endotoxin shock, and ability to induce death when administered in subcutaneous miniosmotic pumps) of native SPE A (data not shown). Thus, these proteins may be useful for consideration as toxoid vaccines.

In certain experiments, most notably in testing mutants K157E and S195A compared to wild-type SPE A with rabbit splenocytes, the highest concentrations of all three proteins were inhibitory to spleen cell proliferation. Although the molecular basis is not known, the high-dose suppression appeared to result from individual variation in rabbit sensitivity to the proteins. In other rabbit lymphocyte experiments with the same mutants, the high-dose suppression was not seen. Similarly, but to a lesser extent, high-dose inhibition was seen when certain mutants were tested with human lymphocytes. In contrast, such variation in response to high doses was much less apparent when mutants were tested with murine lymphocytes, derived from inbred BALB/c mice; the variation that was observed was within the error inherent in the assay.

It is also interesting to note that not all batches of SPE A and mutants were equally stimulatory to lymphocytes at comparable doses. This is attributed to day-to-day variation in the proliferation assay, such as variation in lymphocyte counts and lot of fetal calf serum used in the RPMI medium. The day-to-day variation provided the basis for including positive (wild-type SPE A) and negative (cells only) controls in each assay.

In conclusion, in this work we analyzed several domains of SPE A for their role in lethality and lymphocyte proliferative activities by introducing directed mutations at single and double sites. The results of this study further support the hypothesis that SPE A, similarly to the staphylococcal enterotoxins, may have a disulfide bridge between two of its Cys residues. In particular, Cys 87 and 98 of SPE A, but not Cys 90, may be covalently linked. The disulfide bridge, and not the Cys residues themselves, may participate in biological activities. Furthermore, the results presented here indicate that SPE A lymphocyte proliferative activity and its ability to induce lethality are not completely correlated. We may envision a model for

the action of SPE A in which death is the final point of a very complex toxic process. Mediators such as cytokines, released as a result of both superantigenicity and the enhancement of susceptibility to endotoxin as well as direct toxicity of PTSAGs, are likely to contribute to the capillary leak responsible for hypotension, shock, and death. At the point where death is reached, the host system is overwhelmed by the summation of all of these contributions. However, our results show that there are situations in which the activities of the toxin may be dissociated—for example, by mutagenesis. In these cases, where only one class of contributors to lethality is present (cytokine secreted as a result of superantigenicity, endotoxin enhancement, or direct toxicity), death would be much less likely to occur. This view is supported by our analysis of mutant K157E. This toxin appears to be able to trigger cytokines through superantigenicity (preliminary observation) but is not able to efficiently enhance endotoxin shock or cause lethality when administered in a miniosmotic pump. However, a higher dose of this partly toxic molecule is enough to tip the balance toward death in some of the animals.

ACKNOWLEDGMENTS

This work was supported by USPHS grant HL 36611 from the National Heart, Lung, and Blood Institute.

We gratefully acknowledge D. T. Mitchell and D. H. Ohlendorf for providing Fig. 1 and M. H. Kim and D. L. Murray for valuable discussions and help. We also acknowledge Andreas Podbielski's (Institute for Microbiology, Technical University, Aachen, Germany) help in designing PCR primers for construction of mutant K16N. We thank Tim Leonard for photography work and Melodie Bahan for typing the manuscript.

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Editor: V. A. Fischetti