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Detection and Quantitation of Toxic Shock Syndrome Toxin 1 In Vitro and In Vivo by Noncompetitive Enzyme-Linked Immunosorbent Assay

PATRICIA M. ROSTEN, KAREN H. BARTLETT, AND ANTHONY W. CHOW*

Division of Infectious Diseases, Departments of Medicine and Microbiology, University of British Columbia and Vancouver General Hospital, Vancouver, British Columbia, Canada V5Z 1M9

Received 9 June 1986/Accepted 21 October 1986

Toxic shock syndrome toxin 1 (TSST-1), an exotoxin produced by many *Staphylococcus aureus* strains, is implicated as the prime causal agent of toxic shock syndrome (TSS). A sensitive and specific noncompetitive enzyme-linked immunosorbent assay (ELISA) capable of detecting TSST-1 at concentrations from 0.5 to 16 ng/ml was developed. This assay did not detect other staphylococcal enterotoxins including A, B, C_1, C_2, C_3, D, and E. Possible interactions with protein A were readily eliminated by pretreatment of test samples with 10% normal rabbit serum. The assay was adapted for rapid screening of TSST-1 production by *S. aureus* isolates in culture supernatants in vitro and for detection of TSST-1 in vaginal washings of TSS patients and healthy controls in vivo. All 35 *S. aureus* isolates confirmed to be TSST positive by Ouchterlony immunodiffusion and 59 of 60 isolates confirmed to be TSST-1 negative gave concordant results by ELISA. Interestingly, toxigenic *S. aureus* strains isolated from TSS patients quantitatively produced significantly more TSST-1 in vitro compared with toxigenic control strains ($P < 0.05$, Mann-Whitney rank sum test). TSST-1 could be detected by ELISA in three of four vaginal washings collected within 3 days of hospitalization from three women with acute menstrual TSS, compared with 0 of 17 washings from nine TSS patients hospitalized longer than 3 days ($P = 0.003$, Fisher's exact test) and 1 of 15 washings from 14 healthy control women ($P = 0.016$). This noncompetitive ELISA should be particularly useful for rapid screening of TSST-1 production by *S. aureus* isolates, for the purification and biochemical characterization of TSST-1, and for human and animal studies of the pathogenesis of TSS.

Toxic shock syndrome toxin 1 (TSST-1) is believed to be a major cause of the disease manifestations of toxic shock syndrome (TSS), first described by Todd et al. (18). This toxin, identified as a 22- to 24-kilodalton protein with an isoelectric point (pI of approximately 7.2, is produced by almost all *Staphylococcus aureus* strains isolated from patients with acute menstrual TSS, compared with 16 to 23% of isolates from healthy control women or patients with non-TSS cases of *S. aureus* infection (13). In addition, TSST-1 has been detected in the serum, urine, and vaginal washings (M. E. Melish, F. S. Chen, and M. S. Murata, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, B21, p. 27), as well as breast milk (19) of individuals with typical TSS.

To further explore the role of TSST-1 in the pathogenesis of TSS, we developed a simple, sensitive, and specific enzyme-linked immunosorbent assay (ELISA) capable of detecting TSST-1 at concentrations of 0.5 to 16 ng/ml in culture supernatants and biologic fluids. Such an assay should be particularly useful for rapid screening of TSST-1 production by *S. aureus* isolates in vitro, for the purification and biochemical characterization of TSST-1, and for human and animal studies of the pathogenesis of TSS.

MATERIALS AND METHODS

Immunoreactants. Reference rabbit antitoxin to TSST-1 (1:4-diluted stock) as well as purified reference TSST-1 (50-μg/ml stock) were kindly provided by Merlin Bergdoll (University of Wisconsin, Madison). Additional rabbit anti-TSST-1 antisera were raised by active immunization of New Zealand White rabbits after graded intravenous challenge with purified TSST-1 according to the dose schedule of Bergdoll et al. (2). Immunoglobulin fractions of all rabbit sera to be used were prepared by precipitation with saturated ammonium sulfate by standard procedures (7). Briefly, 1 ml of serum and 0.5 ml of saturated ammonium sulfate were mixed and stirred for 2 h at room temperature. The precipitated protein was centrifuged at 1,400 × *g* for 30 min at room temperature, the supernatant was discarded, and the pellet was dissolved in 1 ml of saline. This procedure was repeated twice, and the final pellet was dissolved in phosphate-buffered saline (PBS; pH 8.0) and dialyzed (12 to 14 kilodalton exclusion) against PBS (pH 8.0) at 4°C for 48 h with four changes of buffer. Protein determinations were performed by the method of Lowry et al. (9).

Ammonium sulfate-precipitated anti-TSST-1 was conjugated to alkaline phosphatase with glutaraldehyde by the method of Voller et al. (20). A 1-mg sample of precipitated anti-TSST-1 and 2.5 mg of alkaline phosphatase enzyme (P-0405; Sigma Chemical Co., St. Louis, Mo.) were mixed, glutaraldehyde (Sigma G-5882) was added to a final concentration of 0.2%, and this was stirred for 2 h at room temperature to allow conjugation. The conjugate was then dialyzed (12- to 14-kilodalton exclusion) against PBS (pH 7.0) for 24 h with two changes of buffer. Further dialysis was performed against 0.05 M Tris hydrochloride (pH 8.0) for 24 h with two changes of buffer. The conjugate was then diluted to 2 ml with 0.05 M Tris hydrochloride containing 1% bovine

* Corresponding author.

serum albumin and 0.02% sodium azide and stored at 4°C in the dark.

ELISA procedure. The noncompetitive, antibody enzyme-conjugated ELISA procedure was used for TSST-1 detection. Ammonium sulfate-precipitated reference rabbit anti-TSST-1 as well as nonimmune control sera (100 μl) diluted at 1:10,000 in 0.05 M carbonate buffer (pH 9.6) were passively adsorbed to the inner wells of polystyrene microtiter plates (Immulon I; Dynatech Laboratories, Inc., Alexandria, Va.) by incubation at 20°C for 18 h. Unbound antitoxin was removed by three 2-min washes with PBS containing 0.05% Tween 20, pH 7.4 (PBS-T). Purified TSST-1 reference standard (serially diluted from 16.0 to 0.5 ng/ml in PBS-T), test samples pretreated with normal rabbit serum (10% [vol/vol] final concentration) to eliminate possible sources of protein A, and additional reagent controls were pipetted in duplicate in 100-μl volumes to their respective wells. This was followed by incubation at 37°C for 2 h and washing to remove unbound toxin. After addition of the conjugate (100 μl) and another 2-h incubation at 37°C, the plates were washed, treated with *p*-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine buffer [pH 9.8], 100 μl) (BDH, Poole, England), and incubated at 37°C for 90 min to allow the enzyme reaction to proceed. Plates were read at 405 nm in a Titertek multiscan spectrophotometer (Flow Laboratories, Inc., McLean, Va.) Linear regression analyses of the standard reference toxin concentrations were calculated by plotting absorbance (405 nm) versus $\log_2$ TSST-1 concentration. TSST-1 concentrations in test samples were predicted from the reference toxin regression equations derived during each assay procedure. The specificity of the ELISA for TSST-1 was examined by inclusion of nonimmune rabbit serum lacking anti-TSST-1, by pretreatment of TSST-1 with rabbit antitoxin, and by examination for possible cross-reactivity with protein A and other staphylococcal enterotoxins, including enterotoxins A, B, C_1, C_2, C_3, D, and E (Toxin Technology Inc., Madison, Wis.) in concentrations of 1,000, 100, and 10 ng/ml.

TSST-1 detection in *S. aureus* culture supernatants. The *S. aureus* isolates tested for in vitro TSST-1 production were vaginal isolates from TSS patients or healthy control women. Culture supernatants were prepared from subcultures of *S. aureus* grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) with shaking at 37°C for 18 to 20 h in ambient atmosphere. Cells were removed by centrifugation (30,000 × *g* for 15 min), and culture supernatant was filter sterilized and frozen at −70°C before testing. Each culture supernatant was tested for TSST-1 both by Ouchterlony immunodiffusion and by ELISA with and without overnight preincubation at 4°C in normal rabbit serum (10%, vol/vol) to eliminate any interfering effect of protein A. Pretreated test samples were centrifuged in a Beckman Microfuge to remove particulate matter before testing in microtiter wells at neat, 1:10, and 1:100 dilutions in PBS-T. Detection of TSST-1 by Ouchterlony immunodiffusion was examined in 1% Noble agar (Difco Laboratories) with reference rabbit antitoxin (1:36 dilution) in the center well.

TSST-1 detection in vaginal washings of TSS patients and healthy controls. Vaginal washings were collected from nine patients who fulfilled the case definition for menstrual TSS according to the Centers for Disease Control and were studied in Vancouver between August 1980 and February 1985 (5). Washings were also collected from 14 healthy control women recruited from the University of British Columbia Student Health Service. Informed consent was obtained from all subjects. Vaginal washings were obtained with 10 ml of sterile pyrogen-free saline from the posterior vaginal fornix, using a plastic pipette and under direct visualization. The washings were immediately centrifuged (90 × *g* for 10 min at 4°C), and the supernatant was filter sterilized and stored at −70°C. Quantitation of TSST-1 in vaginal washings was performed by the above ELISA method with several additional controls. Vaginal washings to be tested for TSST-1 were diluted 1:2, 1:4, and 1:8 in PBS-T and examined in duplicate in 100-μl volumes. Vaginal washings giving positive results were retested with and without 10% normal rabbit serum pretreatment. Pooled vaginal washings devoid of TSST-1 from healthy women (C-0) served as a negative control, and pooled washings with TSST-1 added (50 ng/ml) served as a positive control (C-50). These were diluted 1:2, 1:4, and 1:8 in PBS-T and tested in 100-μl volumes in duplicate. A third control for specificity consisted of the C-50 control to which had been added the same reference rabbit anti-TSST-1 used to coat the microtiter wells; this was also diluted 1:2, 1:4, and 1:8 in PBS-T and tested in 100-μl volumes in duplicate.

Statistical methods. All assays were performed in duplicate. Regression coefficients were calculated by the least-squares method. Statistical comparisons were performed by Fisher's exact test for discrete variables and by the Mann-Whitney rank sum test (two tailed) for continuous variables (16).

RESULTS

Standardization of ELISA procedure. The Immulon I (Dynatech Laboratories) polystyrene microtiter plate was chosen over several other brands, including two other polystyrene plates (Linbro Titertek, Flow Laboratories; and Nunc Immunoplate I, Vangard International, Neptune, N.J.) and a polyvinyl plate (Immulon II; Dynatech Laboratories). The Immunulon I plate was found to be superior for its maximal specific binding of antitoxin and for minimal nonspecific binding of other immunoreactants (data not shown). Binding of specific antitoxin was determined with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Diagnostics). Nonspecific binding of immunoreactants was determined in assays in which the coating rabbit anti-TSST-1 was omitted, and plates were incubated with coating buffer and goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. Optimal dilutions of coating antitoxin and conjugate were determined by a series of checkerboard titrations to achieve the greatest sensitivity and specificity while requiring the least amount of immunoreactants. Optimal working dilutions were 1:10,000 for the coating antitoxin (0.33 μg of protein per well) and 1:750 for the antitoxin conjugate. At these dilutions of antitoxin and in the presence of 16 ng of TSST-1 reference standard per ml, a constant rate of enzymatic hydrolysis for the *p*-nitrophenyl phosphate substrate could be demonstrated over 90 min of incubation at 37°C (data not shown).

Reproducibility of ELISA. A typical standard curve generated with reference TSST-1 over the range of 0.5 to 16.0 ng/ml is shown in Fig. 1. The regression coefficient (r) obtained from 20 separate experiments was 0.985 ± 0.007 (95% confidence limits). Considerable within-day, and day-to-day, variations owing to plate-to-plate differences in binding characteristics were observed (data not shown). These results indicate that test samples and reference TSST-1 standards must be determined on the same plate to obtain reliable results. The coefficient of variation from repeated testing of the same TSST-1 was consistently less than 10%, confirming the reproducibility of the ELISA.

Specificity of ELISA. The specificity of the ELISA was demonstrated in additional experiments in which microtiter wells were coated with a 1:10,000 dilution of ammonium sulfate-precipitated nonimmune rabbit serum that was shown to be negative for anti-TSST-1 by Ouchterlony immunodiffusion (Fig. 1). The fact that no dose-response relationship was produced with this control, as was demonstrated in wells coated with anti-TSST-1 immune serum, further demonstrated the specificity of the ELISA for TSST-1. Other negative controls included wells with no toxin added and wells with toxin that had been pretreated with antitoxin. Furthermore, no false-positive results were obtained with purified preparations of other *S. aureus* enterotoxins including A, B, C_1, C_2, C_3, D, and E at concentrations ranging from 10 to 1,000 ng/ml. A crude preparation of enterotoxin D at a concentration of 1,800 ng/ml did demonstrate false-positive results equivalent to 1.29 ng of TSST-1 per ml (0.07%). This preparation did not immunoprecipitate with rabbit anti-TSST-1 by Ouchterlony immunodiffusion. The false-positive reactivity in the ELISA, presumed to be due to protein A, was completely eliminated when the crude enterotoxin D preparation was pretreated with 10% normal rabbit serum.

Elimination of protein A effect with normal rabbit serum. The interfering effect of protein A and its elimination by pretreatment of test samples with 10% normal rabbit serum were further characterized (Fig. 2). Standard concentrations of reference TSST-1 (0.5 to 16 ng/ml, final concentrations) were prepared in BHI broth and pretreated with protein A (1,000 ng/ml; Sigma P-6650), protein A plus 10% normal rabbit serum, or 10% normal rabbit serum alone. Negative controls included BHI broth without TSST-1 added. Quantitation of TSST-1 in these different experimental groups was done in parallel by our ELISA procedure as described above and compared with positive controls in which reference

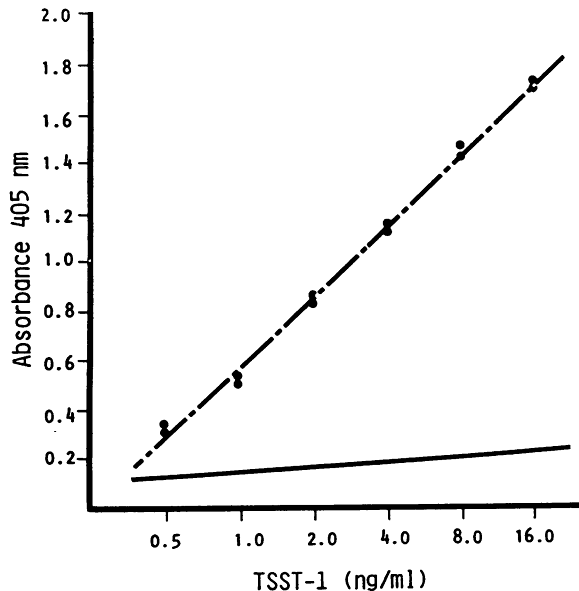

FIG. 1. Reproducibility and specificity of ELISA. Typical curve (-- -- -- --) generated with standard reference TSST-1 over the concentration range of 0.5 to 16.0 ng/ml in wells coated with reference rabbit anti-TSST-1 antibody; regression coefficient (r) = 0.985 ± 0.007 (95% confidence intervals for 20 experiments). Specificity of ELISA was demonstrated in identical experiments in which microtiter wells were coated with a 1:10,000 dilution of rabbit serum devoid of anti-TSST-1 (——).

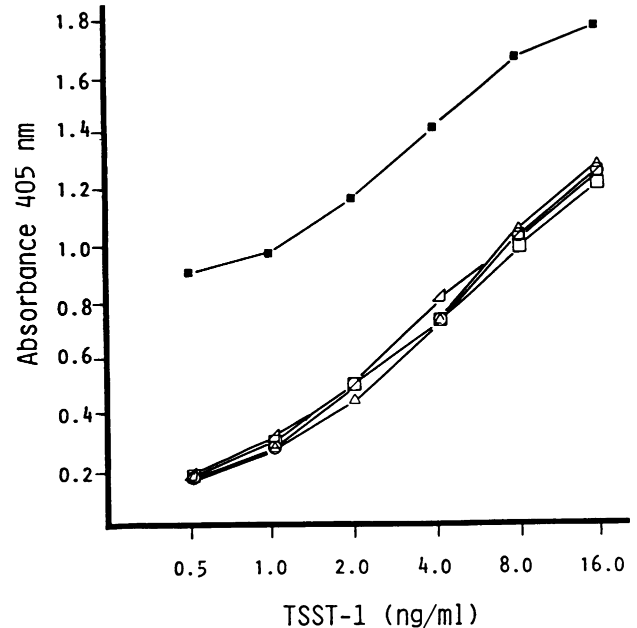

FIG. 2. Comparison of TSST-1 detection by ELISA in PBS-T (○) or BHI broth (△) alone or in BHI broth and pretreated with 1,000 ng of protein A per ml (■), 10% normal rabbit serum (□), or protein A plus 10% normal rabbit serum (◬). The interfering effect of protein A was completely eliminated by the addition of 10% normal rabbit serum without loss of sensitivity when compared with standard curves generated in PBS or BHI broth alone.

TSST-1 standards were prepared in PBS-T. The results clearly demonstrate (i) the interfering effect of protein A in the assay, and (ii) the complete elimination of this effect by the addition of 10% normal rabbit serum, without loss of sensitivity or specificity over the range of reference TSST-1 standard concentrations studied.

Detection of TSST-1 production by culture supernatants of *S. aureus* isolates. The results of testing for TSST-1 production in culture supernatants of 95 *S. aureus* isolates are shown in Fig. 3. Among the 60 isolates confirmed to be negative for TSST-1 by immunodiffusion, 59 also had undetectable toxin by ELISA. Among the 35 isolates confirmed to be positive for TSST-1 by immunodiffusion (24 were isolated from TSS patients), all had detectable toxin by ELISA. Negative controls included uninoculated BHI broth treated in identical fashion. The necessity of pretreating the culture supernatants with 10% normal rabbit serum to absorb out protein A produced by the isolates is clearly illustrated. Of 60 isolates negative for TSST-1 production as tested by immunodiffusion, 42 would be falsely positive by ELISA if culture supernatants were not pretreated with 10% normal rabbit serum to remove the interfering effect of protein A. Similarly, all 35 isolates that were positive for toxin production by immunodiffusion yielded higher values of TSST-1 in untreated compared with treated culture supernatants. Of interest, isolates from TSS patients produced significantly higher concentrations of TSST-1 in culture supernatants compared with toxin-producing strains from non-TSS patients ($P < 0.05$, Mann-Whitney two-tailed rank sum test) (Fig. 4).

Detection of TSST-1 in vaginal washings of TSS patients and healthy control women. A total of 36 vaginal washings from 9 menstrual TSS patients and 14 healthy control women were

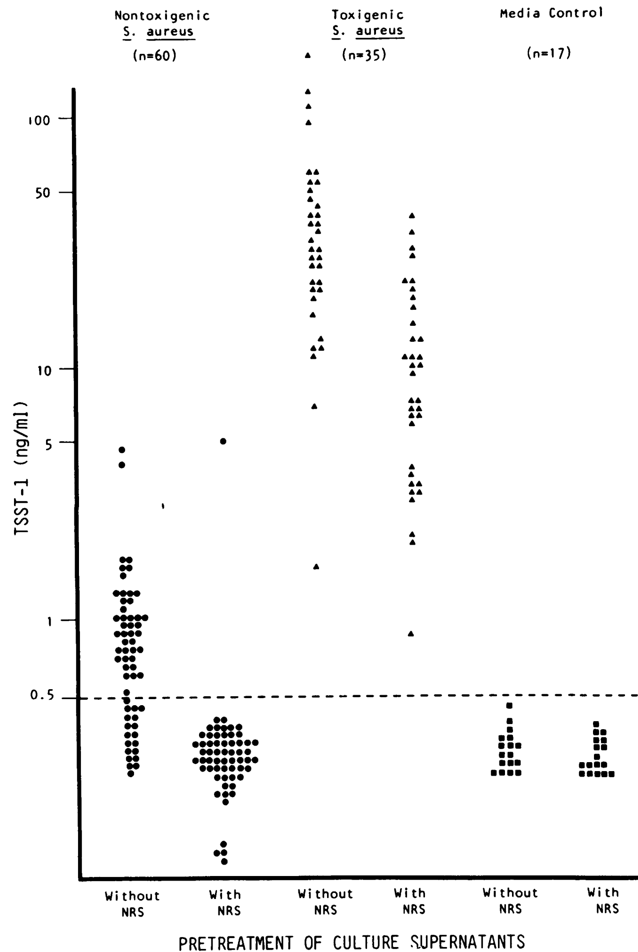

FIG. 3. Effect of 10% normal rabbit serum (NRS) on interference by protein A during detection of TSST-1 in culture supernatants of *S. aureus* by ELISA. *S. aureus* isolates were previously determined by Ouchterlony immunodiffusion to be toxigenic (▲) or nontoxigenic (●). ELISA results of culture supernatants were compared with those of uninoculated medium controls (■) with or without the addition of NRS.

available for quantitation of TSST-1 by ELISA (Table 1). Three of four specimens collected within 3 days of hospitalization from three women with acute TSS had detectable TSST-1, compared with 0 of 17 washings from 9 TSS patients hospitalized longer than 3 days (range, 6 to 63 days; median, 30 days) ($P = 0.003$, Fisher's exact test) and 1 of 15 washings from 14 healthy control women ($P = 0.016$). Specific TSST-1 concentrations detected in vaginal washings from the two women with acute TSS and a single control subject are shown in Table 2. TSST-1 concentrations from vaginal washings of a 29-year-old woman with typical menstrual TSS (subject A) were highest on day 1 of hospitalization and rapidly declined thereafter, coincident with antistaphylococcal therapy and elimination of *S. aureus* in the vaginal flora. Vaginal washings from a 22-year-old healthy control woman (subject C) were initially negative for TSST-1 concurrent with negative vaginal culture for isolation of *S. aureus*. A subsequent washing obtained 1 month later was positive for TSST-1 when her concurrent vaginal culture was also positive for TSST-1-producing *S. aureus*. Her serum antibody titer to TSST-1 was within the 69th percentile of normal anti-TSST-1 titers as determined from 87 healthy women by an ELISA method (P. M. Rosten, K. H. Bartlett, and A. W. Chow, submitted for publication).

DISCUSSION

Several assay techniques have been developed to demonstrate and quantitate TSST-1 in *S. aureus* culture supernatants in vitro, including analytic isoelectric focusing in polyacrylamide gels (15), Ouchterlony immunodiffusion (13), immunoblotting (21), passive latex agglutination (8), radioimmunoassay (Melish, et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1983), and a competitive ELISA with TSST-1 as the enzyme conjugate (12). Analytic isoelectric focusing and immunoblotting are both qualitative assays, and the former lacks specificity (13). Ouchterlony immunodiffusion techniques, including several modifications such as the microslide immunodiffusion test (4), the optimal sensitivity plate method (2), and the single gel diffusion tube method (14), can be used for quantitation of TSST-1, but these generally lack sensitivity, require excessive use of reagents, and cannot be adapted for batch testing of large samples. Although use of radioimmunoassay should improve both sensitivity and specificity, this method requires expensive equipment and handling of radioactive materials. A reversed passive latex agglutination assay has been developed by Igarashi et al. (8)

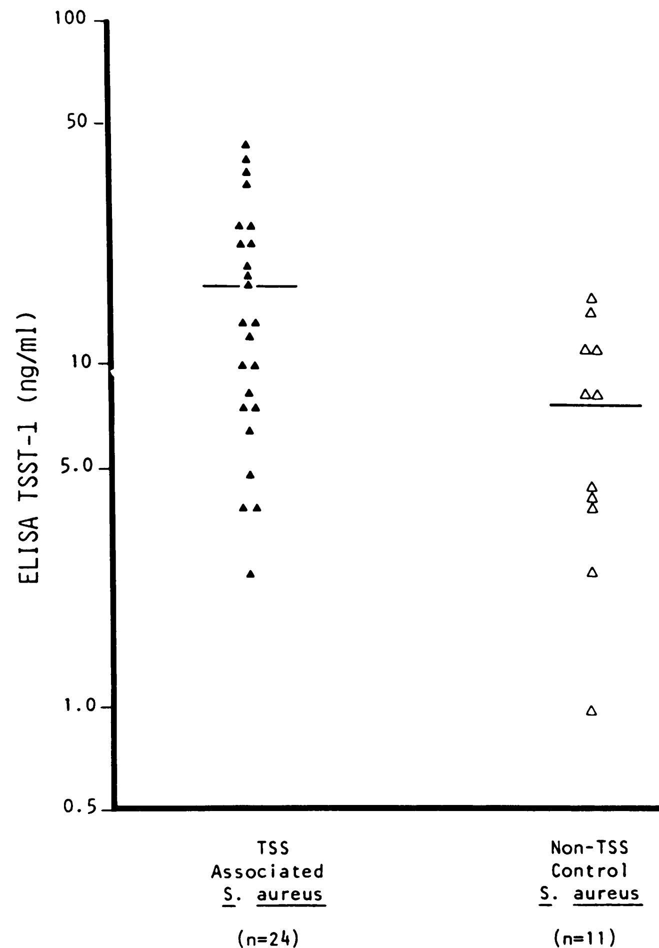

FIG. 4. Quantitative TSST-1 production as measured by ELISA among 24 toxigenic *S. aureus* vaginal isolates from TSS patients (▲) and 11 toxigenic *S. aureus* vaginal isolates from non-TSS healthy women (△) ($P < 0.05$, Mann-Whitney rank sum test).

and can detect nanogram quantities of TSST-1. Although promising, its specificity and possible interference by protein A, however, will require additional evaluation. Most recently, Parsonnet et al. (12) described a competitive ELISA capable of quantitating 0.03 μg of TSST-1 per ml in *S. aureus* culture supernatants. This assay was not influenced by protein A, but did cross-react minimally with staphylococcal enterotoxins A, D, and E. Furthermore, a competitive ELISA with enzyme-labeled antigen requires purified TSST-1 in relatively large amounts for preparation of the enzyme-antigen conjugate. A more important disadvantage of this method is that the competitive ELISA might not be readily adapted for quantitation of TSST-1 in biologic fluids or tissue extracts. This is due to the need to incubate enzyme-labeled TSST-1 directly with biologic fluids or tissue extracts which themselves may contain proteases or enzyme inhibitors and may significantly alter the kinetics and activity of the enzyme conjugated to TSST-1, thus severely affecting the accuracy of the test (6).

The noncompetitive ELISA described here has a number of advantages over the other methods described above. It is sensitive, specific, simple, economical, and does not require handling and disposal of radioactive materials. It obviates the necessity of repeated and elaborate procedures for purification of TSST-1. Importantly, it can be readily adapted to quantitate TSST-1 in biologic fluids and tissue extracts, including urine, serum, and vaginal washings. Its lower limit of sensitivity, 0.5 ng/ml, appears to be adequate for TSST-1 detection in culture supernatants as well as vaginal washings. It does not cross-react with other staphylococcal enterotoxins, and the potential interference by protein A is readily eliminated by pretreatment of samples with 10% rabbit serum. Protein A is produced by most strains of *S. aureus*, but the amount released in culture may vary from strain to strain. Since protein A has the unique ability to bind the Fc portion of immunoglobulins, primarily immunoglobulin G, its presence can readily interfere with the ELISA by binding to both the coating as well as the enzyme-conjugated antitoxin, as is demonstrated in Fig. 2. Berdal et al. (1) determined that the presence of protein A in concentrations greater than 500 ng/ml could affect the detection of staphylococcal enterotoxins A, B, and C from culture supernatants by a similar ELISA technique. These investigators used affinity chromatography to remove protein A before the enterotoxin assays. Our method of protein A adsorption with 10% rabbit serum appears to be equally effective but is much more simple. Since both the sensitivity and specificity of our ELISA method appear satisfactory, we did not feel that the use of monoclonal antibodies to TSST-1 for coating or antibody enzyme conjugation, as was recently described (D. E. Wells, M. W. Reeves, L. M. Graves, and R. M. McKinney, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, V17, p. 391), or the use of immunoglobulin subclasses or Fab-specific preparations will offer any significant advantage. Indeed, the use of polyclonal antitoxin may have provided an element of amplification in the sensitivity of our assay by possibly permitting several different enzyme-labeled antibody epitopes to bind a single TSST-1 molecule with different antigenic domains. Use of polyclonal antitoxin also obviated the need for tissue culture facilities.

TABLE 1. Detection of TSST-1 in vaginal washings of menstrual TSS patients and healthy control women

Subjects	No. positive/total tested	
	Women (P)[a]	Washings (P)[a]
TSS patients	2/12	3/21
≤3 days illness	2/3	3/4
≥3 days illness	0/9 (0.045)	0/17 (0.003)
Control	1/14 (0.063)	1/15 (0.016)

[a] P value by Fisher's exact test, compared with TSS patients tested within 3 days of acute illness.

TABLE 2. Quantitation of TSST-1 in vaginal washings of TSS patients and control women

Washings	TSST-1 detected (ng/ml)	
	With NRS[a]	Without NRS
TSS		
Subject A		
Day 1[b]	15.8	51.2
Day 3	2.2	2.1
Day 7	0	0
Subject B		
Day 1	2.5	2.5
Day 60	0	0
Control (subject C)		
April 1983	0	1.9
May 1983	3.7	8.7

[a] Pretreatment with 10% normal rabbit serum (NRS) to eliminate any interfering effect of protein A.
[b] Day after hospitalization when specimen was collected.

Our finding that toxigenic *S. aureus* strains isolated from TSS patients produced significantly more TSST-1 in vitro compared with toxigenic control strains is of interest. The ability to produce large quantities of TSST-1 in vivo may be an important virulence factor. Conversely, local and mucosal host factors which permit optimal production of TSST-1 in vivo by colonizing or infecting strains of *S. aureus* may be important risk factors of TSS. In this regard, Mills et al. (10, 11) have noted the important role of magnesium ion on TSST-1 production in vitro. They postulated that use of certain tampon brands could have precipitated menstrual TSS since these tampon fibers may avidly bind magnesium ions in vivo and cause a striking increase in TSST-1 production. Alternatively, since our studies indicate that TSST-1 can be detected in vaginal washings of some healthy women, the presence of neutralizing systemic or local antibodies to TSST-1 may be an important protective mechanism in normal individuals. In support of this concept, we (Rosten et al., submitted) and others (2, 3, 17) have demonstrated that anti-TSST-1 is generally low or absent in acute sera of menstrual TSS patients as compared with sera of age-matched control women and that serologic unresponsiveness appears to be a prominent hallmark in such patients.

It is clear that the availability of a simple, sensitive, specific, and reproducible assay as described here, capable of detecting TSST-1 in concentrations as low as 0.5 ng/ml both in vitro and in vivo, should greatly facilitate future studies which are urgently needed to further our understanding of the biologic effects of TSST-1 and the pathogenesis of toxic shock syndrome.

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