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Purification and Characterization of *Staphylococcus aureus* FRI 1169 and 587 Toxic Shock Syndrome Exotoxins

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An exotoxin was purified from a toxic shock toxin (TST)-producing *Staphylococcus aureus* strain, FRI 1169, and another exotoxin was purified from a pyrogenic exotoxin C (PEC)-producing *S. aureus* strain, 587. Both strains had been isolated from toxic-shock syndrome patients. The two exotoxins were purified by the same method of ion-exchange chromatography, chromatofocusing, and gel filtration. After purification, those exotoxins gave a line of identity against an anti-TST serum and also were immunologically similar to TST in a double-diffusion test. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, each exotoxin gave a single band with a relative mobility identical to that of the other. Their molecular weights (24,000), isoelectric points (7.0), amino acid compositions, and NH_2-terminal amino acid sequences (the first four residues) were identical. Both produced fever and enhanced host susceptibility to lethal endotoxin shock in rabbits, comparable with PEC. These findings show that the two exotoxins are the same protein, which is assumed to be TST. When injected into rabbits, the culture supernatant of strain 587 showed biological activity like that described above, whereas the culture supernatant neutralized with anti-TST immunogloblin did not. This showed that PEC-producing strain 587 does not produce any toxin with these biological activities in rabbits except TST.

Toxic shock syndrome (TSS), which occurs most frequently in young women during menstruation, was first described by Todd et al. in 1978 (26). An etiological role for *Staphylococcus aureus* in TSS has been recognized (7, 23). Since the illness was first described, it has been suggested that a toxin or toxins are responsible. Two proteins which are produced by *S. aureus* isolates from TSS patients have been previously identified as toxins that can elicit TSS. One protein was designated staphylococcal enterotoxin F (SEF) by Bergdoll et al. (1), but later was called toxic-shock toxin (TST) by Reiser et al. (17) because its emetic activity in monkeys has not been confirmed. TST is a simple protein with a molecular weight of 24,000 ± 500 and an isoelectric point of 7.0 (17). The other is a protein with a molecular weight of 22,000 and an isoelectric point of 7.2, named pyrogenic exotoxin C (PEC) by Schlivert et al. (21). This exotoxin is characterized by its capacity to induce fever and enhance host susceptibility to lethal endotoxin shock in rabbits (21). The similarity of the two toxins has been debated. Both share their physicochemical properties, which include molecular weight and isoelectric point (2, 17, 21), and are identical by Ouchterlony immunodiffusion (2). Bonventre et al. (2) described a 100% concordance between SEF (TST) and PEC production by isolates from TSS-associated and other sources. Cohen et al. (6) also confirmed this with few exceptions. These two groups (2, 6) reported that SEF (TST) and PEC are the same protein. But Reiser et al. (17) reported that the PEC preparation contained extraneous material in addition to TST.

To clarify the relationship between these toxins, we purified each exotoxin by the same method from a TST (SEF)-producing strain and from a PEC-producing strain. The two strains were the original ones reported to produce TST (SEF) (1, 17) and PEC (21). The physicochemical and biological properties of the two purified exotoxins were compared, and the exotoxins were found to be the same protein (TST). Then, neutralization of the culture supernatant of PEC-producing strain 587 with anti-TST immunoglobulin was tested.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* FRI 1169 that produced TST was kindly provided by M. S. Bergdoll of the Department of Food Microbiology and Toxicology, University of Wisconsin, Madison. *S. aureus* 587 that produced PEC was kindly provided by J. C. Feeley of the Respiratory and Special Pathogens Branch, Centers for Disease Control, Atlanta, Ga. The strains were kept in a lyophilized state before use. Neither of the strains produced staphylococcal enterotoxins A through E.

Column supports. CNBr-activated Sepharose 4B, SP-Sephadex C-25, Sephadex G-25, Sephadex G-75 super fine, Sephacryl S-200 super fine, and polybuffer exchanger PBE 94 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Polybuffer 96 was also obtained from the same source.

Exotoxin production. Each strain was inoculated into a 1,000-ml flask containing 500 ml of sterile medium composed of 4% NZ-Amine A (Sheffield Products, Lyndhurst, N.J.), 0.15% yeast extract (Difco Laboratories, Detroit, Mich.), and 10 mg of nicotinic acid and 0.5 mg of thiamine hydrochloride per liter and adjusted to pH 6.8 with 5 N NaOH. Cultures were then incubated for 24 h at 35°C with shaking at 100 strokes per min. From each seed culture 5-ml amounts were inoculated into 40 1,000-ml flasks, each containing 500 ml of sterilized medium. After incubation for 48 h under the same conditions, each culture was centrifuged at 30,000 × *g* and 4°C with a continuous flow centrifuge (RC-20 FIII; Tomy Seiko Co., Ltd., Tokyo, Japan).

Protein measurements. Protein concentrations were estimated spectrophotometrically by the following formula (12): protein (milligrams per milliliter) = (1.55 × OD_{280}) − (0.76 ×

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OD_{260}), where OD_{280} and OD_{260} are the optical densities at 280 and 260 nm.

Optimal sensitivity plate. Detection of TST in fractions from each purification step was accomplished with a double-diffusion plate, the optimal sensitivity plate as applied by Robbins et al. (18). TST and its antiserum were obtained from M. S. Bergdoll.

Preparation of antisera to the purified exotoxins. Two types of antisera to the purified exotoxins from *S. aureus* FRI 1169 and 587 were prepared by injecting each exotoxin with complete Freund adjuvant (Difco) into rabbits subcutaneously. The exotoxin was injected at 25, 50, 100, 100, and 100 μg per rabbit at intervals of 0, 1, 2, 3, and 5 weeks, respectively. After 7 weeks, blood was taken from the heart with a syringe.

Purification of anti-TST immunoglobulin. Anti-TST immunoglobulin was purified by using antisera to the exotoxin (TST) from *S. aureus* FRI 1169 purified by affinity chromatography by the method of Yamada et al. (27). CNBr-activated Sepharose 4B was coupled with the same exotoxin (TST) from *S. aureus* FRI 1169.

RPLA. For better detection quantitatively and with greater sensitivity than by using optimal sensitivity plates, TST fractions from each purification step were also detected by reversed passive latex agglutination (RPLA). Latex particles (SLD-59, 0.9 μm in diameter) were kindly furnished by Takeda Chemical Industries, Ltd., Osaka, Japan. A 5% latex suspension was diluted 1:20 with phosphate-buffered saline, consisting of 1 volume of 67 mM phosphate buffer at pH 7.3 and 3 volumes of saline with 0.1% NaN_3. Equal volumes of the diluted latex suspension and 60 μg of anti-TST immunoglobulin per ml in phosphate-buffered saline were then mixed and incubated at room temperature for 60 min with gentle and brief swirling every 15 min. These sensitized latex particles were washed once with phosphate-buffered saline and once with a diluent, consisting of 0.83% bovine serum albumin and 0.004% polyvinylpyrrolidone (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in phosphate-buffered saline. They were finally resuspended in the diluent to a concentration of 0.025%. The sensitized latex particles were stored at 4°C.

Then, 25-μl portions of serial twofold dilutions of samples were titrated in microtest plates (N-1182; Nunc Products, Roskilde, Denmark) by the addition of 25 μl of 0.025% sensitized latex particles. After thorough mixing, the plates were incubated in a humidified box for 16 h at room temperature. The endpoint of the titration was examined macroscopically with transmitted light from the bottom.

The minimum amount of TST in samples detected by RPLA was calculated to be approximately 0.5 ng/ml. The sensitized latex particles did not react to staphylococcal enterotoxins A through E.

Staphylococcal enterotoxins A through E. Staphylococcal enterotoxins A through E were purified by ion-exchange chromatography and gel filtration as described by Oda (14, 15). These enterotoxins were stored at −70°C before use.

Immunological properties. The two exotoxins from *S. aureus* FRI 1169 and 587 were compared immunologically in double-diffusion tests by the Ouchterlony method (16). Antibodies (100 μl each) were the two types of undiluted antisera to the exotoxins from *S. aureus* FRI 1169 and 587. Antigens (100 μl each) were 100 μg of the two exotoxins per ml and 50 μg of TST purified by M. S. Bergdoll per ml.

SDS-PAGE. Slab polyacrylamide gel electrophoresis (PAGE) in 0.1% sodium dodecyl sulfate (SDS) was carried out as described by Laemmli (11) with 15% acrylamide. Before electrophoresis the samples with 1% 2-mercaptoethanol were treated at 100°C for 4 min. Gels were stained with Coomassie brilliant blue and then destained. For determination of molecular weight, an electrophoresis calibration kit (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin) was purchased from Pharmacia.

Gel filtration with Sephacryl S-200 super fine. A column of Sephacryl S-200 super fine (2.6 by 90 cm) equilibrated with 0.1 M NH_4HCO_3 was used for the determination of molecular weights. Blue Dextran 2000 and a gel filtration calibration kit (RNase A, chymotrypsinogen A, ovalbumin, and bovine serum albumin) were purchased from Pharmacia.

Determination of isoelectric point. Isoelectric focusing of the samples was performed with a flat-bed isoelectric focusing apparatus (KS 8330 FSE; Marysol Industry, Tokyo, Japan) by using Servalyt Precotes, pH 6 to 9 (Serva Fine Biochemicals Inc., Heidelberg, West Germany).

Amino acid and NH_2-terminal sequence analyses. The samples were hydrolyzed in evacuated, sealed tubes with 100 μl of 6 N HCl or 100 μl of 4 N methanesulfonic acid containing 0.2% tryptamine for 24, 48, and 72 h at 110°C. Excess 4 N methanesulfonic acid was neutralized by adding 100 μl of 3.5 N NaOH, and then 200 μl of 0.02 N HCl was added. Analysis was performed with a Hitachi model 835 amino acid analyzer and by the method of Spackman (24). The samples were oxidized with performic acid for detection of cysteine. The NH_2-terminal sequences were determined manually by the method of Edman (8), and the resulting phenylthiohydantoin amino acid was identified by high-performance liquid chromatography (28).

Biological assays. Pyrogenicity and the capacity to enhance host susceptibility to lethal endotoxin shock in rabbits were measured by a modification of the method of Schlievert et al. (21). Rabbits (Japanese White, male) weighing about 2.5 kg each were purchased from Japan Laboratory Animals, Tokyo, Japan. Healthy rabbits (three per group) were conditioned to a test rack for 3 h daily for 7 days before use. On the day of testing, the animals were placed on the rack for at least 1 h before being administered the exotoxins. The animals were injected intravenously with three different doses of each exotoxin (0.01, 0.05, and 0.1 μg/ml per kg) in pyrogen-free saline. Temperatures were monitored over a 4-h period with rectal thermometers (model EP76-12; Iio Electric Co., Ltd., Tokyo, Japan). One minimum pyrogenic dose was defined as the dose of exotoxin required to produce a 0.5°C/kg increase in rabbit body temperature after the injection.

To evaluate the capacity of the exotoxins to enhance host susceptibility to lethal endotoxin shock, the animals were given a 5-μg/ml dose of endotoxin per kg (lipopolysaccharide derived from *Salmonella typhimurium* L-3629; Sigma Chemical Co., St. Louis, Mo.) in pyrogen-free saline 4 h after they received the exotoxins. The 50% lethal dose of endotoxin alone was about 500 μg/kg.

***Limulus* amebocyte lysate test.** To assess contamination of endotoxin in the samples injected into rabbits, *Limulus* amebocyte lysate tests were performed by using Pre-gel-S (Teikoku Hormone Mfg. Co., Tokyo, Japan) as reported by Levin et al. (13). The lysate sensitivity of Pre-gel-S was 0.25 Endotoxin units per ml.

Neutralization test. Culture supernatant of strain 587 prepared under the conditions described above was filtered through a membrane (Acrodisc, 0.45 μm pore size; Gelman Science Inc.., Ann Arbor, Mich.) and then diluted 1:10 with sterile saline. Ten volumes of the diluted culture supernatant

and 1 volume of anti-TST immunoglobulin (0.83 mg/ml) in 0.15 M phosphate buffer at pH 7.2 were then mixed and incubated at 35°C for 1 h. In controls, sterilized saline was substituted for anti-TST immunoglobulin. The samples were sterilized by Acrodisc before intravenous injection (1 ml/kg) into rabbits (three per group). Pyrogenicity and the capacity to enhance host susceptibility to lethal endotoxin shock in rabbits were measured by the same method described above. TST in the samples was assayed by RPLA.

RESULTS

Purification procedure. All the chromatography processes were performed at 4°C.

(i) Ion-exchange chromatography on SP-Sephadex C-25. The procedure was a modification of the method of Oda (14, 15). Approximately 19 liters of culture supernatant (8 μg of exotoxin per ml) of *S. aureus* FRI 1169 was diluted with 80 liters of distilled water and adjusted to pH 5.0 with 6 N HCl. Then, about 400 ml of SP-Sephadex C-25 equilibrated with 0.01 M phosphate-citrate buffer (PCB), pH 5.0, was added. The mixture was stirred for 1 h at room temperature, after which the liquid was separated from the resin by decanting. The resin was layered on 600 ml of C-25 resin, previously equilibrated with the same buffer, in a chromatographic tube, 5 cm in diameter. The resin was washed with 3 liters of 0.01 M PCB at pH 5.0. The exotoxin was eluted with an increasing linear gradient of PCB by using 1,000 ml of 0.01 M PCB at pH 5.0 and 1,000 ml of 0.02 M PCB at pH 8.7, flowing at 60 ml/h (Fig. 1A).

Culture supernatant (1.5 μg of exotoxin per ml) of *S. aureus* 587 was treated in the same manner (Fig. 1B).

The exotoxin in fractions was detected by optimal sensitivity plates and RPLA, and the fractions with high RPLA titer ($>10^5$) were combined.

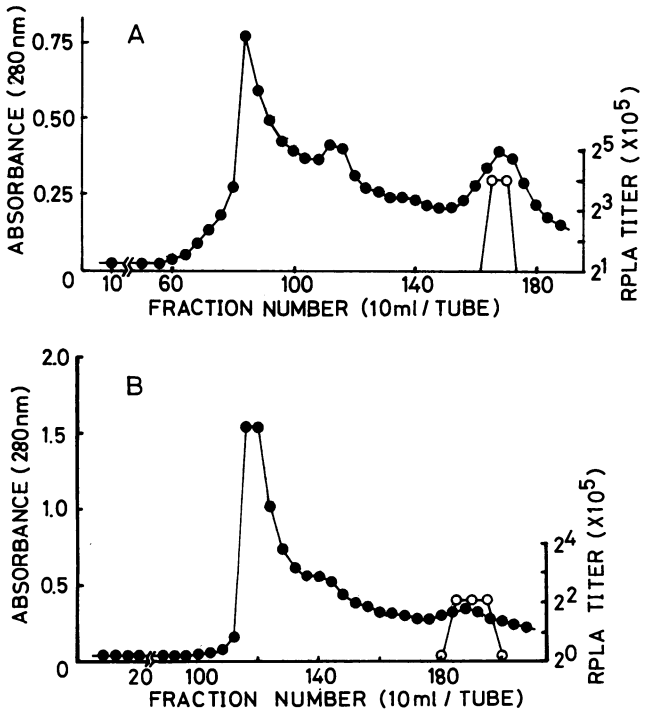

FIG. 1. Ion-exchange chromatography on SP-Sephadex C-25. (A) Exotoxin from strain FRI 1169. (B) Exotoxin from strain 587. Symbols: ●, absorbance at 280 nm; ○, RPLA titer. The conditions for chromatography are described in the text.

(ii) Lyophilization of exotoxin fractions. Each of the combined fractions was concentrated by rechromatography with SP-Sepadex C-25. The exotoxin fractions derived from strain FRI 1169 were diluted with an equal volume of distilled water and adjusted to pH 5.0 with 6 N HCl. The exotoxin solutions were applied to a SP-Sephadex C-25 column (1.5 by 20 cm) previously equilibrated with 0.01 M PCB at pH 5.0. The solutions were eluted stepwise from the resin with 0.02 M PCB, pH 8.7, at a flow rate of 50 ml/h. The fractions with high RPLA titer ($>10^5$) were pooled and then layered carefully on a column of Sephadex G-25 (2.5 by 40 cm) equilibrated with 0.1 M NH_4HCO_3 so as not to contain large amounts of salts for chromatofocusing. The exotoxin solutions were eluted with the same buffer flowing at 170 ml/h. The exotoxin fractions were pooled and lyophilized.

The combined fractions from the ion-exchange chromatography step (i) derived from strain 587 were treated in the same manner.

(iii) Chromatofocusing on PBE 94. Each of the lyophilized fractions was dissolved in about 6 ml of 25 mM ethanolamine hydrochloride, pH 9.4, and layered on a column of PBE 94 (1 by 40 cm) equilibrated with the same buffer. For elution of the exotoxin, 300 ml of Polybuffer 96-acetic acid at pH 7.0 was used as described by Pharmacia (*Chromatofocusing with Polybuffer and PBE*, technical pamphlet), flowing at 25 ml/h (Fig. 2). Fractions with high RPLA titer ($>10^5$) were pooled.

Gel filtration through Sephadex G-75 super fine. Each exotoxin fraction was applied to a column of Sephadex G-75 super fine (2.6 by 90 cm) equilibrated with 0.1 M NH_4HCO_3.

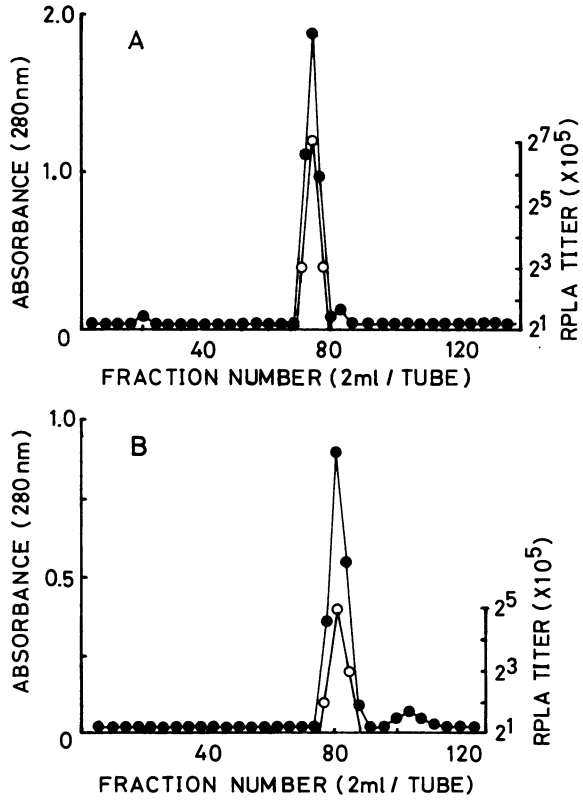

FIG. 2. Chromatofocusing on PBE 94. (A) Exotoxin from strain FRI 1169. (B) Exotoxin from strain 587. Symbols: ●, absorbance at 280 nm; ○, RPLA titer. The conditions for elution are described in the text.

The same buffer was used for elution of the exotoxin at 6 ml/h (Fig. 3). Fractions with high RPLA titer ($>10^5$) were pooled and lyophilized.

Total recoveries of the two exotoxins derived from strains FRI 1169 and 587 were approximately 40%. The total purifications of the exotoxins from strains FRI 1169 and 587 were 4,500-fold and 21,000-fold over the culture supernatant, respectively.

Comparison of some properties of the two exotoxins. (i) Test of purity. Each purified exotoxin from strains FRI 1169 and 587 was homogeneous on the basis of immunological and electrophoretic tests. Each exotoxin gave a single precipitin line in double-diffusion optimal sensitivity plates with anti-TST serum (Fig. 4). Both were immunologically similar to TST. The exotoxins gave a single band in SDS-PAGE with identical relative mobilities (Fig. 5).

(ii) Immunological property. The two exotoxins and TST all gave a line of identity against two types of antisera to each exotoxin by double-diffusion test (Fig. 6). The test showed immunologically that the two exotoxins are identical and similar to TST.

(iii) Molecular weight and isoelectric point. The molecular weights of the two exotoxins were both calculated to be 24,000 from their mobilities on SDS-PAGE (Fig. 7). The molecular weights were determined from gel filtration of Sephacryl S-200 superfine (data not shown). Isoelectric focusing of the two exotoxins gave an isoelectric point of 7.0.

(iv) Amino acid composition and NH_2-terminal amino acid sequence. The amino acid compositions of the two exotoxins were essentially identical (Table 1). No half-cystine was detected, whereas 1.75 residues of half-cystine per mol were detected under the same condition in the purified preparation of staphylococcal enterotoxin A, which is known to have two residues of half-cystine per mol by its primary structure (19). The NH_2-terminal amino acid sequences of the exotoxins from strains FRI 1169 and 587 were deter-

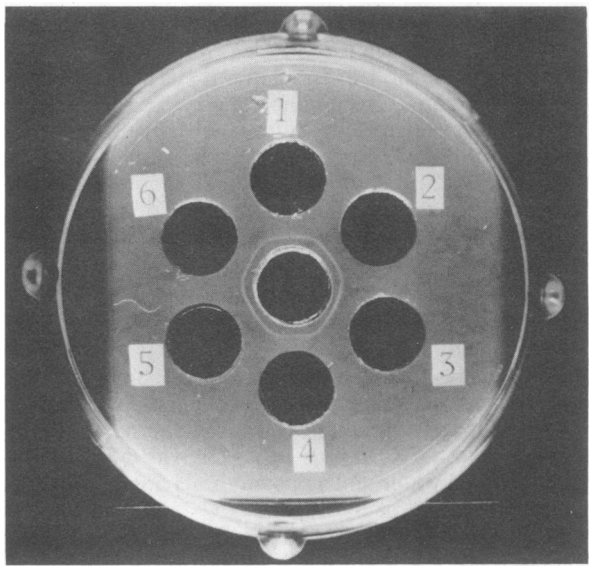

FIG. 4. Double-gel diffusion test of each exotoxin from strains FRI 1169 and 587 on optimal sensitivity plates. Wells: 1 and 4, TST (4 μg/ml); 2, exotoxin from FRI 1169 (5 μg/ml); 3, exotoxin from strain 587 (10 μg/ml); 5, exotoxin from strain FRI 1169 (10 μg/ml); 6, exotoxin from strain 587 (5 μg/ml); center, anti-TST serum. Each well contained 40 μl of reagent.

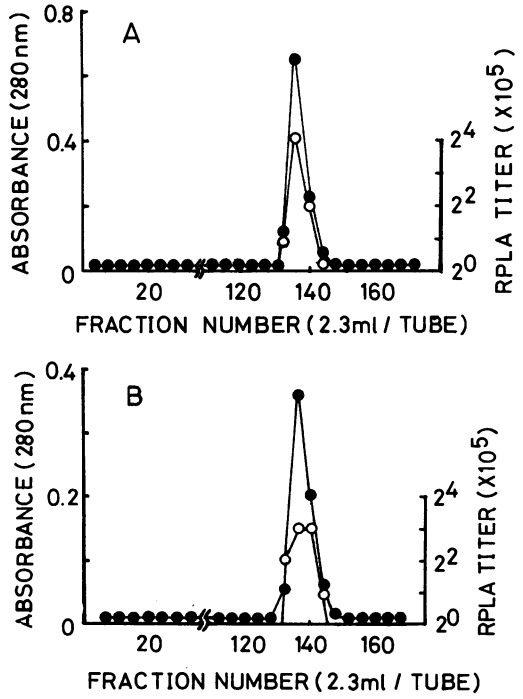

FIG. 3. Gel filtration on Sephadex G-75 super fine. (A) Exotoxin from strain FRI 1169. (B) Exotoxin from strain 587. Symbols: ●, absorbance at 280 nm; ○, RPLA titer. The conditions for elution are described in the text.

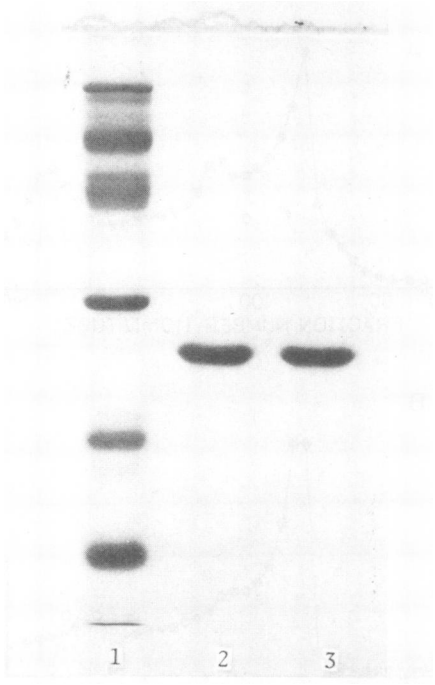

FIG. 5. SDS-PAGE of each exotoxin from strains FRI 1169 and 587. Lanes: 1, electrophoresis calibration kit (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin); 2, exotoxin from strain FRI 1169 (12.5 μg per gel); 3, exotoxin from strain 587 (12.5 μg per gel).

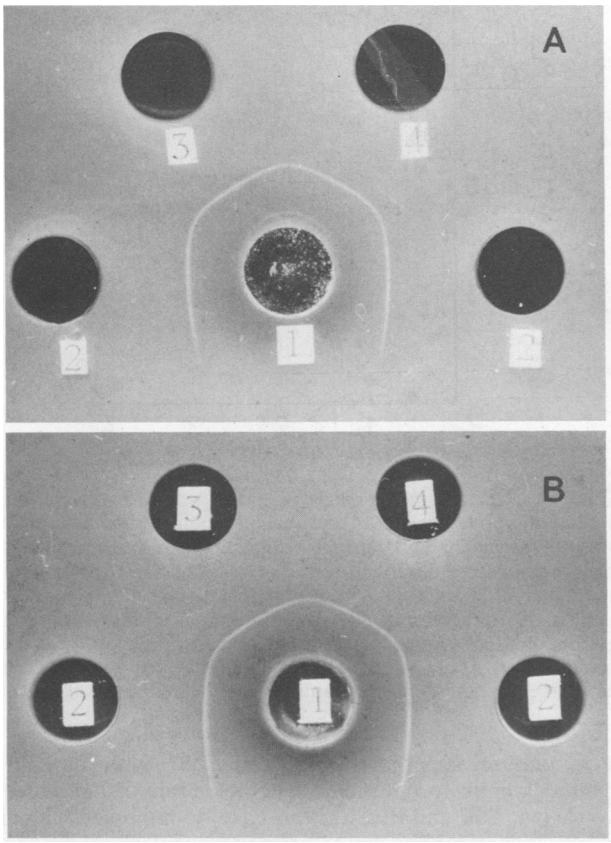

FIG. 6. Immunodiffusion test of two exotoxins from strains FRI 1169 and 587. Wells: 1, antiserum against (A) exotoxin from strain FRI 1169 and (B) exotoxin from strain 587; 2, TST (50 μg/ml); 3, exotoxin from strain FRI 1169 (100 μg/ml); 4, exotoxin from strain 587 (100 μg/ml). Each well contained 100 μl of reagent. Sera were undiluted.

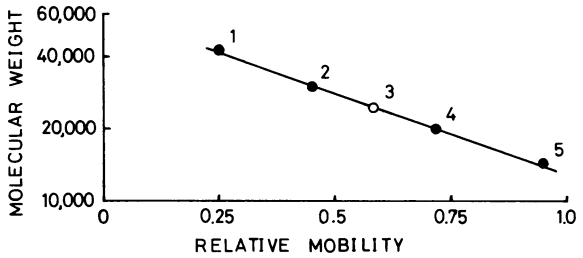

FIG. 7. Estimation of molecular weight of exotoxins from strains FRI 1169 and 587 by SDS-PAGE. Numbers correspond to the following: 1, ovalbumin; 2, carbonic anhydrase; 3, exotoxins from strains FRI 1169 and 587; 4, trypsin inhibitor; 5, α-lactalbumin.

mined up to nine amino acids (Ser-Thr-Asn-Asp-Asn-Ile-Lys-Asp-Leu-) and four amino acids (Ser-Thr-Asn-Asp-), respectively. Their first four NH_2-terminal amino acids were identical.

(v) Biological property. Each exotoxin induced fever in rabbits, with maximal response at approximately 4 h after injection (Fig. 8). All of the samples injected into rabbits were negative by *Limulus* amebocyte lystate test. Each also enhanced host susceptibility to lethal endotoxin shock in rabbits (Fig. 8). None of the animals administered the exotoxin or endotoxin alone died.

Neutralization test. Intravenous injection of culture supernatant of strain 587 into rabbits induced fever and enhanced host susceptibility to lethal endotoxin shock, whereas the culture supernatant neutralized with anti-TST immunoglobulin produced much less fever and did not enhance host susceptibility to lethal endotoxin shock (Fig. 9). The animals administered the control culture supernatant had maximum fever (0.75°C) 4 h after injection, whereas those which received the neutralized culture supernatant had maximum fever (0.20°C) 2 h after injection. The former group all died after the endotoxin injection, whereas all of the latter group survived. It was not possible to detect TST by RPLA in the neutralized culture supernatant, but approximately 50 μg of TST per ml was detected in the culture supernatant of the control.

DISCUSSION

We purified by the same method two single exotoxins from *S. aureus* FRI 1169 and 587, which had been isolated from patients suffering from TSS. *S. aureus* FRI 1169 was first reported by Bergdoll et al. (1) as a staphylococcal enterotoxin F-producing strain. This toxin was subsequently renamed TST by Reiser et al. (17) because it does not cause vomiting in monkeys. *S. aureus* 587 produces PEC and was first reported by Schlievert et al. (21).

The two purified exotoxins gave a line of identity against two types of antisera to each exotoxin in double-diffusion tests. The physicochemical properties of the two exotoxins, including molecular weights, isoelectric points, amino acid compositions, and NH_2-terminal amino acid sequences (the first four residues), were identical. The two exotoxins produced fever and enhanced host susceptibility to lethal endotoxin shock in rabbits. These findings confirmed that the two exotoxins are the same protein.

Regarding the relationship between the two exotoxins and TST, the two gave a line of identity to TST in double-diffusion tests with anti-TST serum and were shown immunologically to be identical to TST. Data on the physico-

TABLE 1. Amino acid composition of exotoxins from strains FRI 1169 and 587

Amino acid	Exotoxin from strain (residues per mole):[a]	
	FRI 1169	587
Asp	26.5 (27)	27.0 (27)
Thr	21.4 (21)	20.5 (21)
Ser	21.2 (21)	22.2 (22)
Glu	18.2 (18)	18.4 (18)
Pro	10.5 (11)	10.5 (11)
Gly	11.9 (12)	11.9 (12)
Ala	3.3 (3)	3.2 (3)
Half cystine	0[b] (0)	0[b] (0)
Val	5.5 (6)	5.6 (6)
Met	2.2 (2)	1.6 (2)
Ile	17.7 (18)	17.7 (18)
Leu	16.5 (17)	15.8 (16)
Tyr	9.7 (10)	9.8 (10)
Phe	7.6 (8)	7.7 (8)
Lys	24.6 (25)	25.2 (25)
His	5.3 (5)	4.7 (5)
Trp	2.5 (3)	3.4 (3)
Arg	4.4 (4)	4.3 (4)

[a] Residue values are based on a molecular weight of 24,000. Numbers in parentheses are the rounded values.

[b] Not detectable as cysteic acid by performic acid oxidation.

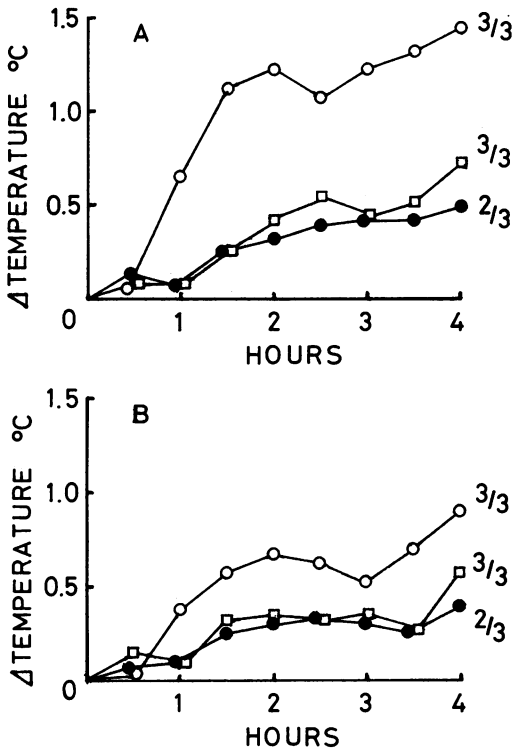

FIG. 8. Pyrogenicity and capacity to enhance susceptibility to lethal endotoxin shock in rabbits for the exotoxins from strains FRI 1169 (A) and 587 (B). Symbols: ○, 0.1 μg/ml per kg; □, 0.05 μg/ml per kg; ●, 0.01 μg/ml per kg. Endotoxin (5 μg/ml/kg) was given intravenously at 4 h. Fractions at right are the number of rabbits that died in each group per number in each group. Temperature is the mean of each group.

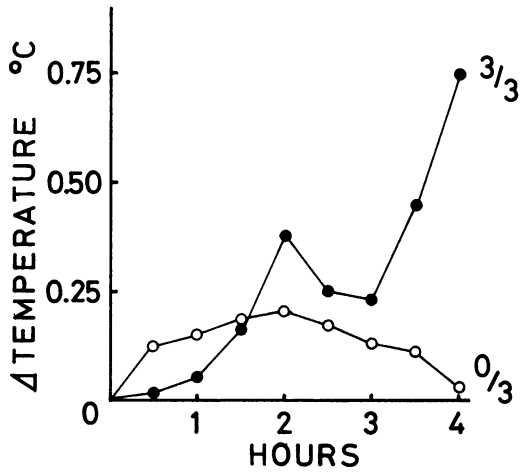

FIG. 9. Decrease in pyrogenicity and capacity to enhance host susceptibility to lethal endotoxin shock in rabbits for the neutralized culture supernatant of strain 587 with anti-TST immunoglobulin. The conditions are described in the text. Symbols: ○, the culture supernatant of strain 587 neutralized with anti-TST immunoglobulin (1 ml/kg); ●, the culture supernatant of strain 587 in control (1 ml/kg). Endotoxin (5 μg/ml per kg) was given intravenously at 4 h. Fractions at right are the numer of rabbits that died in each group per number of rabbits. Temperature is the mean of each group.

chemical properties of two exotoxins were essentially identical to those reported by Reiser et al. (17) and Bonventre et al. (2), especially to those of the former report. The two exotoxins were identical to TST (17) in molecular weight, isoelectric point, amino acid composition with the exception of a half-cystine residue, and the NH_2-terminal amino acid (serine). In light of these facts, we surmise that the exotoxins which we purified from *S. aureus* FRI 1169 and 587, respectively, are TST as reported by Reiser et al. (17).

Our data give further confirmatory information on amino acid composition, NH_2-terminal amino acid sequence, and some biological properties in rabbits. It is suggested that TST might be composed of a single polypeptide chain because of its single NH_2-terminal amino acid of serine.

However, our amino acid analyses of the two exotoxins from strains FRI 1169 and 587 showed that these toxins have no half-cystine. Moreover, we could not detect half-cystine (0.3 residues per mol) in the preparation which R. E. Reiser et al. purified from strain FRI 1169 (unpublished data), whereas we could detect 2 residues of half-cystine per mol in preparations of staphylococcal enterotoxin A and amino terminal peptide (residues 1 through 42) of bovine prothrombin, which contain two residues of half-cystine per toxin or peptide.

It became clear in this study that strain 587, which Schlievert et al. reported (21) produces PEC, also produces TST. However, since our purification method was purposed for producing TST, we conducted an experiment to neutralize a culture supernatant of strain 587 with anti-TST immunoglobulin to determine whether strain 587 produces two toxins, TST and PEC. In this case, to minimize biological activities in rabbits due to unknown substances, we used type-specific immunoglobulin to TST purified by affinity chromatography instead of whole serum, and a culture supernatant of strain 587 was diluted 1:10 with a sterile saline.

As a result, we confirmed that biological activities, such as pyrogenicity and capacity to enhance host susceptibility to lethal endotoxin shock in rabbits, shown by a culture supernatant of strain 587 were completely neutralized by anti-TST immunoglobulin.

This shows that the substance which has these biological activities in rabbits in a culture supernatant of strain 587 is neutralized with anti-TST immunoglobulin and that the substrate is identical to TST and further that no substance with these biological activities other than TST exists in culture supernatants of strain 587.

We compared the amino acid composition of TST purified from strain 587 with that of PEC as reported by Schlievert et al. (20) and found that they were greatly different. Moreover, when we compared pyrogenicity and the capacity to enhance host susceptibility to lethal endotoxin shock in rabbits, we discerned that the minimum pyrogenic dose of the exotoxin purified from strain 587 was 0.018 μg/kg and the minimum lethal dose (dead/total, 3/3) was 0.01 to 0.05 μg/kg. In the case of PEC as reported by Schlievert et al. (20, 21), they were 0.1 to 0.15 and 1.0 to 5.0 μg/kg, respectively. Thus, the exotoxin purified by us had much higher biological activities than PEC. These results, as Reiser et al. (17) state, suggest the possibility that the PEC which Schlievert et al. (21) purified, was a mixture of toxin and other contaminating substances.

TST purified by us did not elicit emesis in cynomolgus monkeys (*Maccaca irus*; body weight, ca. 2 kg each) at up to 50 μg per monkey by a feeding test (unpublished data). This fully concurred with the report of Reiser et al. (17). More-

over, TST is chemically different from the previously well-characterized staphylococcal enterotoxins A (19), B (10), C_1 (9), C_2 (9), D (5), and E (3). TST has no half-cystine in contrast with them and has a completely different NH_2-terminal sequence from those of staphylococcal enterotoxin B (10) and C_1 (22). This evidence proves the designation staphylococcal enterotoxin F to be unsuitable.

On the other hand, it was previously reported that staphylococcal enterotoxins (4, 25) produced fever and enhanced host susceptibility to lethal endotoxin shock in rabbits, similar to TST. We also confirmed that staphylococcal enterotoxins A, B, and C_2 had similar activities in rabbits by using the same method described above (unpublished data). These findings show that TST may have a region in its structure in common with staphylococcal enterotoxins, regardless of having no emetic activity to monkeys.

TST purified by us was distinguished by the following methods. One of these is the use of chromatofocusing. In the sample isolated without this process, four to five bands were seen with SDS-PAGE, but by employing chromatofocusing, bands other than that of TST disappeared. In addition, in the case of concentration of an effluent from SP-Sephadex chromatography, it was proved that when TST was concentrated with UM-10 membrane (Amicon Co., Lexington, Mass.), a considerable quantity of TST passed through the membrane. To prevent this, a minicolumn (1.5 by 20 cm) of SP-Sephadex was adjusted and the effluent was applied to the minicolumn, and after adsorbing TST, it was concentrated by a method of eluting it with a small amount of eluant.

A problem that remains to be solved in the future is whether TST produced from a large number of *S. aureus* separated from TSS patients can actually cause TSS.

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