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Improved Purification and Biologic Activities of Staphylococcal Toxic Shock Syndrome Toxin 1

WINNIE W. S. KUM, KEVIN B. LAUPLAND, RAYMOND H. SEE, AND ANTHONY W. CHOW*

Departments of Medicine and Microbiology, Division of Infectious Diseases, University of British Columbia, Vancouver General Hospital, and the Canadian Bacterial Diseases Network, Vancouver, British Columbia, Canada V5Z 3J5

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An improved method for producing highly purified toxic shock syndrome toxin 1 (TSST-1) by preparative isoelectric focusing in a Bio-Rad Rotofor cell and then chromatofocusing is described. Purification to homogeneity was confirmed by silver staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 50 μg of protein was loaded), by immunoblotting with polyclonal rabbit antiserum raised against the crude culture supernatant used for purification, and by autoradiography after iodination and SDS-PAGE. Biologic activity was demonstrated by mitogenicity and cytokine induction (tumor necrosis factor alpha [TNF-α], interleukin 1-β [IL-1β], and IL-6) of human peripheral blood mononuclear cells (PBMCs) and by lethality in New Zealand White rabbits following subcutaneous infusion. In contrast to commercial TSST-1 preparations, our TSST-1 preparation required the presence of both monocytes and T cells for the induction of TNF-α and IL-1β from human PBMCs. A 46-kDa contaminating protein in the commercial TSST-1 preparation, identified as staphylococcal lipase, was likely responsible for the induction of TNF-α and IL-1β from human monocytes in the absence of T cells, a biologic activity falsely attributed to purified TSST-1. Our improved purification procedure for TSST-1 provides a high yield and is both more rapid and less labor intensive than previously reported methods. Furthermore, our studies clearly demonstrate the need for stringent methods of purity assessment of TSST-1 preparations before ascribing to them their potent biologic activities.

Toxic shock syndrome toxin 1 (TSST-1) is implicated as the major cause of toxic shock syndrome (TSS) (2, 6, 14, 24, 31). The biologic activities of TSST-1 include nonspecific mitogenicity of T lymphocytes (5), induction of various cytokines including several interleukins (interleukin-1 [IL-1], IL-2, and IL-6), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) from human peripheral blood mononuclear cells (PBMCs) (8, 9, 13), suppression of immunoglobulin synthesis (30), and enhancement of host susceptibility to lethal endotoxin shock (31). However, the exact mechanism(s) by which TSST-1 exerts these effects remains poorly understood. The availability of highly purified TSST-1 preparations is essential for elucidating the pathogenic role of TSST-1 in TSS. Several groups of investigators have reported different purification procedures for TSST-1 (3, 4, 12, 20, 22, 26, 27, 29). However, variations in the molecular sizes (20 to 24 kDa), isoelectric points (6.8 to 7.2), amino acid compositions, and biologic activities of different TSST-1 preparations have been reported. We previously observed considerable variability when different TSST-1 preparations from several investigators and commercial sources were examined under standard conditions and strongly emphasized the need for stringent purity assessment of TSST-1 preparations (29). We also reported additional modifications of existing purification procedures including ion-exchange chromatography, gel filtration, reverse-phase high-performance liquid chromatography, and preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (29). However, these procedures were both labor intensive and time-consuming, while the final yield of purified TSST-1 was low (16%). Here, we report a further improved, time-efficient, and less labor intensive procedure for obtaining highly purified TSST-1. The final yield was tripled (43%), while the time requirement for purifying 10 to 15 mg of TSST-1 was shortened, from 4 weeks by the previous procedure down to 7 to 10 days by the method described here. In addition, we demonstrate that, contrary to other reports, purified TSST-1 requires T cell participation to stimulate human peripheral blood monocyte production of TNF-α, IL-1β, and IL-6 and that a contaminating 46-kDa protein, identified as staphylococcal lipase, is responsible for the induction of these cytokines in the absence of T cells. Our studies of the biologic properties of purified TSST-1 further demonstrate the need for continued vigilance and stringent methods of purity assessment for TSST-1 preparations.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strain and TSST-1 purification. (i) Preparation of culture supernatant. *Staphylococcus aureus* MN8, originally isolated from the vagina of a woman with typical menstrual TSS, was used for toxin production. All reagents and glassware used for bacterial culture and purification of TSST-1 were maintained in pyrogen-free condition to prevent endotoxin contamination (29). Briefly, the organism was cultured for 18 h in a dialyzable brain heart infusion broth at 37°C in a controlled-environment incubator-shaker (New Brunswick Scientific, Edison, N.J.). The culture supernatant (10 liters) was centrifuged at 15,000 × *g* for 30 min at 4°C (L8-M ultracentrifuge; Beckman Instruments, Palo Alto, Calif.). The spun supernatant was desalted and concentrated 10-fold by using the Amicon Spiral Cartridge concentrator (molecular mass cutoff, 10 kDa; model CH2PRS; Acrylic Reservoir RA2000; Spiral Cartridge S1 Y10; Amicon, Beverly, Mass.).

The concentrated supernatant was then filter sterilized and stored at 4°C until use.

(ii) Preparative isoelectric focusing (PIEF). Forty-five milliliters of concentrated crude supernatant was mixed with 2% (wt/vol) Pharmalyte (pH 6.7 to 7.7; Sigma Chemical Co., St. Louis, Mo.) and focused on the Rotofor cell (Bio-Rad Laboratories, Richmond, Calif.) for 4 h at 12 W of constant power and 15°C. The initial conditions were 550 V and 22 mA; at equilibrium, the settings were 1,050 V and 10 mA. The electrolytes in the anode and cathode chambers were 0.1 M H_3PO_4 and 0.1 M NaOH, respectively. Twenty fractions were collected and their pH readings were recorded, and aliquots of the fractions were assayed for TSST-1 by an enzyme-linked immunosorbent assay (ELISA) previously developed in our laboratory (28). Fractions containing TSST-1 with concentrations greater than 50 μg/ml were pooled, dialyzed against several changes of deionized water (total volume, 12 liters) for 48 h at 4°C, and lyophilized.

(iii) Chromatofocusing. TSST-1 fractions partially purified by PIEF were further purified by chromatofocusing (7, 16) by using a pH 6 to 8 gradient polybuffer exchanger (PBE 94; Pharmacia Fine Chemicals, Uppsala, Sweden). Lyophilized and partially purified TSST-1 preparations were reconstituted in 10 ml of Tris-acetate buffer (25 mM; pH 8.3) and applied to a column (K15/30, 1.5 by 30 cm; Pharmacia) containing 80 ml of PBE 94 equilibrated with Tris-acetate buffer. Elution of TSST-1 was accomplished with polybuffer 96-acetate (pH 6.0; Pharmacia) at a flow rate of 36 ml/h. Fractions were assayed for TSST-1 by ELISA, and those containing TSST-1 were pooled, dialyzed against several changes of deionized water (total volume, 12 liters) for 72 h at 4°C, and lyophilized.

(iv) Comparison with a previously established purification procedure. We have previously established a purification procedure for TSST-1, which was essentially modified from that of Igarashi et al. (12), in which we used ion-exchange chromatography and then chromatofocusing. This purification procedure was compared with the further improved and rapid procedure described here. Briefly, after two SP-Sephadex C-25 ion-exchange chromatography procedures, the fractions containing TSST-1 at concentrations greater than 100 μg/ml were pooled, dialyzed against several changes of NH_4HCO_3 (0.1 M; pH 7.5) for 48 h at 4°C, lyophilized, and subjected to chromatofocusing as described above.

Assessment of purity. (i) Silver staining after SDS-PAGE. The purified toxin preparations were analyzed on an SDS–14% PAGE system (15). A commercial preparation of TSST-1 purified from *S. aureus* FRI-1169 was purchased from Toxin Technology Inc. (Madison, Wis.) and was included for comparison. Samples were prepared by boiling for 4 min in sample buffer (Tris-HCl [0.175 M; pH 6.8], 4% SDS, 20% glycerol, and 0.004% bromophenol blue); 50 μg of protein was loaded per well. The molecular size markers included were myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa) (Amersham Canada Limited, Oakville, Ontario, Canada). Protein bands were visualized by silver staining (Bio-Rad) (19).

(ii) Immunoblotting with rabbit antisera raised against crude *S. aureus* MN8 culture supernatant. Female New Zealand White rabbits (20 weeks old; R and R Rabbitry, Stanwood, Wash.) were immunized against crude culture supernatant from *S. aureus* MN8 by six repeated subcutaneous injections at 2-week intervals (100 μg of culture supernatant in Freund's complete adjuvant [1:1] for the first injection and in incomplete adjuvant thereafter). For immunoblot analysis, TSST-1 preparations were subjected to SDS-PAGE (10 μg of protein was loaded) and were electrophoretically transferred to nitrocellulose by using the Semi-Dry Electroblotter A (Ancos, Dimension Laboratories, Inc., Mississauga, Ontario, Canada). The nitrocellulose blot was incubated overnight in 5% skim milk (Difco Laboratories, Detroit, Mich.) containing 1% polyclonal rabbit antiserum raised against the crude culture supernatant from *S. aureus* MN8. The blot was then treated with 0.1% biotinylated goat anti-rabbit immunoglobulin G (GIBCO/BRL Life Technologies, Inc., Gaithersburg, Md.) in Tris-buffered saline (TBS) for 2 h; this was followed by 30 min of incubation with 0.1% streptavidin–horseradish peroxidase conjugate (GIBCO/BRL) in TBS. Color development was accomplished with 4-chloronaphthol (GIBCO/BRL) substrate.

Autoradiography after iodination and SDS-PAGE. The purities of the TSST-1 preparations were further assessed by visualization after iodination and autoradiography. TSST-1, which was purified from the crude culture supernatant of *S. aureus* MN8, was iodinated by the modified chloramine T procedure (33) and was subjected to SDS-PAGE (160 pg of protein was loaded) as described above. At the end of the run, the gel was fixed in a solution containing methanol, acetic acid, and distilled water (4:1:5), dried, and subjected to autoradiography on Kodak X-Omat RP film with double intensifying screens at −70°C for 1 day.

Assessment of biologic activity. (i) Mitogenicity assay. Fresh human PBMCs from healthy adult donors were obtained by centrifugation over Histopaque 1.077 (Sigma) leukopheresis packs (33). Cells at the interface were washed three times in Hanks balanced salt solution and were suspended at a concentration of 3×10^6 cells per ml in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah)–2 mM L-glutamine (GIBCO/BRL)–20 μg of polymyxin B sulfate (Sigma) per ml. A total of 3×10^5 cells were cultured in 200-μl volumes with various concentrations of TSST-1 in round-bottom 96-well tissue culture plates (Falcon Labware; Becton-Dickinson Canada Inc., Mississauga, Ontario, Canada) for 3 days at 37°C in 5% CO_2. At 48 h, cells were pulsed with 1 μCi of [^{3}H]thymidine (5 Ci/mmol; Amersham) and, 18 h later, were harvested onto glass fiber filter paper with an automatic harvester (Skatron, Sterling, Va.). Samples were counted in a liquid scintillation counter (LS1800; Beckman).

(ii) Induction of TNF-α, IL-1β, and IL-6. Human PBMCs and fractionated human monocytes or T cells were prepared as described by See et al. (34). Briefly, fresh human PBMCs were prepared as described above. Mononuclear cells were first separated into T and non-T cell populations by rosetting with sheep erythrocytes. Monocytes were then separated from B lymphocytes by density centrifugation over Percoll (Pharmacia Fine Chemicals, Dorval, Quebec, Canada). The purities of the monocyte preparations were ≥95%, as assessed by nonspecific esterase staining. For isolation of purified human T lymphocytes, erythrocyte-rosetted cells were first treated with ammonium chloride to lyse sheep erythrocytes, washed three times, and subjected to antibody-directed complement lysis to remove contaminating monocytes and B cells by using the combination of an antibody to the human leukocyte antigen (HLA)-DR antigen (L243), an antibody to the monocyte-specific CD11b antigen (OKM1), and pooled rabbit complement. Purified human T cells were >98% $CD2^+$ cells and <2% HLA-DR^+ cells as determined by flow cytometric analysis. For cytokine induction studies, 5×10^5 monocytes, T cells, or both (mixed 1:1)

TABLE 1. Recovery and relative purities of TSST-1 fractions during various stages of purification

Procedure and fraction	Sp act (mg of TSST-1/mg of protein)[a]	% Recovery[b]
Current (PIEF and chromatofocusing) procedure[c]		
Crude culture supernatant[d]	0.019 ± 0.007	100.0
PIEF	0.267 ± 0.004	52.7 ± 16.8
Chromatofocusing	1.013 ± 0.011	84.7 ± 10.6
Overall recovery relative to that in crude culture supernatant		42.9 ± 8.6
Previous (ion-exchange chromatography and chromatofocusing) procedure[e]		
Crude culture supernatant	0.003 ± 0.002	100.0
First ion exchange	0.268 ± 0.155	82.5 ± 6.7
Second ion exchange	0.472 ± 0.113	75.8 ± 5.9
Chromatofocusing	0.875 ± 0.012	76.2 ± 0.9
Overall recovery relative to that in crude culture supernatant		42.6 ± 1.7

[a] TSST-1 was quantitated by ELISA (28); total protein was quantitated as described by Lowry et al. (18). Values are means ± standard deviations.
[b] Relative to the purification step, unless stated otherwise. Values are means ± standard deviations.
[c] Values are averages of two separate experiments.
[d] Concentrated 10-fold.
[e] Values are averages of three separate experiments.

were cultured in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 20 μg of polymyxin B sulfate per ml, in the presence or absence of 10 μg of different TSST-1 preparations per ml, at 37°C in 5% CO_2. After 24 h, supernatants of toxin-stimulated cell cultures were collected, microcentrifuged at 800 × *g* for 5 min, and frozen at −70°C until analysis. The presence of TNF-α in culture supernatants was assayed by an ELISA previously developed in our laboratory (34). Briefly, flat-bottom 96-well microtiter plates (Immulon I; Dynatech Laboratories, Inc., Alexandria, Va.) were coated overnight with goat anti-human TNF-α (R & D Systems, Minneapolis, Minn.) in carbonate buffer (pH 9.6). Test samples or human recombinant TNF-α standards (R & D Systems) in phosphate-buffered saline with 3% bovine serum albumin were added in triplicate and incubated at 37°C for 90 min. The plates were washed and incubated with biotinylated goat anti-human TNF-α at 37°C for 90 min. After washing and incubation with streptavidin-alkaline phosphatase at 37°C for 20 min, an ELISA amplification system (GIBCO/BRL) was then used to increase the sensitivity of the assay. Coloration was quantitated at an optical density at 495 nm with a Titertek Multiscan MC spectrophotometer (Flow Laboratories). The sensitivity limit of this ELISA was 62 pg/ml; no cross-reactivity was observed with human TNF-β, IL-1α, IL-1β, or IFN-γ. In separate parallel experiments, this TNF-α ELISA correlated strongly with the L929 cytotoxicity assay (correlation coefficient, 0.84; $n = 13$; $P < 0.001$) (34). Human IL-1β was quantitated by using an ELISA kit purchased from R & D Systems (sensitivity limit, 120 pg/ml). The presence of IL-6 was determined by a [^{3}H]thymidine uptake assay with the IL-6-dependent murine hybridoma subclone B9 as described previously (1). Briefly, B9 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum–2 mM L-glutamine–8 pM human recombinant IL-6 (rh-IL-6; R & D Systems) for 3 days at 37°C in 5% CO_2. Test samples or rh-IL-6 standards were added in triplicate to 96-well round-bottom plates (Falcon Labware). Then, 5×10^3 washed B9 cells were added to each well, and the plates were incubated at 37°C in 5% CO_2 for 3 days. Cells were then pulsed with 0.5 μCi of [^{3}H]thymidine (5 Ci/mmol; Amersham) for 6 h, harvested, and counted in a liquid scintillation counter (LS1800; Beckman). The sensitivity limit of this assay was 12.5 pg/ml.

(iii) Lethality in rabbits. The subcutaneous infusion model of Parsonnet et al. (23) was used to determine the lethal effect of TSST-1 in vivo. The purified toxin was administered to male New Zealand White rabbits (18 to 20 weeks old; R and R Rabbitry) by constant subcutaneous infusion with implanted miniosmotic pumps (Alza Corp., Palo Alto, Calif.). Six groups of rabbits were infused with various concentrations of TSST-1 over a period of 7 days. The 50% lethal dose was calculated as described by Reed and Muench (25).

RESULTS

Comparative yield of TSST-1. The yield and specific activity of TSST-1 obtained at various stages of our current and previously established purification procedures are summarized in Table 1. The overall yield by the current (PIEF and chromatofocusing) procedure was 42.9%, which was comparable to that by our previous (ion-exchange and chromatofocusing) procedure (42.6%). However, the new method was much less labor intensive, and the time requirement for the purification of 10 to 15 mg of TSST-1 was considerably reduced, from 4 weeks by the previous procedure to approximately 7 to 10 days by the current procedure. In a single 4-h run, the PIEF step with the Rotofor cell eliminated most extraneous proteins while retaining 53% of TSST-1 from the crude culture supernatant of *S. aureus* MN8 (Table 1 and Fig. 1B, lane 3).

Purity assessment of TSST-1. The purities of our TSST-1 preparations were evaluated by several methods, as follows: (i) silver staining after SDS-PAGE (50 μg of protein was loaded) (Fig. 1A), (ii) immunoblotting with polyclonal rabbit antiserum raised against the crude culture supernatant of *S. aureus* MN8 during various stages of purification (10 μg of protein was loaded) (Fig. 1B), and (iii) autoradiography after iodination and SDS-PAGE (160 pg of protein was loaded) (Fig. 1C). All these methods revealed one homogeneous protein (molecular size, 22 kDa). In contrast, at least eight bands (molecular size range, 15 to 50 kDa) were visualized by silver staining of the commercial TSST-1 preparation (Fig. 1A, lane 1). Immunoblotting of the commercial preparation (purified from *S. aureus* FRI-1169) with the rabbit

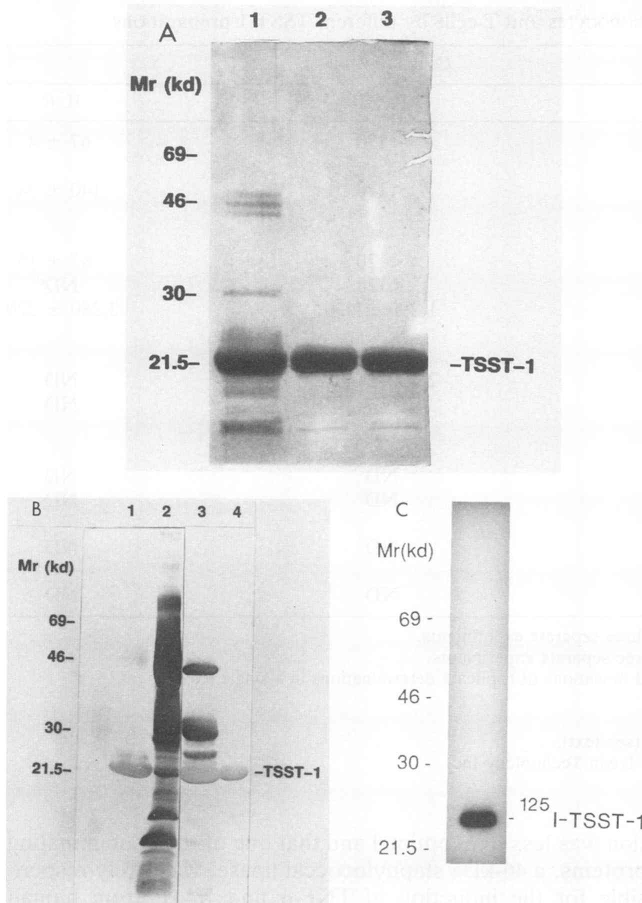

FIG. 1. (A) Purities of TSST-1 preparations assessed by silver staining after SDS-PAGE. Lane 1, commercial TSST-1 (Toxin Technology Inc.); lane 2, purified TSST-1 (PIEF and chromatofocusing method); lane 3, purified TSST-1 (ion-exchange chromatography and chromatofocusing method). (B) Purities of TSST-1 preparations by immunoblotting with rabbit antisera raised against crude culture supernatant from *S. aureus* MN8. Lane 1, commercial TSST-1 purified from *S. aureus* FRI-1169 (Toxin Technology Inc.); lane 2, crude culture supernatant from *S. aureus* MN8; lane 3, after PIEF; lane 4, after chromatofocusing. (C) Purities of TSST-1 preparations by autoradiography of ^{125}I-labeled TSST-1 purified by the PIEF and chromatofocusing method (specific activity, 2,856 Ci/mmol; 160 pg was loaded for SDS-PAGE).

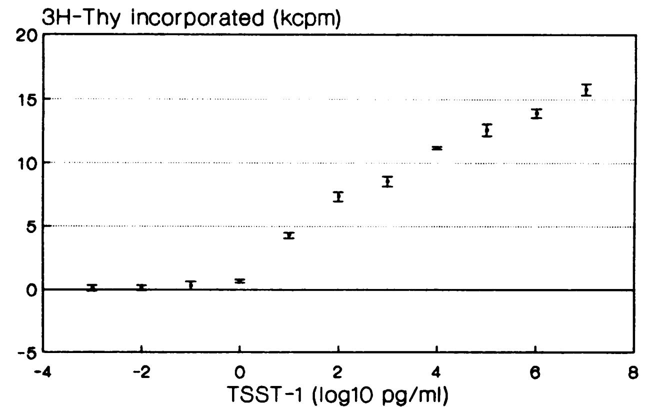

FIG. 2. Dose-dependent mitogenic activity of purified TSST-1 (PIEF and chromatofocusing method) on human PBMCs in vitro. PBMCs (3×10^5) were incubated with various concentrations of TSST-1 for 48 h at 37°C and pulsed with 1 μCi of [^{3}H]thymidine (^{3}H-Thy) as described in the text. Each value represents the mean ± standard deviation for triplicate determinations. Background activity was subtracted.

antiserum raised against *S. aureus* MN8 revealed at least two different weakly immunoreactive bands (molecular size range, 24 to 50 kDa) in addition to TSST-1 (Fig. 1B, lane 1). Immunoblotting of the same preparation with pooled normal human serum revealed at least eight different bands (molecular size range, 15 to 50 kDa) (data not shown).

Biologic activities of purified TSST-1. Our TSST-1 preparations were endotoxin-free, as measured by the *Limulus* amoebocyte lysate test (sensitivity limit, 10 pg/ml) (17). This TSST-1 preparation retained its biologic activity, including (i) induction of mitogenicity of human PBMCs in a dose-dependent manner (Fig. 2); (ii) induction of TNF-α, IL-1β, and IL-6 from coculture of human monocytes and T cells (mixed 1:1) (Table 2); and (iii) lethality in New Zealand White rabbits following subcutaneous infusion with a 50% lethal dose of 45 μg/kg (Fig. 3).

Of interest, our purified TSST-1 preparation did not induce TNF-α, IL-1β, or IL-6 from human monocytes alone unless T cells were also present (Table 2). In contrast, as observed previously by us and others (34, 35), the commercial TSST-1 preparation (Toxin Technology Inc.) was able to induce TNF-α and IL-1β production in human monocytes alone in the absence of T cells (Table 2). When the commercial TSST-1 preparation was further purified to homogeneity by a chromatofocusing step and was then incubated with human monocytes alone, little or no detectable TNF-α was found unless T cells were also present (Table 2). Finally, two impurity peaks contained within the commercial TSST-1 preparation were eluted from the chromatofocusing column. One of these (impurity peak 1 but not impurity peak 2) was able to induce TNF-α in human monocytes in the absence of T cells (Table 2). Impurity peak 1, a 46-kDa protein, was subsequently identified by N-terminal amino acid sequence analysis to be a staphylococcal lipase. Staphylococcal lipase was purified to homogeneity from the culture supernatant of *S. aureus* MN8 by a combination of isoelectric focusing in the Bio-Rad Rotofor cell and then gel filtration through a Sephadex G-75 column (data not shown). This purified staphylococcal lipase strongly stimulated the induction of TNF-α, IL-1β, and IL-6 from human monocytes alone in a dose-dependent manner and in the absence of T cells (data not shown).

Since other investigators who did not use the commercial TSST-1 preparation (Toxin Technology Inc.) have also reported that T cells were not required for TNF-α and IL-1 induction in human monocytes (21, 22), the discrepancy between our results and those of other authors may lie in the purity of fractionated monocytes (e.g., contamination with T cells) as well as the purity of the TSST-1 preparations. To investigate this, we assessed the ability of commercial as well as our purified TSST-1 preparations to induce the production of TNF-α and IL-1β in the human monocytic cell line THP-1 (American Type Culture Collection, Rockville, Md.), which obviates the possibility of T cell contamination. Our purified TSST-1 preparations did not induce these cytokines from THP-1, whereas the commercial TSST-1 preparation did (data not shown). Furthermore, THP-1 cells and human monocytes stimulated with purified TSST-1 did not exhibit transcriptional activation of the IL-1β gene

TABLE 2. Induction of TNF-α, IL-1β, and IL-6 in human monocytes and T cells by different TSST-1 preparations

Stimulus and cells	Concn (pg/ml)		
	TNF-α[a]	IL-1β[b]	IL-6[c]
RPMI + monocytes	<62	<120	67 ± 4
RPMI + monocytes + T cells	<62	<120	140 ± 74
Our TSST-1 with:			
Monocytes	<62	<120	87 ± 15
T cells	<62	<120	ND[d]
Monocytes + T cells	2,600 ± 1,100	1,290 ± 430	2,280 ± 220
TSST-1 (TT)[e] with:			
Monocytes	1,620 ± 190	2,400 ± 40	ND
Monocytes + T cells	1,500 ± 46	ND	ND
Further purified TSST-1 (TT)[f] with:			
Monocytes	83 ± 37	ND	ND
Monocytes + T cells	3,050 ± 450	ND	ND
Impurity peak 1[g] + monocytes	1,350 ± 600	ND	ND
Impurity peak 2[g] + monocytes	<62	ND	ND

[a] TNF-α was measured by ELISA; results are means ± standard deviations of three separate experiments.
[b] IL-1β was measured by ELISA; results are means ± standard deviations of three separate experiments.
[c] IL-6 was measured by the B9 proliferation assay; results are means ± standard deviations of triplicate determinations in a single experiment.
[d] ND, not done.
[e] TSST-1 (TT), commercial TSST-1 purchased from Toxin Technology Inc.
[f] TSST-1 from Toxin Technology Inc. was further purified by chromatofocusing (see text).
[g] Impurity peaks 1 and 2 were eluted during further purification of TSST-1 from Toxin Technology Inc.

unless T cells were present and in physical contact with monocytes (15a).

DISCUSSION

Several groups have reported different purification procedures for TSST-1 (3, 4, 12, 20, 22, 26, 27, 29). However, those investigators seldom reported stringent methods of purity assessment of their preparations. We have previously shown the inadequacy of Coomassie blue or silver staining after SDS-PAGE as the sole criterion for determining the purity of TSST-1 (29). Here, we demonstrated by several techniques that the purity of a commercial TSST-1 preparation was less than optimal and that one of the contaminating proteins, a 46-kDa staphylococcal lipase, was likely responsible for the induction of TNF-α and IL-1β from human monocytes alone in the absence of T cells, a biologic activity which was falsely attributed to purified TSST-1. We demonstrated that induction of both TNF-α and IL-1β in human monocytes could be detected only if T cells were also present and in physical contact with the monocytes (34). The induction of IL-6 was also dramatically enhanced compared with that in controls only in the presence of both T cells and monocytes. After further purification to homogeneity by a chromatofocusing step, we observed that the further purified commercial TSST-1, like our purified TSST-1, also required the presence of both monocytes and T cells for TNF-α induction. Finally, we showed conclusively that the presence of impurities in the commercial TSST-1 preparation, subsequently identified by N-terminal amino acid sequence analysis to be a staphylococcal lipase, was responsible for the induction of TNF-α in human monocytes alone in the absence of T cells.

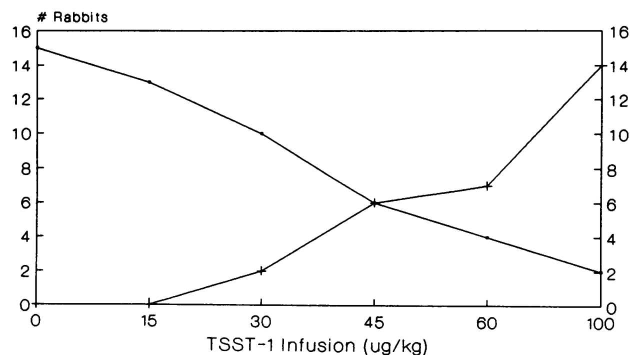

FIG. 3. Dose-dependent lethal activity of purified TSST-1 (PIEF and chromatofocusing method) in New Zealand White rabbits following subcutaneous infusion by using implanted miniosmotic pumps containing various concentrations of TSST-1. The 50% lethal dose, determined by the point of intersection of the two curves, was 45 μg/kg of body weight. ●, rabbits that survived; +, rabbits that died.

Trede et al. (35) recently reported that a commercial TSST-1 preparation (also from Toxin Technology Inc.) induced transcriptional activation of TNF-α and IL-1β genes in human monocytes and the monocytic cell line THP-1. We confirmed this finding with the commercial TSST-1 preparation (data not shown), but were unable to induce either TNF-α or IL-1β from human monocytes alone when stimulated with our purified TSST-1 (34). Other investigators who did not use the commercial TSST-1 preparation have also reported the production of TNF-α and IL-1β in human monocytes alone in the absence of T cells (8, 13, 21, 22, 32). The discrepancy between our results and those of the other workers may lie in the purity of fractionated monocytes (e.g., contamination with T cells) as well as in the purity of TSST-1 preparations. Importantly, those authors used plas-

tic adherence-purified monocytes for the induction of TNF and IL-1 by TSST-1. Thus, a few contaminating T cells in the monocyte preparations may be sufficient for triggering TNF and IL-1 production in response to the toxin. We routinely fractionate our monocytes from human PBMCs first by rosetting with sheep erythrocytes to separate T cell and non-T cell populations and subsequently by density centrifugation over Percoll. The purities of our monocyte preparations were ≥95% as assessed by nonspecific esterase staining and flow cytometric analysis. Finally, we demonstrated conclusively by using the monocytic cell line THP-1, which eliminated any possibility of T cell contamination, that purified TSST-1 is unable to induce the production of TNF-α or IL-1β in the absence of T cells, while the commercial TSST-1 preparation can (15a).

Our findings are in agreement with those observed with another superantigen, staphylococcal enterotoxin A, in which TNF and IL-1 production in human monocytes are also T cell dependent (10, 11). These findings further emphasize the critical importance of using highly purified preparations for studies of the biologic activities of these potent members of the superantigen family.

Here, we described a simplified, further improved, and more rapid procedure for the purification of TSST-1 with a Rotofor cell for PIEF and then chromatofocusing of culture supernatants. Since both of these purification steps are based in part on the pI of TSST-1, there is the possibility that two different proteins with the same pI could be copurified by our procedure. This did not occur with *S. aureus* MN8, since a single homogeneous 22-kDa protein was visualized either by silver staining after SDS-PAGE (after loading 50 μg of protein), by immunoblotting with polyclonal rabbit antiserum raised against the crude culture supernatant of *S. aureus* MN8, or by autoradiography after iodination and SDS-PAGE. This improved purification method was comparable to our previous procedure modified from that of Igarashi et al. (12) in producing a similar yield of highly purified TSST-1, but our procedure is more rapid and less labor intensive. The time requirement for purifying 10 to 15 mg of TSST-1 was shortened considerably, from 4 weeks by the ion-exchange and chromatofocusing procedure down to 7 to 10 days by the current procedure. Furthermore, the final yield was threefold higher than that achieved with an earlier method, also described by us, using ion-exchange chromatography, gel filtration, reverse-phase high-performance liquid chromatography, and preparative SDS-PAGE (29). The ready availability of highly purified TSST-1 preparations by these techniques should greatly facilitate ongoing studies for the further elucidation of the biologic properties of this staphylococcal exoprotein and its putative role in the pathogenesis of TSS.

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REFERENCES

1. **Aarden, L. A., E. R. De Groot, O. L. Schaap, and P. Lansdorf.** 1987. Production of hybridoma growth factor by human monocytes. Eur. J. Immunol. **17:**1411–1416.
2. **Bergdoll, M. S., B. A. Crass, R. F. Reiser, R. N. Robbins, and J. P. Davis.** 1981. A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock syndrome Staphylococcus aureus isolates. Lancet **i:**1017–1021.
3. **Blomster-Hautamaa, D. A., B. N. Kreiswirth, R. P. Novick, and P. M. Schlievert.** 1986. Resolution of highly purified toxic-shock syndrome toxin-1 into two distinct proteins by isoelectric focusing. Biochemistry **25:**54–59.
4. **Blomster-Hautamaa, D. A., and P. M. Schlievert.** 1988. Preparation of toxic shock syndrome toxin-1. Methods Enzymol. **165:**37–43.
5. **Calvano, S. E., F. W. Quimby, A. C. Antonacci, R. F. Reiser, M. S. Bergdoll, and P. Dineen.** 1984. Analysis of the mitogenic effects of toxic shock toxin on human peripheral blood mononuclear cells in vitro. Clin. Immunol. Immunopathol. **33:**99–110.
6. **de Azavedo, J. C. S., and J. P. Arbuthnott.** 1984. Toxicity of staphylococcal toxic shock syndrome toxin-1 in rabbits. Infect. Immun. **46:**314–317.
7. **Ende, I. A., G. Terplan, B. Kickhofen, and D. K. Hammer.** 1983. Chromatofocusing—a new method for purification of staphylococcal enterotoxins B and C1. Appl. Environ. Microbiol. **46:** 1323–1330.
8. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1988. Nonpurulent response to toxic shock syndrome toxin 1 producing *Staphylococcus aureus*: relationship to toxin-stimulated production of tumor necrosis factor. J. Immunol. **140:**949–953.
9. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. Infect. Immun. **57:**291–294.
10. **Fischer, H., M. Dohlsten, M. Lindvall, H.-O. Sjogren, and R. Carlsson.** 1989. Binding of staphylococcal enterotoxin A to HLA-DR on B cell lines. J. Immunol. **142:**3151–3157.
11. **Gjorloff, D., H. Fischer, G. Hedlund, J. Hansson, J. S. Kenny, A. C. Allison, H.-O. Sjogren, and M. Dohlsten.** 1991. Induction of interleukin-1 in human monocytes by the superantigen staphylococcal enterotoxin A requires the participation of T cells. Cell. Immunol. **137:**61–71.
12. **Igarashi, H., H. Fujikawa, H. Usami, S. Kawabata, and T. Morita.** 1984. Purification and characterization of *Staphylococcus aureus* FRI 1169 and 587 toxic shock syndrome enterotoxins. Infect. Immun. **44:**175–181.
13. **Ikejima, T., S. Okusawa, J. W. M. van der Meer, and C. A. Dinarello.** 1988. Induction by toxic shock syndrome toxin-1 of a circulating tumor necrosis factor-like substance in rabbits and of immunoreactive tumor necrosis factor and interleukin-1 from human mononuclear cells. J. Infect. Dis. **158:**1017–1025.
14. **Kass, E. H., and J. Parsonnet.** 1987. On the pathogenesis of toxic shock syndrome. Rev. Infect. Dis. **9:**S482–S489.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.

15a. **Lee, V. T. P., and A. W. Chow.** Unpublished data.

16. **Lei, Z., R. F. Reiser, and M. S. Bergdoll.** 1988. Chromatofocusing in the purification of staphylococcal enterotoxin D. J. Clin. Microbiol. **26:**1236–1237.
17. **Levin, J., and F. B. Bang.** 1984. The role of endotoxin in the extracellular coagulation of limulus blood. Bull. Johns Hopkins Hosp. **115:**265–274.
18. **Lowry, O. H., N. J. Rosebrough, L. A. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
19. **Merrill, C. R., D. Goldman, and M. L. Van Keuren.** 1984. Gel protein stains: silver stain. Methods Enzymol. **104:**441–447.
20. **Notermans, S., and J. B. Dufrenne.** 1982. A simple purification method for enterotoxin F produced by *Staphylococcus aureus* and some properties of the toxin. Antonie Leeuwenhoek **48:** 447–455.
21. **Parsonnet, J., and Z. A. Gillis.** 1988. Production of tumor necrosis factor by human monocytes in response to toxic shock syndrome toxin-1. J. Infect. Dis. **158:**1026–1033.
22. **Parsonnet, J., Z. A. Gillis, and G. B. Pier.** 1986. Induction of interleukin-1 by strains of *Staphylococcus aureus* from patients with nonmenstrual toxic shock syndrome. J. Infect. Dis. **154:** 55–63.
23. **Parsonnet, J., Z. A. Gillis, A. G. Richter, and G. B. Pier.** 1987.

A rabbit model of toxic shock syndrome that uses a constant, subcutaneous infusion of toxic shock syndrome toxin 1. Infect. Immun. **55:**1070–1076.

24. **Rasheed, J. K., R. J. Arko, J. C. Feeley, F. W. Chandler, C. Thornsberry, R. J. Gibson, M. L. Cohen, J. D. Jeffries, and C. V. Broome.** 1985. Acquired ability of *Staphylococcus aureus* to produce toxic shock-associated protein and resulting illness in a rabbit model. Infect. Immun. **47:**598–604.
25. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
26. **Reeves, M. W., R. J. Arko, F. W. Chandler, and N. B. Bridges.** 1986. Affinity purification of staphylococcal toxic shock syndrome toxin-1 and its pathologic effects in rabbits. Infect. Immun. **51:**431–439.
27. **Reiser, R. F., R. N. Robbins, G. P. Khoe, and M. S. Bergdoll.** 1983. Purification and some physicochemical properties of toxic-shock toxin. Biochemistry **22:**3907–3912.
28. **Rosten, P. M., K. H. Bartlett, and A. W. Chow.** 1987. Detection and quantitation of toxic shock syndrome toxin-1 in vitro and in vivo by a noncompetitive enzyme-linked immunosorbent assay. J. Clin. Microbiol. **25:**327–332.
29. **Rosten, P. M., K. H. Bartlett, and A. W. Chow.** 1989. Purification and purity assessment of toxic shock syndrome toxin 1. Rev. Infect. Dis. **11:**S110–S116.
30. **Schlievert, P. M.** 1983. Alteration of immune function by staphylococcal pyrogenic exotoxin type C. Possible role in toxic-shock syndrome. J. Infect. Dis. **151:**65–72.
31. **Schlievert, P. M., K. N. Schandsx, B. B. Dan, G. P. Schmid, and R. D. Nishimura.** 1981. Identification and characterization of an exotoxin from *Staphylococcus aureus* associated with toxic-shock syndrome. J. Infect. Dis. **143:**509–516.
32. **Scholl, P., A. Diez, W. Mourad, J. Parsonnet, R. S. Geha, and T. Chatila.** 1989. Toxic shock syndrome toxin 1 binds to major histocompatibility complex class II molecules. Proc. Natl. Acad. Sci. USA **86:**4210–4214.
33. **See, R. H., G. Krystal, and A. W. Chow.** 1990. Binding competition of toxic shock syndrome toxin 1 and other staphylococcal exoproteins for receptors on human peripheral blood mononuclear cells. Infect. Immun. **58:**2392–2396.
34. **See, R. H., W. W. S. Kum, A. H. Chang, S. H. Goh, and A. W. Chow.** 1992. Induction of tumor necrosis factor and interleukin-1 by purified staphylococcal toxic shock syndrome toxin 1 requires the presence of both monocytes and T lymphocytes. Infect. Immun. **60:**2612–2618.
35. **Trede, N. S., R. S. Geha, and T. Chatila.** 1991. Transcriptional activation of IL-1β and tumor necrosis factor-α genes by MHC class II ligands. J. Immunol. **146:**2310–2315.