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Presence of Toxic Shock Toxin in Toxic Shock and Other Clinical Strains of *Staphylococcus aureus*

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Toxic shock toxin (TST), also known as pyrogenic exotoxin C (Schlievert et al., J. Infect. Dis. 143:509–516, 1981) and staphylococcal enterotoxin F (Bergdoll et al., Lancet i:1017–1021, 1981), was purified from toxic shock strains of *Staphylococcus aureus* by preparative isoelectric focusing and by chromatofocusing. Neither method produced an absolutely pure protein as determined by silver staining of sodium dodecyl sulfate-acrylamide gels, although chromatofocusing was the better method of the two. Three molecular weight variants of the protein were found in the two toxic shock syndrome strains that were studied, regardless of the purification method that was used. An isoelectric point of 7.15 and molecular weights of 21,400, 22,100, and 23,200 were determined for the different forms of the protein from electrophoresis data. A sedimentation coefficient of 2.3S was determined by sucrose gradient centrifugation, and a Stokes radius of 2×10^{-7} cm was determined by gel filtration. An average molecular weight of 18,900 for all of the TST forms was calculated from these data by the Stokes-Einstein equation. A survey for TST in 32 control and 46 toxic shock strains of *S. aureus* by isoelectric focusing and by agarose gel double immunodiffusion with specific rabbit antiserum revealed that the isoelectric focusing method tends to overestimate the number of TST-positive strains because of the detection of non-TST, neutral staphylococcal proteins. Based on immunodiffusion data, the association of TST with toxic shock strains was found to be 100% in vaginal isolates and 62% in non-vaginal isolates. In the control strains, TST was found in 16% of the vaginal strains and 23% of the non-vaginal strains. The value of this toxin as a marker for toxic shock and its relationship to the pathogenesis of this disease are discussed.

Toxic shock syndrome (TSS) is an acute, severe, multisystem disease that is seen most frequently in young women during menses (12, 41, 47); ca. 15% of reported TSS cases are not related to menstruation (30). Bacterial cultures from TSS patients have consistently yielded *Staphylococcus aureus*, and it is now accepted that this organism is responsible for the disease (12, 30, 43). The development of TSS does not require a deep tissue invasive infection (19); thus, the disease is thought to be mediated by an *S. aureus* toxin or toxins. Two *S. aureus* exoproteins have been reported to be uniquely associated with TSS and to have a variety of toxin-like activities that could be related to the pathogenesis of TSS. Also, both have been characterized as having a neutral isoelectric point and a molecular weight of 20,000 to 22,000 (5, 40). Although it was originally thought that these proteins were distinct, more recent evidence has shown that both are in fact the same protein (7, 10). Although there are still some questions about the biological activity of this protein and its relationship to TSS (7; P. Garbe, M. Reeves, P. Hayes, R. Arko, K. Rasheed, and C. Broome, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 23rd, Las Vegas, Nev., abstr. no. 235, 1983), the protein has been designated as toxic shock toxin (TST) (32).

As a part of a study of the toxins of TSS *S. aureus* isolates, we purified TST by the preparative isoelectric focusing (IEF) method used by Schlievert et al. (40) and found that preparations made by this method were not pure. A simpler and more effective purification method that utilizes chromatofocusing is described in this study. In addition, we used antiserum produced with highly purified TST to detect the production of TST by TSS and non-TSS strains of *S. aureus* with a double immunodiffusion (ID) assay. Results obtained with this ID method were compared with results obtained by analytical IEF.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. TSS strains were selected from a collection of *S. aureus* strains which had been isolated from patients with definite TSS and were sent to the Centers for Disease Control for study. Only those isolates were used that came from cases which met the revised case definition of TSS (31). The 26 non-vaginal TSS isolates used in this study were recovered in pure culture from abscesses, blood, and surgical wounds of men and non-menstruating women. There was no evidence of other types of infection in these cases, and the isolated strains were considered to be the only possible etiological agent involved. The criteria for TSS have been revised to include blood isolates of *S. aureus* (9). Non-TSS non-vaginal strains were selected at random from clinical strains that had been submitted for phage typing, and normal vaginal strains were selected from a group of strains isolated from apparently healthy individuals. All strains were stored in defibrinated rabbit blood at −65°C. For the production of TST, frozen stock was inoculated into brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and grown overnight at 35°C on a rotary shaker at 100 rpm. A brain heart infusion culture was used as inoculum for the defined medium listed in Table 1. One milliliter of culture was used to inoculate 120 ml of defined medium in a 500-ml flask. Growth was at 35°C with shaking. The composition of the defined medium (Table 1) was based on the reported amino acid requirements of *S. aureus* for optimal enterotoxin production (53). The medium was supplemented with trace metals which are generally required for bacterial nutrition (15, 28). Sodium acetate was used to replace glucose as a substrate to avoid the inhibitory

TABLE 1. Composition of *S. aureus* defined medium[a]

Compound	Final concn (mg/liter)	Final concn (mM)
Buffer and salts		
3-(*N*-Morpholino)-propanesulfonic acid	10,446	50.00
KH_2PO_4	272	2.00
$NaC_2H_3O_2$ (anhydrous)	4,102	50.00
$Na_3C_6H_5O_7 \cdot 2H_2O$	500	1.70
$NaHCO_3$	500	5.95
Vitamins		
Thiamine hydrochloride	0.5	0.0015
Nicotinic acid	10.0	0.0812
Calcium pantothenate	2.0	0.0077
Trace metals		
$CaCl_2$	0.555	0.0050
$CoCl_26H_2O$	0.476	0.0020
$CuSO_4 \cdot 5H_2O$	0.025	0.0001
$FeSO_4 \cdot 7H_2O$	5.560	0.0200
$MgSO_4$ (anhydrous)	84.280	0.7000
$MnCl_2 \cdot 4H_2O$	0.020	0.0001
$NaMoO_4 \cdot 2H_2O$	1.210	0.0050
$NiSO_4 \cdot 6H_2O$	0.526	0.0020
NH_4VO_3	1.170	0.0100
$ZnSO_4 \cdot 7H_2O$	28.754	0.1000
Amino acids		
L-Alanine	2,500	26.94
L-Arginine hydrochloride	800	3.82
L-Asparagine monohydrate	600	4.00
L-Aspartic acid	600	4.51
L-Cysteine hydrochloride monohydrate	250	1.43
L-Glutamine	600	4.11
L-Glutamic acid	600	4.08
L-Glycine	2,500	33.30
L-Histidine hydrochloride monohydrate	450	2.16
L-Isoleucine	600	4.57
L-Leucine	500	3.81
L-Lysine hydrochloride	600	3.30
L-Methionine	150	1.01
L-Phenylalanine	200	1.21
L-Proline	2,500	21.72
L-Serine	3,000	28.55
L-Threonine	2,500	20.99
L-Tryptophan	50	0.25
L-Tyrosine	150	0.83
L-Valine	400	3.41

[a] The final medium was adjusted to pH 6.5 with solid KOH, made to volume with deionized water, and sterilized by membrane filtration (0.45-μm filter; Millipore Corp.).

effect of glucose on TST production (38). Bacterial growth and TST production in the defined medium were equal to, and in some cases greater than, that observed with fresh beef heart dialysate medium which had been prepared as described by Schlievert et al. (40).

TST precipitation. Cells were removed from cultures grown in defined medium by centrifugation (12,000 × *g* for 60 min). Two hundred milliliters of cold (−20°C) absolute ethanol was added to 50 ml of culture supernatant, and the mixture was kept at 4°C for 3 days. The precipitate was collected by centrifugation (12,000 × *g* for 60 min) and was dissolved in 1 ml of distilled water. Each sample was dialyzed against three changes of 200 volumes of 1% glycine at 4°C. Insoluble material was removed by centrifugation, and the samples were stored at −20°C for later analysis. Ethanol precipitation of TST has been shown to be almost quantitative under these conditions (38).

IEF. IEF was conducted in thin-layer polyacrylamide gels on an LKB 2117 Multiphor (LKB Instruments, Stockholm, Sweden) as previously described (51). Samples (25 μl) were focused in a gradient (pH 3.5 to 9.5) (Ampholine Pagplate; LKB) and were stained with Coomassie brilliant blue R250 (Sigma Chemical Co., St. Louis, Mo.). The isoelectric point of TST was estimated by its position relative to the positions of the major proteins of horse myoglobin (Sigma) which have isoelectric points of 7.3 and 6.8 (26).

Purification of TST. All purification steps were conducted at 4°C. TST was purified from the culture supernatant of the defined medium by ethanol precipitation and preparative IEF as previously described (40, 52). Gel fractions were eluted with distilled water, and the eluates were assayed for pH and for TST by IEF. Those fractions which contained TST were pooled and concentrated by dialysis against 20 M polyethylene glycol (Union Carbide Corp., New York, N.Y.). The concentrated material was dialyzed against three changes of phosphate-buffered saline and stored at −20°C.

TST was also purified by chromatofocusing (46). Cell-free culture supernatants of the defined medium were passed through an Amicon hollow fiber apparatus run in the concentration mode with a 50,000-molecular-weight cutoff (Amicon Corp., Lexington, Mass.). The concentrate was washed with 10 volumes of distilled water containing 0.2% sodium azide and was discarded. The filtrate and wash fluids were pooled and passed through the Amicon hollow fiber apparatus in the same manner with a 5,000-molecular-weight cutoff. The filtrate from this pass was discarded, and the concentrate was dialyzed against four changes of 200 volumes of 0.025 M Tris which had been adjusted to pH 8.5 with glacial acetic acid. Chromatofocusing gel PBE-94 (Pharmacia Fine Chemicals, Uppsala, Sweden) was suspended in Tris buffer (pH 8.5), poured into a column (1.5 by 90 cm), and washed with 10 column volumes of buffer. Loading of the Amicon 5,000- to 50,000-molecular-weight concentrate (360 ml) onto the column resulted in a protein-to-gel ratio of 1.5 mg/ml of settled gel. After it was loaded, the column was washed with 2 column volumes of Tris buffer and was then eluted with 10 column volumes of Polybuffer-96 (Pharmacia) which had been diluted to a ratio of 1:13 with distilled water and adjusted to pH 6.0 with acetic acid. The eluted fractions were assayed for TST by IEF, and those that were positive were pooled and dialyzed against three changes of 200 volumes of Tris buffer (pH 8.5). The pool was loaded onto a second column of PBE-94 (0.9 by 70 cm) which resulted in a protein-to-gel ratio of 0.5 mg/ml of settled gel. The column was washed with buffer and eluted with Polybuffer-96 as before. Fractions containing TST were pooled, concentrated with polyethylene glycol to a protein concentration of 4 mg/ml, and dialyzed against three changes of 200 volumes of phosphate-buffered saline. The pool was applied to a column of Sephadex G-75 superfine (1.5 by 90 cm; Pharmacia) and eluted with phosphate-buffered saline. The fractions containing TST were pooled, concentrated with polyethylene glycol, dialyzed against phosphate-buffered saline, and stored at −20°C.

Determination of molecular weight. The Stokes radius of TST was estimated by gel filtration of purified TST in Sephadex G200 (Pharmacia) as described by Reichert et al. (29). A sedimentation coefficient was determined by centrifugation of TST on a 5 to 20% linear sucrose gradient (21), with horse myoglobin as the standard ($s_{20,w}$ = 2.04S; *CRC Handbook of Biochemistry, Selected Data for Molecular Biology, 1968 ed.*). TST was detected in the gradient fractions by IEF. The molecular weight and frictional ratio of

TST were calculated by the Stokes-Einstein equation from the gel filtration and sedimentation data by the method of Siegel and Monty (45). TST molecular weight was also estimated by electrophoresis in a vertical polyacrylamide gel slab gradient (140 by 250 by 0.75 mm; 3.3 to 20%) in 0.1% sodium dodecyl sulfate as described by Tsang et al. (49). Gels were stained with silver nitrate by a modification (48) of the method of Morrissey (22), and molecular weights were calculated by the method of Shapiro et al. (44).

Antibody production. Purified TST in 0.02 M phosphate-buffered saline (pH 7.2) was emulsified in an equal volume of Freund incomplete adjuvant (Difco) and inoculated into 10 intradermal sites in the back of a New Zealand White female rabbit. The initial inoculation contained 100 μg of protein. Three intramuscular booster inoculations of 200 μg of protein were given at 14-day intervals after the initial inoculation. Rabbits were bled from an ear vein beginning 20 days after the last booster inoculation. Antibody production was assayed by double ID (24) in 1% purified agarose (Calbiochem-Behring, La Jolla, Calif.) in a buffer that contained 150 mM sodium chloride, 12 mM potassium phosphate (dibasic), 1 mM magnesium chloride, 15 mM sodium azide, and 14 mM sodium citrate. For the ID assay, 3 ml of hot buffered agarose was spread on a glass side (50 by 75 mm) and allowed to cool. A center hole with a 4-mm diameter and six surrounding holes with 3-mm diameters were cut out of the agar. Anti-TST rabbit serum (15 μl) was placed in the center well, and 12 μl of antigen sample was placed in each of the surrounding wells. Based on analysis of dilutions of purified TST, the limit of sensitivity of this assay was determined to be 5 μg of TST per ml of sample.

Protein assay. Protein concentration was estimated with the Bradford reagent (8). Absorbance was read at 595 nm, and bovine albumin (fraction V; Sigma) was used as the standard.

RESULTS

Purification. TST purified by the preparative IEF method (40, 52) focused as a single band between pH 7.1 and 7.2 and appeared to be homogeneous when analyzed for isoelectric point on IEF gels stained with Coomassie blue (Fig. 1, lanes 5 to 10). When these same preparations were analyzed for molecular weight on thin-layer acrylamide gels, however, it was found that they contained as many as 5 to 14 protein bands, with molecular weights ranging from 13,000 to 124,000 (Fig. 2, lanes 2 and 5). The degree of purification obtained with this method varied with the strain. Two major bands were found at the position in which TST was expected (Fig. 2, arrow). Their molecular weights were estimated to be 21,400 and 22,100, based on their relative mobilities in these gels.

Because the preparative IEF method did not yield a homogeneous TST, chromatofocusing was examined as an alternative method of purification. Preliminary experiments showed that the efficiency of this method was greatly improved when high-molecular-weight proteins were first removed from the culture supernatant by Amicon filtration. The G-75 filtration step after chromatofocusing removed most of the remaining extraneous proteins, but again, two major bands were found at the TST position which appeared to be identical to the bands found in the preparative IEF sample (Fig. 2, lanes 3 and 6). Analysis of these bands with a TST monoclonal antibody in an enzyme-linked immunoelectrotransfer blot system (49) showed that both of these bands as well as the small band above them (23,200 molecular weight) reacted with the antibody (D. E. Wells, M. W. Reeves, and R. M. McKinney, manuscript in preparation). Work is now in progress to attempt to separate these bands further and to determine more accurately their molecular weights and their reactivity with this antibody. In both the chromatofocusing and preparative IEF TST preparations, several very faint bands were noted in the 30,000- to 40,000- and 13,000- to 20,000-molecular-weight ranges; however, all of these bands faded very quickly when the gels were treated to remove background staining (48). The molecular weights and isoelectric point of the chromatofocusing-purified TST were identical to those determined for the TST obtained by preparative IEF (Fig. 1 and 2). Antiserum produced in rabbits to the chromatofocusing-purified TST reacted in the agarose ID assay to form a band of identity with TST produced by both purification methods (Fig. 3A). This antiserum also showed a faint reaction with a non-TST antigen with the crude ethanol precipitate of strain 033 (Fig. 3A). The ID method did not show two precipitin bands with the purified TST preparation. ID analysis of both TST preparations with rabbit antiserum to toxic shock antigen (33) and with our anti-TST serum showed that a single band of identity was formed with both antisera which would indicate that our TST has immunological identity with staphylococcal enterotoxin F and pyrogenic exotoxin C (data not shown; antiserum to toxic shock antigen was kindly provided by H. L. Ritz).

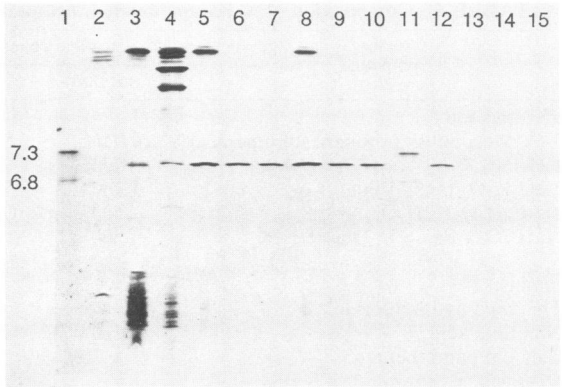

FIG. 1. IEF in a pH gradient of 3.5 (bottom) to 9.5 (top). All samples were applied to the gel in 25 μl. Lanes 1 and 11, horse myoglobin (pI = 7.3 and 6.8); lane 2, strain D-8339 (non-TSS, vaginal) grown for 18 h; lane 3, strain D-8339 grown for 72 h; lane 4, strain D-0322 (non-TSS, non-vaginal) grown for 18 h; lane 5, ethanol-precipitated TST from strain 189 (TSS, vaginal); lane 6, TST from strain 189 prepared by preparative IEF; lane 7, TST from strain 189 prepared by chromatofocusing; lane 8, ethanol-precipitated TST from strain 033 (TSS, vaginal); lane 9, TST from strain 033 prepared by preparative IEF; lane 10, TST from strain 033 prepared by chromatofocusing; lanes 12 to 15, chromatofocused TST from strain 033 (600 μg of protein per ml) diluted to ratios of 1:5, 1:10, 1:20, and 1:40, respectively. Concentration of protein in the samples applied to lanes 6, 7, 9, and 10 was ca. 600 μg/ml.

A sedimentation coefficient of 2.3S was determined by sucrose-gradient centrifugation, and a Stokes radius of 2×10^{-7} cm was determined by gel filtration for the chromatofocusing-purified TST. We calculated a molecular weight of 18,900 from these data, using an assumed partial specific volume of 0.725 (45). A frictional ratio of 1.14 was calculated from the same data which indicated that the protein(s) is essentially spherical in shape (45). Since only single TST peaks were seen in the gel filtration and sucrose-gradient centrifugation experiments, these data are the average values for the TST bands seen in Fig. 2.

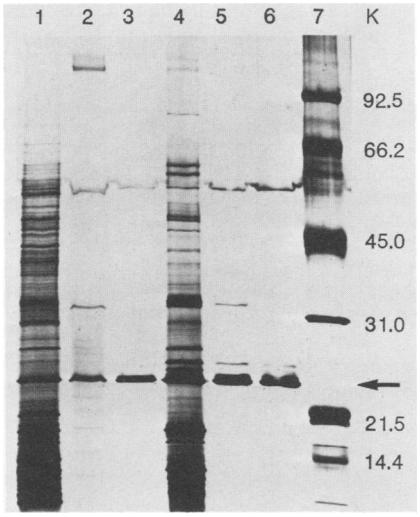

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crude and purified TST. Samples applied to lanes 2, 3, 5, and 6 contained 1 to 2 μg of protein; samples applied to lanes 1, 4, and 7 contained 5 μg of protein. Lane 7, molecular weight standards in thousands (K) lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase B (92,500); lane 1, ethanol-precipitated TST from strain 033; lane 2, strain 189 TST from preparative IEF; lane 3, strain 189 TST from chromatofocusing; lane 4, ethanol-precipitated TST from strain 189; lane 5, strain 033 TST from preparative IEF; lane 6, strain 033 TST from chromatofocusing. The stained band seen at 58K in all of the samples is an artifact of the gel system. Arrow denotes two major bands at the position in which TST was expected.

Survey of strains for TST. Examination of 26 non-vaginal TSS *S. aureus* strains for TST protein by IEF showed that the association of this protein with TSS was lower than that seen in vaginal TSS strains; 75 versus 100% (Table 2). When all of the TSS and non-TSS strains were examined for TST by ID with rabbit antiserum made to the chromatofocusing-purified protein and by IEF, it was found that some of the strains that were positive by IEF did not react with the antiserum. For example, strains D-8339 and D-0322 produced an IEF band similar to TST (Fig. 1, lanes 3 and 4) but did not react with antiserum to TST (Fig. 3B). These results (Table 2) showed that the IEF assay tended to overestimate the presence of TST in all strains except vaginal TSS strains. The protein was clearly associated with vaginal TSS strains by both assay methods, but in the non-vaginal TSS strains the association was only 62% (Table 2; ID data); the IEF assay estimate for TST was 75% with these strains. With non-vaginal non-TSS strains, the percentage of TST-positive strains by IEF was 39% compared with 23% by ID. With non-TSS vaginal strains, the estimates were 32% by IEF versus 16% by ID. For the total of TSS case strains, the IEF estimate for TST-positive strains was 87%, whereas by ID it was 78%. For the total non-TSS strains, the estimation of TST by IEF was also higher, 34 versus 19%.

During growth experiments with the defined medium, it was found that some strains that were negative for a neutral protein in the IEF assay after 18 h of growth became positive for a neutral protein when the growth period was extended to 72 h (Fig. 1, lanes 2 and 3). Analysis for TST by ID, however, showed that this 72-h protein did not react with antiserum to TST (Fig. 3B). Analyses for TST in TSS and non-TSS strains grown for 72 h showed that many TST-negative strains became positive by the IEF assay after this extended growth period; however, none of these "late" neutral proteins could be identified as TST by the ID assay (Table 2). The strains that were positive for the protein by ID after 72 h of growth were the same strains which had been positive at 18 h. The extended growth period, however, did increase the amount of TST produced by these positive strains. This increase was observed as a larger TST band in IEF and ID gels (data not shown).

The lack of correlation between the IEF and ID results suggested that the ID assay was less sensitive for TST than was the IEF assay. A chromatofocusing-purified TST was diluted in 1% glycine to the point that the protein could not be detected by IEF (Fig. 1, lanes 12 to 15). When these same dilutions were examined by ID, it was found that the ID assay was actually far more sensitive than the IEF assay (Fig. 1 and 3B). Repeated freezing and thawing of purified TST, however, caused a loss of ID reactivity, with no apparent loss of detectable protein by IEF (data not shown). This loss of ID activity indicates that immunological analyses for TST in biological specimens may be affected when specimens are repeatedly thawed and frozen.

DISCUSSION

TSS has been associated with the isolation of *S. aureus* strains which produce a characteristic exoprotein with a neutral isoelectric point and a molecular weight between 20,000 and 22,000 (2, 5, 6, 40). The association of the disease with strains having this marker protein (TST) has been reported to be as high as 83 to 100% (2, 5–7, 13, 14, 39–41). The presence of TST in non-TSS strains has varied from 0 to 63%, including 4 to 40% in vaginal isolates from healthy carriers and 0 to 36% in non-vaginal non-TSS isolates (2, 5–7, 10, 13, 14, 20, 39–41). The association of TST with non-vaginal TSS strains has not been as well studied but may be as high as 92% (based on data given in Table 1 of reference 5). The results of the present study have shown that TST was found in 100% of vaginal isolates from clearly defined TSS cases, in 62% of non-vaginal TSS isolates, in 16% of vaginal non-TSS isolates, and in 23% of non-vaginal non-TSS isolates (Table 2; ID data). Because of the low number of strains used in this study, these data cannot be extrapolated to reflect with absolute accuracy the presence of TST in a larger population of strains; however, the data compare well with results from other studies. The lower incidence of this toxin in non-vaginal isolates from clearly defined cases of TSS and the widely varying presence of TST in non-TSS strains indicate that the toxin may have little or no diagnostic value in this disease. Also, the fact that some non-vaginal strains from TSS cases are lacking TST suggests that it must not be the only staphylococcal toxin involved in the pathogenesis of this disease. This is supported by the study by Kapral, who found that a particular staphylococcal epidermal toxin was found in 73% of 52 TSS strains and in only 18% of 106 control strains (17).

The most widely used methods for the identification of TSS marker protein are analytical IEF and agarose gel ID. In one study, both methods were used with no reported discrepancy in the results obtained with either method (7). Our experience with IEF has shown that this method is subject to a substantial amount of error (Fig. 1 and 3B), particularly when certain types of growth conditions are used (Table 2). This was not unexpected, since *S. aureus* produces a number of neutral exoproteins, such as alpha toxin variant and exfoliative toxin (35), and several of the enterotoxins (4).

Cohen et al. (10) in a similar study found that strains which were positive by the IEF assay were not always positive by a solid-phase radioimmunoassay and that the production of TST by some strains depended on the medium used for growth. The ID assay used in this study proved to be highly specific and more sensitive than IEF. The level of sensitivity was similar to that of the ID procedure of Schlievert and Blomster (38) but was less than that described for the method of Robbins et al. (34). ID methods do not have the sensitivity of a radioimmunoassay, but they do not involve the cost and problems associated with radiolabeled materials. The ability of our ID method to detect TST-positive strains was examined early in this study by analysis of several coded strains provided by the Special Pathogens Laboratory, Centers for Disease Control, and in every case our results matched the strain designation as a TST-positive or -negative strain.

The defined medium used in this study proved to be very effective for the growth of these strains and the production of this protein. TST production in this medium ranged from 5 to 20 μg/ml of culture filtrate, depending on the strain. This compares well with previous reports of production of 2.4 to 19.2 μg of TST/ml in beef heart medium (38) and 1.5 to 8 μg of TST/ml in 4% NZ-amine A medium with 0.15% yeast extract (16). Complex infusion media are generally used for the growth of *S. aureus*, but defined media are preferred because they provide better quality control and a cleaner starting material for the purification of exoproteins. Also, they can be used to study the nutritional factors that control the synthesis of these proteins. Growth of our cultures for longer than 18 h resulted in an increase in the amount of TST produced by these strains, but it did not increase the number of TST-positive strains detected in this study. However, the use of an extended growth period in combination with a highly sensitive immunological assay method may prove to be very useful for identifying strains which produce very low amounts of this protein.

TABLE 2. Analysis for TST in 18- and 72-h cultures of TSS and non-TSS strains of *S. aureus* by ID and IEF

Isolate	No. of positive strains/no. of strains tested (% positive) in:			
	18-h Culture		72-h Culture	
	ID assay	IEF assay	ID assay	IEF assay
TSS case, vaginal	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)
TSS case, non-vaginal	16/26 (62)	20/26 (75)	9/15 (60)	13/15 (87)
Non-TSS, vaginal	3/19 (16)	6/19 (32)	3/19 (16)	12/19 (63)
Non-TSS, non-vaginal	3/13 (23)	5/13 (39)	3/13 (23)	7/13 (54)
Total TSS strains	36/46 (78)	40/46 (87)	29/35 (83)	33/35 (94)
Total non-TSS strains	6/32 (19)	11/32 (34)	6/32 (19)	19/32 (59)

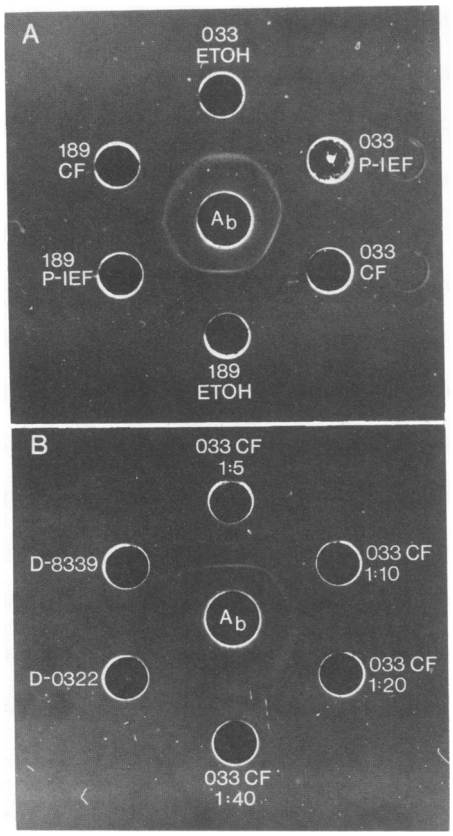

FIG. 3. Agarose gel ID with specific rabbit antiserum to TST purified by chromatofocusing (center wells). (A) Crude ethanol (ETOH)-precipitated TST and TST purified by preparative IEF (P-IEF) and chromatofocusing (CF) from strains 033 and 189 (TSS case, vaginal isolates). (B) Dilutions to ratios of 1:5, 1:10, 1:20, and 1:40 of strain 033 TST purified by chromatofocusing (600 μg of protein per ml) and crude ethanol precipitates of strains D-8339 (72 h of growth) and D-0322 (18 h of growth).

Attempts to purify the protein by preparative IEF (40) were not as successful as had been expected. Silver nitrate is a far more sensitive stain for proteins in acrylamide gels than is Coomassie blue (18, 22; Fig. 1 and 2), which may account for the reason we have found contaminants in these IEF preparations that were not reported in earlier studies in which Coomassie blue staining was utilized (7, 40). This lack of purity may be the reason that the amino acid analysis of IEF-purified TST (40) does not match that of TST purified by other methods (16, 32). The method of chromatofocusing followed by gel filtration proved to be more effective than did the preparative IEF method (Fig. 2); however, absolute purity again was not achieved. Igarashi et al. (16) have also used chromatofocusing and gel filtration, but it is not clear whether our preparations are of similar purity since they did not silver stain their acrylamide gels. We have made attempts to purify this protein further by chromatography on hydroxylapatite, DEAE-cellulose, and carboxymethyl cellulose in various buffers, but they were unsuccessful. Experiments with affinity chromatography with specific antisera are in progress.

Two major and one minor TST bands with apparently identical isoelectric points and very similar molecular weights were observed with both strains in our 250-mm gels (Fig. 2). The two major forms of this protein differed in molecular weight by only 700, which represents ca. five to seven amino acids. The minor TST band differed from the lower-molecular-weight TST about 1,800, or 13 to 18 amino acids. It is not clear at this time why this protein should exist in these different forms, or if all of them have the same toxin activity, or if they are present in other *S. aureus* strains. The presence of multiple molecular forms of exoproteins is not unusual in *S. aureus* (27). The small difference in molecular weights would indicate that these forms are not aggregates of a single protein. It is conceivable that these different proteins

are the result of incomplete removal of an N-terminal signal sequence of amino acids which is generally found in bacterial proteins which are secreted (11). Signal sequences can be longer than 20 amino acids and are usually removed by cleavage once the protein has passed through the membrane (11). If such is the case, then several N-terminal amino acids should be present; however, both Igarashi et al. (16) and Reiser et al. (32) have reported that only serine could be detected as the N-terminal amino acid of TST. It is interesting to note that the TST proteins purified by these investigators differ in chemical composition by ca. five amino acid residues (16, 32).

The molecular weights and isoelectric point determined in acrylamide gels for TST in this study are similar to those previously reported (5, 7, 23, 40). Curiously, an average molecular weight of 18,900 was determined when hydrodynamic methods were used which did not involve denaturation of the proteins. The sodium dodecyl sulfate-acrylamide gel method tends to overestimate the molecular weight of a protein that does not bind as much sodium dodecyl sulfate as the standard proteins used in the assay (42). Bonventre et al. (7) have reported that the estimated molecular weight of TST varied with the concentration of acrylamide used in the gel, a phenomenon which is typical of proteins which do not bind sodium dodecyl sulfate as well as expected (42). These data suggest that the true molecular weights of these proteins may be less than originally reported. This lower molecular weight may have to be taken into consideration when estimating the amino acid content of TST.

The fact that multiple forms of this protein have not been reported before raises an important question about the methods that have been used to assess the purity of TST preparations. Generally, these have involved electrophoresis on an acrylamide gel which was then stained with Coomassie blue. We have found this method to be far less sensitive than silver staining (Fig. 1 and 2). The degree of purity, or lack of it, of different TST preparations may help explain the different biological activities that have been reported for this protein. In an early report on TST, it was stated that the purified protein caused emesis in monkeys (5); in a later study in which the same purification method was used, it was observed that this biological property was lost (32). The 50% lethal dose of purified TST for rabbits has been reported to be as low as 2 μg/kg when given subcutaneously and 60 μg/kg when given intravenously (23). TST purified by the preparative IEF method has a reported intravenous 50% lethal dose for rabbits of 10 to 30 μg/kg (37). In contrast, Reiser et al. (32) have reported that their purified TST was not lethal in rabbits when given intravenously at 50 μg/kg. Also, they did not observe an enhancing effect by TST on endotoxin lethality in either mice or rabbits (32). This is in sharp contrast to reports that IEF-purified TST enhances endotoxin shock and lethality (36, 40). During our immunization procedure, rabbits were inoculated subcutaneously with chromatofocusing-purified TST with Freund incomplete adjuvant at concentrations as high as 100 μg/kg, and no deaths occurred. A rise in body temperature of 1 to 2°C within 24 h was generally observed in these rabbits. It is certainly possible that this lack of lethality was due to a very slow release of the protein from the adjuvant. These results emphasize the difficulty in assessing the biological activity of protein preparations which may vary in purity and suggest that the biological activities described for this protein (5, 31, 36, 37, 40) should be viewed with caution until it can be shown with certainty that the protein is homogeneous.

The relationship of TST to the pathogenesis of toxic shock is still unclear. The toxin is clearly associated with vaginal isolates but is sometimes missing in TSS strains isolated from other sites. The presence of TST protein in the breast milk of a woman with toxic shock and with vaginal colonization with TST-positive *S. aureus* cells has been previously reported (50), but this is not clear evidence that this toxin alone was responsible for the disease. Other staphylococcal toxins may have been present as well. Pollack et al. (25) have reported that successful vaginal colonization of TST-positive *S. aureus* cells in rhesus monkeys did not result in clinical signs of toxic shock, even though there was evidence of in vivo production and release of the protein (25). Very recently, Arnow et al. (1a) reported that colonization of TST-positive *S. aureus* cells in the patients and nursing staff of a hospital burn unit during a 4-month period did not result in a TSS-like illness, even though there was evidence of an antibody response to TST in one of the patients. A nurse with recurrent episodes of TSS was identified as the source of the TST strain, and it was noted that when she eventually developed a high antibody titer to TST, no further relapses occurred. Arnow et al. concluded that colonization with a TST-positive strain does not result in illness either because TST production may not be high enough or because TST alone may not be sufficient to cause the disease (1a). In a study by Barbour (2), it was shown that TSS strains with TST were generally less lethal than control strains without the protein. The presence of TST does not increase adherence to the vaginal mucosa or enhance resistance to killing by neutrophils (3). These studies do not make it clear whether the disease is mediated by more than one staphlococcal toxin or whether the presence of TST is absolutely vital to the development of the disease. The role of this protein in toxic shock will be more clearly defined when a homogeneous protein is available for use in suitable animal models, such as the rabbit model described previously by Arko et al. (1) and by Scott et al. (41).

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