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Resolution of Highly Purified Toxic-Shock Syndrome Toxin 1 into Two Distinct Proteins by Isoelectric Focusing†

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ABSTRACT: Highly purified toxic-shock syndrome toxin 1 (TSST-1) was prepared by differential precipitation with ethanol and resolubilization in water followed by successive electrofocusing in pH gradients of 3-10, 6-8, and 6.5-7.5. TSST-1, thus isolated, migrated as two distinct protein bands with isoelectric points of 7.08 (TSST-1a) and 7.22 (TSST-1b). When tested by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, both toxins migrated as homogeneous bands with molecular weights of 22 000. The gel bands were visualized by silver staining. The two toxins have nearly identical amino acid compositions and are immunologically identical as shown by Ouchterlony reactivity against TSST-1 hyperimmune serum. TSST-1a and TSST-1b have the same biological activities as TSST-1: the capacity to induce fever, enhancement of host susceptibility to lethal endotoxin shock, nonspecific T lymphocyte mitogenicity, and suppression of immunoglobulin M synthesis against sheep erythrocytes. These two proteins have been isolated from several different TSS-associated *Staphylococcus aureus* strains. The data suggest that the differences in isoelectric point result either from the presence of a cofactor or from alternative conformations. Since only two bands appear, microheterogeneity as a result of deamination or acetylation is unlikely.

Toxic-shock syndrome (TSS) is a multisystem illness characterized by acute onset of high fever, hypotension or dizziness, rash, desquamation of skin upon recovery, and variable multisystem involvement (Todd et al., 1978; Davis et al., 1980; Shands et al., 1980; Tofte & Williams, 1981). *Staphylococcus aureus* producing TSST-1 have been isolated from nearly 100% of menstrual-associated TSS patients (Schlievert et al., 1981; Bergdoll et al., 1981). TSST-1 has been cited by several investigators as a major toxin most likely responsible for the symptoms of TSS (Schlievert et al., 1981; Bergdoll et al., 1981;

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de Azavedo & Arbuthnott, 1984; Rasheed et al., 1985).

In 1981, TSST-1 was identified and characterized independently by Schlievert et al. as pyrogenic exotoxin C (PEC) and by Bergdoll and colleagues as enterotoxin F (SEF). Later PEC and SEF were shown to be immunologically and biochemically identical (Bonventre et al., 1983; Bergdoll & Schlievert, 1984; Igarashi et al., 1984). Other laboratories subsequently reported on the isolation of this TSS-associated toxin (Notermans & Dufrenne, 1982; Reiser, et al., 1983; Igarashi et al., 1984). Variations in the molecular weight (20000–24000), the isoelectric point (6.8–7.2), and the activity of certain biological functions of TSST-1 have been reported. This diversity may be the result of differences in the assay methods employed by each laboratory or may be due to the presence of low levels of contaminants.

In this report, we examine the purity of TSST-1, isolated by a modification of the procedure previously described by Schlievert et al. (1981). Purity was assessed by highly sensitive silver staining and Western blot analysis. Furthermore, we report the resolution of purified TSST-1 into two separate bands, TSST-1a and TSST-1b. The two proteins have different isoelectric points, 7.08 and 7.22, respectively. We demonstrate that the two TSST-1 proteins are immunologically and functionally identical. The biological activities which are evaluated include the capacity to induce fever, enhancement of host susceptibility to lethal shock by endotoxin, nonspecific T lymphocyte mitogenicity, and suppression of IgM synthesis against sheep erythrocytes. Finally, we investigate the occurrence of TSST-1a and TSST-1b by examining several different TSS-associated *S. aureus* strains.

Materials and Methods

All reagents and glassware used in the purification of TSST-1 and all biological assays were maintained pyrogen free.

Bacterial Strains. Four different strains of *Staphylococcus aureus* were used as the source of TSST-1a and TSST-1b. Strains MN8, 587, and RN4282 were isolated from patients who met the established criteria for TSS (Shands et al., 1980). In these strains, the TSST-1 structural gene was contained in the bacterial genome. Strain RN4512 produces toxin due to the insertion of plasmid pRN6201 which contains one copy of the TSST-1 structural gene. This strain did not contain a TSST-1 gene in its bacterial genome (Kreiswirth et al., 1983). All strains were maintained in the lyophilized state in the presence of 20% nonfat dry milk.

Toxin Purification. TSST-1 was prepared by a modification of methods previously described (Schlievert et al., 1981). Briefly, each *S. aureus* strain was cultured overnight in a dialyzable beef heart medium with shaking at 37 °C. TSST-1 was precipitated by adding 4 volumes of absolute ethanol at 4 °C (cells were not removed prior to ethanol addition). The precipitate was collected by centrifugation, and the residual alcohol was removed by brief lyophilization (30 min). The pellet was suspended in distilled water and centrifuged; this was repeated twice, and both supernatant fluids were pooled. The pooled supernatant fluids were then dialyzed against water overnight at 4 °C. The resultant preparation was then subjected to isoelectric focusing in a pH 3–10 gradient using commercial ampholytes (LKB-Produkter, Stockholm, Sweden). The visible band containing TSST-1 was harvested and refocused in a pH 6–8 gradient, yielding highly purified TSST-1. Direct visualization of the TSST-1 band results from a change in the refractive index of focused toxin.

TSST-1a and TSST-1b were isolated by one additional electrofocusing step. After TSST-1 was focused on the pH 6–8 gradient, approximately half of the Sephadex gel (G-75-50, Sigma Chemical Co., St. Louis, MO) was removed from the anode end. The gel remaining on the cathode end, containing the TSST-1 band, was repoured and then focused overnight using the remaining pH gradient (approximately pH 6.5–7.5).

After electrofocusing in a pH 6–8 or 6.5–7.5 gradient, protein bands were located by the zymogram print method (Winter et al., 1975). Discrete bands were scraped off the plate and were eluted from the gel by using pyrogen-free water. The ampholytes were removed by dialysis against water for 4 days with daily changes. Protein concentration was determined by the Bradford protein dye binding assay (Bio-Rad Laboratories, Richmond, CA). A typical purification of TSST-1a and -1b from strain MN8 yielded approximately 2 mg of each toxin per liter of culture fluid. For strains other than MN8, 200 μg of each toxin was obtained per liter of culture fluid.

Antiserum. TSST-1 hyperimmune antiserum was raised against the purified toxin in rabbits as previously described (Schlievert et al., 1977). Serum was filter-sterilized (0.20 μm) and stored at 4 °C.

Assays for Purity. The purified toxin samples were analyzed on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) system (Laemmli, 1970). The gels were stained by using the silver stain method described by Oakley et al. (1980). Samples were prepared for SDS–PAGE by boiling for 1 min in sample buffer [10% glycerol, 0.0625 M tris(hydroxymethyl))aminomethane hydrochloride (Tris-HCl) (pH 6.8), 2% SDS, and 0.0025% bromophenol blue]. β-Mercaptoethanol was omitted from the sample buffer to eliminate the band it produced at approximately M_r 60000 during silver staining (Merrill et al., 1984). The protein standards were diluted in sample buffer containing β-mercaptoethanol. Molecular weight standards used on all SDS–PAGE gels were ovalbumin (43000), α-chymotrypsinogen (25700), β-lactoglobulin (18400), lysozyme (14300), bovine trypsin inhibitor (6200), and insulin (3000).

Western blot analysis was performed following the procedure described by Blake et al. (1984). Briefly, the proteins from a 15% SDS–PAGE gel were transferred electrophoretically to nitrocellulose. The nitrocellulose blot was then preincubated for 45 min with 3% gelatin in Tris–phosphate buffer (TBS; 20 mM Tris and 0.5 M NaCl, pH 7.5). The blot was washed for 45 min in TBS–Tween 20 (0.05%) followed by a 1.5-h incubation with TSST-1 hyperimmune rabbit serum (1:100 dilution). The blot was washed twice for 5 min each with TBS–Tween 20 and then incubated for 1.5 h with a $1:10^3$ dilution of goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate (Sigma Chemical Co.). After addition of the substrate solution (5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium ion), color development was seen at the sites of antibody binding.

To show complete transfer of the protein bands from the SDS–PAGE gels to the nitrocellulose, we visualized the blotted bands on a duplicate nitrocellulose sheet by staining with 1% amido black (Hancock & Tsang, 1983).

Ouchterlony immunodiffusion (Ouchterlony, 1962) was performed in agarose [a 1% solution in phosphate-buffered saline (0.005 M sodium phosphate and 0.15 M NaCl, pH 7.0)] on microscope slides. Wells, punched in the gel in a hexagonal pattern, contained 20 μL of rabbit TSST-1 hyperimmune serum or 20 μL of purified toxin at 100 μg/mL. Slides were incubated at 37 °C and read after 6 h. The slides were soaked in water for 2 days to remove salts, air-dried, and finally

stained with Coomassie brilliant blue R250 overnight. The background was destained by using water–ethanol–glacial acetic acid (8:3:1).

Amino Acid Analysis. The amino acid compositions of TSST-1a and TSST-1b were determined by using an amino acid analyzer (Dionex D-330 amino acid/peptide analyzer, column dimensions 0.32 cm × 14 cm, Dionex DC-5A resin). The toxin samples were hydrolyzed (6 N HCl at 100 °C) for 22 h under a N_2 atmosphere. For the cysteic acid determination, dimethyl sulfoxide (Me_2SO) was added to the acid prior to hydrolysis. Cysteine was oxidized to cysteic acid, and the cysteic acid value was normalized against a regular run (Spencer & Wold, 1963). For proline determination, a wavelength of 440 nm was used. The proline value was then normalized against a regular run (Benson, 1972, 1973).

The predicted amino acid composition was derived from the DNA sequence of the TSST-1 structural gene [strain RN4512 (pRN6201)].

Biological Assays. Pyrogenicity and the capacity to enhance host susceptibility to endotoxic shock were measured as described by Kim & Watson (1970). In brief, rabbits (three per group) were given intravenous injections of purified toxin solutions diluted in phosphate-buffered saline. The temperature responses were recorded over a 4-h period by using rectal thermometers. Three different toxin doses (10, 1.0, and 0.1 μg/kg) were used to determine the minimum pyrogenic dose (MPD) at 4 h; 1 MPD/kg was defined as the dose of toxin required to produce a 0.5 °C increase in rabbit body temperature after 4 h (Schlievert et al., 1981). Enhancement of host susceptibility to lethal shock was demonstrated by challenging rabbits with $^1/_{500}$th LD_{50} (1 μg/kg) of endotoxin (derived from *Salmonella typhimurium*) given intravenously, 4 h after purified toxin was administered. The results are reported as the number of rabbits dead/total number of rabbits in the group (Schlievert et al., 1981; Schlievert, 1982).

Nonspecific T lymphocyte proliferation was measured by the quantitation of [^{3}H]thymidine incorporation into cellular DNA. The procedure used was previously described by Poindexter & Schlievert (1985a). Basically, 2.5 × 10^5 mouse splenocytes, in 200-μL volumes, were cultured in the presence of purified toxin in quadruplicate in RPMI 1640 medium supplemented with 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 200 mM L-glutamine, 100 units of penicillin, 100 μg of streptomycin/mL, and 2% heat-inactivated fetal calf serum (Grand Island Biological Co.). The cultures were maintained in 96-well tissue culture plates and incubated at 37 °C under an atmosphere of 7.5% CO_2 and 92.5% air in a humidified incubator. On day 3, 1 μCi of [^{3}H]thymidine was added to each well. Eighteen hours later, the cells were harvested by using a MASH II apparatus (Microbiological Associates, Bethesda, MD) which collected the cellular DNA onto glass fiber filters. The samples were counted for ^{3}H incorporation in a scintillation counter (Model LS; Beckman Instruments, Fullerton, CA). The data were reported as the average counts per minute of four replicate samples ± the standard deviation (SD).

Toxin-induced suppression of IgM-secreting cells was measured by a modified Jerne plaque assay described by Schlievert (1983). Briefly, 2.5 × 10^6 mouse splenocytes, in 250-μL volumes, were incubated in triplicate with 4 × 10^5 sheep erythrocytes (SRBC) and varying amounts of purified toxin. Culture conditions were the same as those for the mitogenicity assay, mentioned above. Maximum suppression occurs after 4 days of exposure to nanogram levels of TSST-1 (Schlievert, 1983). Therefore, cultures were assayed at day 4 for direct plaque-forming cells (PFC) against sheep erythrocytes. Results were reported as PFC ± SD/10^7 cells.

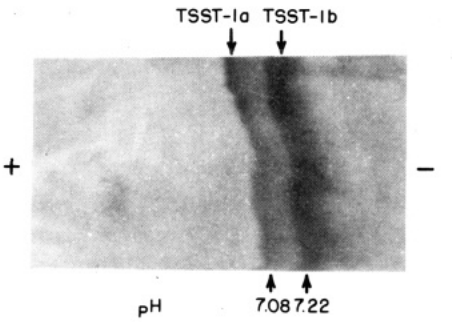

FIGURE 1: Zymogram print of highly purified TSST-1 which was resolved into TSST-1a (p*I* = 7.08) and TSST-1b (p*I* = 7.22) by electrofocusing in a pH gradient of approximately 6.5–7.5. Protein bands were located on the filter paper print after being stained with Coomassie brilliant blue R250. TSST-1 was isolated from *S. aureus* strain MN8.

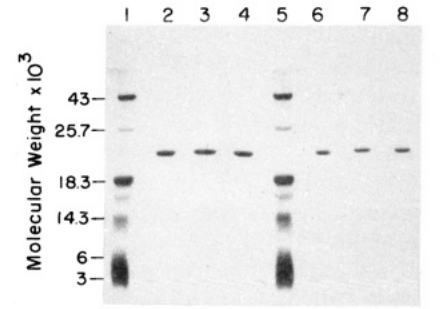

FIGURE 2: SDS–polyacrylamide gel electrophoresis. Lanes 1 and 5, low molecular weight standards; lane 2, purified TSST-1 (3.5 μg); lane 3, TSST-1a (3.5 μg); lane 4, TSST-1b (3.5 μg); lane 6, purified TSST-1 (1.5 μg); lane 7, TSST-1a (1.5 μg); lane 8, TSST-1b (1.5 μg). The protein bands were detected by silver staining. The proteins used as molecular weight standards were ovalbumin (43K), α-chymotrypsinogen (25.7K), β-lactoglobulin (18.4K), lysozyme (14.3K), bovine trypsin inhibitor (6.2K), and insulin (3K).

RESULTS

Purification of TSST-1, -1a, and -1b. Schlievert et al. (1981) published the isolation of TSST-1 (PEC); the purity was assessed by Coomassie blue staining of SDS–PAGE gels. The modified purification procedure has two additional electrofocusing steps: a pH 6–8 gradient which yields a highly pure preparation of TSST-1 and a pH 6.5–7.5 gradient which resolves the TSST-1 preparation into two separate bands, TSST-1a (p*I* = 7.08) and TSST-1b (p*I* = 7.22), shown in Figure 1. An isoelectric point (p*I*) of 7.2 was previously reported for TSST-1 isolated from the pH 3–10 gradient (Schlievert et al., 1981). These results show that TSST-1 focused on a narrow pH gradient actually has two molecular forms.

The purity of the above preparations, which were derived from *S. aureus* strain MN8, was evaluated on a 15% SDS–PAGE gel (Figure 2). The level of purity was determined by silver staining. The gel shows that all three toxins (TSST-1, -1a, and -1b)) migrated as homogeneous bands with molecular weights of 22 000. Concentrated solutions of TSST-1, 1 mg/mL (14 μg/lane), also resulted in a single band on SDS–PAGE gels (gels not shown). These results verify that our purification procedure yields a highly pure preparation of TSST-1 with a molecular weight identical with that previously reported by Schlievert et al. (1981).

The SDS–PAGE gel bands were electrophoretically transferred to nitrocellulose for Western blot analysis (Figure

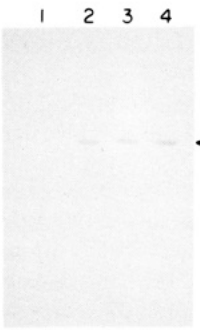

FIGURE 3: Western blot analysis. Lane 1, low molecular weight standards; lane 2, TSST-1 (3.5 μg); lane 3, TSST-1a (3.5 μg); lane 4, TSST-1b (3.5 μg). Antiserum, rabbit anti-TSST-1 serum. Conjugate serum, goat anti-rabbit Ig–alkaline phosphatase conjugate.

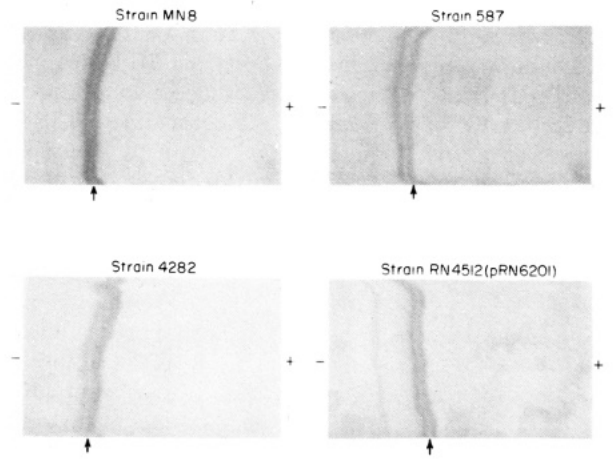

FIGURE 4: Isolation of TSST-1a and TSST-1b from four different *S. aureus* preparations by isoelectric focusing on a pH 6–8 gradient. Zymogram prints were stained with Coomassie brilliant blue R250.

3). The immunoblot shows all three proteins were immunologically reactive to the rabbit antiserum, lanes 2, 3, and 4. Controls were run to ensure that the TSST-1 hyperimmune serum was not nonselectively binding to protein bands. Lane 1 contains the molecular weight standards; no bands were detected upon the addition of substrate to this control. A duplicate lane containing the standards was stained with 1% amido black to demonstrate that all molecular weight bands were effectively transferred to the nitrocellulose (blot not shown). Lanes 1–4 were also incubated with normal rabbit serum. The control serum did not react with any transferred bands in the immunoblotting analysis (blot not shown).

Characterization of TSST-1a and -1b. TSST-1a and TSST-1b were isolated from four different *S. aureus* strains to evaluate whether the occurrence of the two TSST-1 proteins was a phenomenon restricted to *S. aureus* strain MN8. The isoelectric focusing patterns of all four strains in a pH 6–8 gradient are shown in Figure 4. Strain RN4512 (pRN6201), containing only cloned TSST-1, also yielded both 1a and 1b, thus suggesting that the two proteins are not the result of two slightly different TSST-1 structural genes existing in the bacterial genome.

The two bands were harvested from all four strains and were assayed for Ouchterlony reactivity. A single clear line of identity was obtained between TSST-1, TSST-1a, and TSST-1b, demonstrating that the toxins are immunologically identical.

The amino acid compositions for TSST-1a and TSST-1b are listed in Table I. The proteins have nearly identical amino acid compositions. In addition, comparisons between the compositions of 1a and 1b with the predicted values derived

Table I: Amino Acid Compositions (Residues per Mole) of TSST-1a and -1b, Isolated from Strain MN8, As Compared to the Inferred Amino Acid Composition of RN4512 (pRN6201)

amino acid	TSST-1a[a]	TSST-1b[a]	RN4512 (pRN6201)[b]
aspartic acid	26	27	23
threonine	21	20	18
serine	20	20	19
glutamic acid	20	20	17
proline	10	8	10
glycine	13	14	10
alanine	4	5	7
half-cystine	0	0	0
valine	5	5	5
methionine	0	0	1
isoleucine	15	15	15
leucine	14	16	16
tyrosine	10	8	11
phenylalanine	7	7	8
histidine	5	5	6
lysine	23	24	22
tryptophan	ND[c]	ND[c]	3
arginine	4	5	4
	total 197	199	195

[a] Residues per mole values are based on a molecular weight of 22 000. [b] Residues per mole inferred from the DNA sequence of the TSST-1 structural gene. [c] ND, not determined.

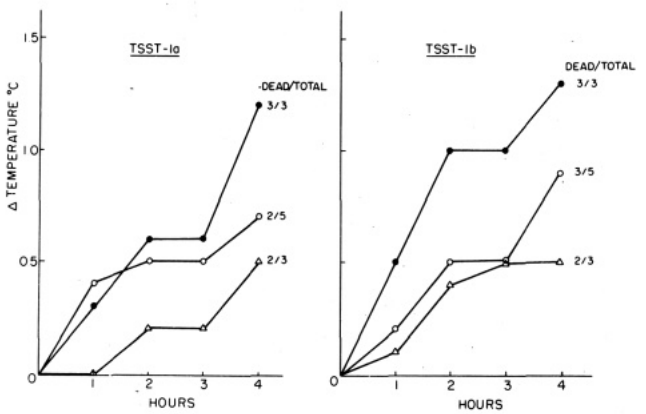

FIGURE 5: TSST-1a- and TSST-1b-induced pyrogenicity and enhanced susceptibility to lethal endotoxin shock in rabbits (three per group). Exotoxin was given intravenously (iv) at 0 h: 10 μg/kg (●); 1.0 μg/kg (○); 0.1 μg/kg (△). Temperature reported is the mean value of each group. Endotoxin, 1 μg/kg, was given iv at the 4-h time point. Fractions at the right list the number of the rabbits in each group that died per number of rabbits in each group. The LD_{50} of endotoxin alone given to rabbits was approximately 500 μg/kg.

from the known TSST-1 gene sequence were also in good agreement. The composition of the two toxins also correlates closely with compositions previously reported for TSST-1 (Igarashi et al., 1984; Reiser et al., 1983), excluding the differences in cysteine residues reported to be present by Reiser et al. (1983).

Biological Properties. The two TSST-1 proteins were examined for their ability to induce fever and to enhance host susceptibility to lethal endotoxin shock (Figure 5). Both toxins, 1a and 1b, induced a rise in temperature to similar levels, at equal doses. The rise in fever at each dose (10, 1, and 0.1 μg/kg) is comparable to that produced by highly purified TSST-1 at equivalent doses. The MPD calculated for TSST-1a is 0.13 μg/kg and is 0.14 μg/kg for TSST-1b. Schlievert (1984) reported an MPD of 0.15 μg/kg for TSST-1.

To evaluate the toxins for enhanced susceptibility to endotoxin, rabbits were given 1 μg/kg endotoxin 4 h after they received TSST-1a or -1b. This endotoxin dose is $^1/_{500}$th of the reported LD_{50} for rabbits (Schlievert, 1982). At toxin doses of 10 and 1.0 μg/kg, both TSST-1a and TSST-1b en-

Table II: Comparisons of Lymphocyte Mitogenicity[a] Induced by TSST-1, -1a, and -1b, As Measured by Incorporation of [^{3}H]Thymidine into DNA

	mean cpm × 10^3 ± SD × 10^3[b] for		
toxin dose (ng)	TSST-1	TSST-1a	TSST-1b
1000.0	220 ± 26	180 ± 21	220 ± 12
100.0	210 ± 9	200 ± 9	210 ± 19
10.0	220 ± 17	210 ± 15	210 ± 25
1.0	210 ± 25	192 ± 11	220 ± 19
0.1	180 ± 10	127 ± 29	140 ± 18
0.01	70 ± 7	25 ± 5	33 ± 9
0.001	5 ± 2	4 ± 2	5 ± 1
0	1 ± 0	2 ± 1	2 ± 0

[a] Cultures contain 2.5 × 10^5 murine splenocytes per 200 μL. [b] cpm ± SD, average counts per minute of quadruplicate samples ± standard deviation.

Table III: In Vitro Suppression by TSST-1, -1a, and -1b of the Direct Plaque-Forming (IgM) Cell (PFC) Response to Sheep Erythrocytes after 4 Days of Culture

treatment	dose (ng/culture)[a]	PFC ± SD/10^7 splenocytes	suppression (%)[b]
no toxin	0	1030 ± 370	
TSST-1	1000	220 ± 120	79
	100	190 ± 80	82
	10	450 ± 40	56
	0.1	440 ± 100	57
TSST-1a	1000	150 ± 170	85
	100	330 ± 70	68
	10	210 ± 160	80
	0.1	650 ± 140	37
TSST-1b	1000	210 ± 80	80
	100	420 ± 270	60
	10	350 ± 190	66
	0.1	700 ± 350	32

[a] Culture contained 2.5 × 10^6 splenocytes in 250 μL. [b] Percent of suppression = (1 − experimental PFC/control PFC) × 100.

hanced the susceptibility to lethal endotoxin shock. The degree of enhancement elicited by TSST-1a and -1b is equivalent to that of TSST-1 preparations (Schlievert, 1982).

To examine the mitogenic ability of the two proteins, mouse splenocytes were cultured with various concentrations of TSST-1, -1a and -1b. Proliferative responses were measured in a 4-day assay by the incorporation of [^{3}H]thymidine into cellular DNA (Table II). Significant responses could be generated by all three toxins at amounts as low as 0.01 ng/2.5 × 10^5 cells. These results are comparable to those obtained by Poindexter & Schlievert (1985a).

TSST-1a and TSST-1b were evaluated for the capacity to suppress the IgM antibody response generated by SRBC stimulation of murine splenocytes. The response was measured by quantitating the number of direct (IgM) plaque-forming cells (PFC) per culture. The effective dose range for suppression of the 4-day PFC responses by TSST-1, -1a, and -1b is shown in Table III. Substantial suppression was produced by all three toxins at doses ranging from 1000 to 10 ng per culture. Toxin doses as low as 0.1 ng per culture still showed 30–50% suppression of the PFC response. These results are similar to the in vitro TSST-1-induced suppression results previously reported (Schlievert et al., 1981). The presence of toxin in the cell cultures did not affect cell viability.

Discussion

The data presented in this paper show that TSST-1 was purified to homogeneity by double isoelectric focusing, as seen by silver staining of SDS–PAGE gels (Figure 2). Further, purified TSST-1 was resolved, after isoelectric focusing in a pH gradient of 6.5–7.5, into two immunologically and functionally identical proteins which differed only in isoelectric point. This report is the first study to demonstrate the isolation of two TSST-1 proteins.

The results showed that both TSST-1 proteins, designated TSST-1a and TSST-1b, have molecular weights of 22 000, which agrees with the TSST-1 value previously reported by Schlievert et al. (1981). Also, the sequence-predicted amino acid composition yields a molecular weight of 22 268, which is analogous to our value. The two toxins formed a line of identity with purified TSST-1 when reacted against TSST-1 antiserum. Both TSST-1a and TSST-1b produced fever and enhanced host susceptibility to lethal endotoxin shock in rabbits. Earlier, Igarashi et al. (1983) reported an MPD of 0.018 μg/kg for TSST-1 isolated from strain 587. In comparison to the MPD reported by Schlievert & Kelly (1982), Igarashi et al. concluded that they had isolated a toxin with a higher biological activity and inferred that our preparation was contaminated, therefore lowering the MPD obtained. SDS–PAGE analysis showed that a single TSST-1 band is produced, even by electrophoresis of concentrated TSST-1 solutions (1 mg/mL). The high level of toxin purity coupled with our use of pyrogen-free glassware and water implies that the apparent higher biological activity seen by Igarashi et al. is generated by contaminants, pyrogens and/or endotoxin present in distilled water and unburned glassware, or by differences in animals.

The amino acid composition of 1a and 1b correlates well with the composition obtained from the TSST-1 sequence, as well as those previously published except that reported by Schlievert et al. (1981). The composition reported by Schlievert et al. (1981) most likely contained ampholytes remaining from the isoelectric focusing which interfered with the amino acid analysis (Poindexter & Schlievert, 1985b). The TSST-1 samples analyzed here were dialyzed against water for 4 days to eliminate ampholyte interference. Reiser et al. (1983) found that TSST-1 contained two half-cystine residues. No cysteine residues were observed in TSST-1 in this study, in the predicted sequence, or in all other reported amino acid compositions (Igarashi et al., 1983; Schlievert et al., 1981). The lack of cysteine residues in TSST-1, as well as the lack of emetic activity, differentiates TSST-1 from staphylococcal enterotoxins which characteristically contain two half-cystine residues (Bergdoll et al., 1973).

TSST-1a and TSST-1b were isolated from several different TSS-associated *S. aureus* strains, showing the isolation of these two toxins was not restricted to strain MN8. Interestingly, both toxins were produced by strain RN4512 (pRN6201), which produces toxin from the plasmid-encoded TSST-1 structural gene. These results imply that the differences in isoelectric point are not necessarily the consequence of two different structural genes. Further, TSST-1 does not contain any carbohydrate residues (Igarashi et al., 1983); therefore, differences in the degree of glycosylation cannot explain the difference in p*I*. Since we consistently isolate only two bands, microheterogeneity, resulting from modification of particular sites such as deamination or acetylation, is unlikely because these processes usually generate several bands (three to six) of varying p*I* (Metzger et al., 1975). It is possible that one TSST-1 form associates with a cofactor (AMP, NADH) which causes the differences seen. Related streptococcal toxins have been shown to associate with AMP (Schlievert et al., 1979). The differences in p*I* values also could arise from minimal proteolytic processing or the presence of a blocked N-terminus. Finally, the microheterogeneity observed could be explained by the existence of two alternate conformations of TSST-1

which maintain the structure of immunological and functional sites but differ in total protein charge. Whatever mechanism is generating the difference in p*I*, that mechanism is regulated in such a way to consistently generate nearly equivalent amounts of TSST-1a and TSST-1b.

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