

Toxicity of Recombinant Toxic Shock Syndrome Toxin 1 and Mutant Toxins Produced by *Staphylococcus aureus* in a Rabbit Infection Model of Toxic Shock Syndrome

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Menstrually associated toxic shock syndrome (TSS) is attributed primarily to the effects of staphylococcal exotoxin toxic shock syndrome toxin 1 (TSST-1). A region of the 194-amino-acid toxin spanning residues 115 through 144 constitutes a biologically active site. Several point mutations in the TSST-1 gene in that region result in gene products with reduced mitogenic activity for murine T cells. In this study we evaluated the toxicity of recombinant TSST-1 and several mutants of TSST-1 made by transformed *Staphylococcus aureus* during in vivo growth in a rabbit infection model of TSS. The toxicities of the transformed strains of *S. aureus* for rabbits correlated with the mitogenic activities of the recombinant toxins. An isolate originally obtained from a patient with a confirmed case of TSS (*S. aureus* 587) implanted in a subcutaneous chamber served as a positive control. TSST-1 produced in vivo led to lethal shock within 48 h, and a TSST-1-neutralizing antibody (monoclonal antibody 8-5-7) administered to rabbits challenged with *S. aureus* 587 prevented fatal illness. Rabbits infected with transformed *S. aureus* RN4220 expressing wild-type toxin (p17) or mutant toxins retaining mitogenic activity for T cells succumbed within a similar time frame. Blood chemistries of samples obtained from infected animals before death indicated abnormalities in renal and hepatic functions similar to those induced by parenteral injection of purified staphylococcal TSST-1. Mutant toxin 135 (histidine modified to alanine at residue 135) possessed only 5 to 10% of the mitogenic activity of wild-type toxin. Rabbits challenged with transformed *S. aureus* RN4220 expressing mutant toxin 135 exhibited only mild transient illness. Mutant toxin 135 retained reactivity with monoclonal antibody 8-5-7 and by several criteria was conformationally intact. Toxin from a double mutant, 141.144, with alanine substitutions at residues 141 (histidine) and 144 (tyrosine), also was devoid of mitogenic activity. In this case, antibody recognition was lost. Mutant toxins 115 and 141 were found to possess approximately half-maximal mitogenic activity. Rabbits challenged with *S. aureus* RN4220 expressing either 115 or 141 toxin succumbed to lethal shock. We conclude that the ability of TSST-1 to activate murine T cells in vitro and its expression of toxicity leading to lethal shock in rabbits are related phenomena.

Toxic shock syndrome (TSS) toxin 1 (TSST-1) belongs to a family of staphylococcal exotoxins classified as superantigens (17). Characteristics of superantigens include massive T-cell activation with preferential utilization of specific V β elements of the T-cell receptor (11). Furthermore, superantigens are not processed like conventional protein antigens, although superantigens bind to major histocompatibility complex (MHC) class II molecules before interaction with the V β element of T cells (19). The activation of large numbers of mature T cells is believed to be significant in the pathogenesis of TSS (10).

The important role of TSST-1 in the pathogenesis of menstrually associated TSS is based on strong experimental evidence. In a previous study we found that virtually all *Staphylococcus aureus* strains isolated from cases of confirmed menstrual TSS produced TSST-1, as measured by gene probe and colony immunoblot assays (6, 7). The limitations imposed by studies of human TSS prompted the development of animal models of the disease. Two such models with rabbits as experimental animals are widely accepted for the study of TSS pathogenesis. The toxin model developed by Parsonnet et al. (21) utilizes a subcutaneously implanted osmotic pump to deliver a measured quantity of

TSST-1 at a constant rate over several days. Abnormalities of several organ systems mimic the clinical profile seen in human TSS. Using the rabbit toxin model, we have shown that TSST-1-induced illness and mortality are prevented by specific anti-TSST-1 monoclonal antibody (5). A second model of TSS utilizes live bacteria to elicit the shock syndrome. In this case, viable *S. aureus* cells are introduced into a diffusible chamber implanted subcutaneously (24) or intravaginally (9). An infection model developed by Scott et al. (24) employs a polyethylene whiffle ball surgically implanted under the skin in the dorsolateral aspect of the rabbit. Bacteria introduced through a hole in the encapsulated whiffle ball several weeks later establish a localized infection. A large inoculum of a TSST-1-producing strain of *S. aureus* results in a fatal illness within 1 or 2 days. The syndrome is precipitated by TSST-1, since neutralizing monoclonal antibody is fully protective (1). Thus, the rabbit infection model of TSS mimics the course of events occurring in the human illness; i.e., establishment of a focal staphylococcal infection, dissemination of TSST-1 from the infected chamber, and development of toxic shock involving multiple organ systems. This mimicry of the events occurring in human TSS prompted us to use the rabbit infection model of TSS developed by Scott et al. (24). Using this animal model, we compared the toxicity of a genetically engineered host strain of *S. aureus* harboring the unmodified

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TSST-1 gene with those of strains carrying a TSST-1 gene modified by site-directed mutagenesis. The data confirm and extend the results of our previous study suggesting that specific histidine and tyrosine residues near the carboxyl terminus of the TSST-1 protein are critical for biological activity of TSST-1 (3).

MATERIALS AND METHODS

Purified staphylococcal TSST-1. Purified staphylococcal TSST-1 was a gift of J. Parsonnet, Dartmouth Medical Center, Hanover, N.H. Details of the purification procedure were published previously (22).

TSST-1 monoclonal antibody. A TSST-1-specific monoclonal antibody (MAb 8-5-7) was developed as described elsewhere (5). MAb 8-5-7 neutralizes TSST-1-induced proliferation of murine T cells, ablates the lethal effects of toxin for rabbits, and reacts with toxin in Western immunoblotting assays and enzyme-linked immunosorbent assays (ELISAs) (5). Polyclonal rabbit anti-TSST-1 serum was purchased from Toxin Technologies, Sarasota, Fla.

Bacterial strains. *S. aureus* RN4220 was obtained from B. Kreiswirth and R. Novick, Public Health Research Institute, New York, N.Y. This strain does not produce TSST-1 or staphylococcal alpha-toxin.

Plasmid and expression vector. The cloned TSST-1 gene was obtained originally from B. Kreiswirth and R. Novick in the form of plasmid pRN6550 in *Escherichia coli* AB259 (14). A 1.6-kb *Bgl*I fragment encoding the TSST-1 gene was excised from pRN6550 and inserted into a pUC19/pBD64-based plasmid. This shuttle vector was then used to express the recombinant toxins in either *E. coli* AB259 or *S. aureus* RN4220. Mutations on the TSST-1 gene were made by site-directed mutagenesis in which tyrosine or histidine residues were replaced by alanine. Plasmids encoding unmodified and mutant TSST-1 genes were used to transform competent *E. coli* AB259 (3) or for electroporation of *S. aureus* RN4220. Colonies were selected for resistance to ampicillin (100 µg/ml) or kanamycin (25 µg/ml). Wild-type TSST-1 expressed in *E. coli* AB259 or *S. aureus* RN4220 was indistinguishable from staphylococcal TSST-1. The mutant toxins are all full-length proteins, several of which exhibit reduced mitogenicity for murine T cells (3).

Electroporation of *S. aureus* RN4220. The plasmids were electroporated into *S. aureus* RN4220 as described by Kraemer and Iandolo (13). The gene pulser (Bio-Rad, Rockville Centre, N.Y.) settings were 250 kV, 200 Ω, and 25 µF, and the concentration of plasmid DNA used was between 1.0 and 2.0 µg per 40-µl sample. Washed cells of *S. aureus* were suspended in 500 mM sucrose to a concentration of 10⁹ CFU/ml and placed in 0.2-cm electroporation cuvettes (Bio-Rad), which were cooled on ice for 20 min before and after pulsing. Selection of transformed *S. aureus* was made on nutrient agar plates containing kanamycin (25 µg/ml). Isolated colonies were grown overnight to test for recombinant TSST-1 or mutant toxin by immunoblotting and competitive ELISA with MAb 8-5-7 (5).

Growth of *S. aureus* by the membrane-over-agar method. To increase the sensitivity of assays for TSST-1 produced by the electroporated *S. aureus* RN4220 strains, the organisms were grown by the biphasic membrane-over-agar method described by Robbins et al. (23). This procedure effectively concentrates staphylococcal products in a small volume of dialysate separated from the nutrient agar surface by a semipermeable membrane.

Purification of recombinant TSST-1 and mutant toxins from

***S. aureus* culture supernatants.** Staphylococcal proteins were precipitated from the concentrated culture supernatants with saturated ammonium sulfate. Pellets were resuspended in 50 mM sodium acetate, pH 5.0 (buffer A), and dialyzed against the same at 4°C overnight. Dialyzed supernatants were filtered through a 0.2-µm-pore-size filter and injected onto a fast protein liquid chromatography (FPLC) Mono S column equilibrated with buffer A. Toxin was eluted with a linear gradient of 0.5 M NaCl. Toxin eluted as a single peak that was homogeneous on Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Quantitation of purified toxins was with a Bradford protein assay (Bio-Rad).

Mitogen assay of purified recombinant toxins. TSST-1 and cloned gene products purified from culture supernatant fluids were evaluated for mitogenic potential in a lymphocyte proliferation assay as previously described (3). Single-cell suspensions of spleens excised from BALB/c mice were adjusted to 10⁷ splenocytes per ml of RPMI 1640 supplemented with 10% fetal calf serum (Hazleton Biologicals, Lenexa, Kans.). Data are presented as the means of triplicate determinations ± standard deviations.

Rabbit infection model. The rabbit infection model of TSS described by Scott et al. (24) was used in these experiments. In this model, a sterile polyethylene chamber (golf whiffle ball) is surgically implanted subcutaneously in rabbits. After encapsulation with fibrous tissue, a process requiring 5 to 6 weeks, the chamber contains a transudate fluid rich in inflammatory elements. The staphylococcal inoculum is then introduced into one of the holes of the plastic ball. Bacteria remain localized within the encapsulated chamber, and TSST-1 and other exoproducts are disseminated systemically.

In our studies, New Zealand White rabbits weighing between 2.0 and 3.0 kg were used. A gas-sterilized polyethylene whiffle ball was surgically implanted subcutaneously in the left dorsolateral region of each rabbit. After 5 to 6 weeks, encapsulation was confirmed by drawing a sample of transudate from the chamber interior; the presence of amber fluid containing numerous cellular elements indicated that encapsulation had occurred and that rabbits could then be used in challenge experiments. The establishment of a focal staphylococcal infection followed by a systemic toxemia mimicking TSS provides a reasonable model of the human disease. In our experiments, the transformed strains of *S. aureus* RN4220 were grown in Luria broth containing kanamycin (25 µg/ml) to provide selective pressure. Cells were washed and resuspended in phosphate-buffered saline to a concentration of 2.0 × 10⁹ CFU/ml. Rabbits received 1.0 × 10⁹ CFU of staphylococci introduced through one of the holes in the ball located by palpation. Simultaneously, 5.0 mg of sterile dextran sulfate (molecular weight, 5 × 10⁵) in solution was injected to retard phagocytosis of bacteria by neutrophils (4). The strategy here was to provide for extended extracellular survival of *S. aureus* and to favor the production of recombinant TSST-1 or mutant toxins. Body temperature and food and water intake were also monitored. Blood was drawn daily from a marginal ear vein for serum chemistry analysis.

Protection of rabbits with MAb 8-5-7. In some cases, rabbits were treated with MAb 8-5-7 to provide protection against the systemic effects of TSST-1 and to confirm that death of unprotected rabbits was caused by TSST-1. Rabbits were given 1.5 mg of purified MAb 8-5-7 in 1.0 ml of phosphate-buffered saline intravenously on days -1, 0, and +1 of challenge with staphylococci.

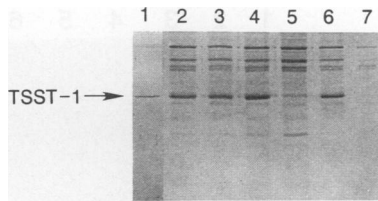


FIG. 1. Coomassie blue-stained SDS-PAGE gel of culture supernatant fluids of transformed *S. aureus* 4220 strains. Lanes: 1, TSST-1 standard (0.25 µg); 2, 4220-135; 3, 4220-141; 4, 4220-115; 5, 4220-80.82; 6, 4220-p17; 7, 4220 (untransformed host strain).

Blood chemistry. Blood urea nitrogen (BUN), serum glutamic pyruvic transaminase (SGPT), and triglycerides in rabbit serum samples were measured in a Hitachi 737 multi-chemistry analyzer.

RESULTS

Expression of wild-type and mutant toxins by *S. aureus* RN4220. Figure 1 shows a Coomassie blue-stained gel of culture supernatant fluids of transformed *S. aureus* RN4220 strains grown by the membrane-over-agar method (23). All of the lanes show protein bands corresponding to the 22-kDa band exhibited by the TSST-1 standard (lane 1). This confirms that wild-type (p17) and mutant toxins were secreted by the staphylococci during growth. We estimate that secretion of recombinant TSST-1 by transformed *S. aureus* RN4220-p17 exceeds by severalfold the quantity of toxin expressed by *E. coli* AB259-p17 (3).

Figure 2 shows corresponding immunoblots of the stained gel. In this case, anti-TSST-1 MAb 8-5-7 (Fig. 2B) is shown to bind with purified staphylococcal TSST-1 and the wild-type (p17) gene product (lanes 4 and 6). Of the mutant toxins, 80.82, 135, and 141 are recognized by MAb 8-5-7. Mutant toxins 115 (lane 3) and 141.144 (data not shown) do not bind with the monoclonal antibody. These results are consistent with observations made with the *E. coli* AB259 recombinant products (3). On the other hand, Fig. 2A shows that all of the toxin mutants are recognized by polyclonal antiserum.

Mitogenicity of recombinant TSST-1 and mutant toxins. In a prior structure-function study of TSST-1, we found that replacement of specific tyrosine and/or histidine residues by alanine results in a significant loss of mitogenic activity for mouse spleen cells (3). Figure 3 shows the mitogenic activities of purified staphylococcal TSST-1 and several recombinant toxins purified by FPLC. Mitogenic activity was measured at concentrations between 0.1 and 100 ng/ml. The data show that staphylococcal TSST-1 and wild-type recombinant TSST-1 (p17) possess comparable mitogenic potencies for murine T cells. Mutant toxins 115 and 141 demonstrated mitogenic activity at a reduced level. Mutant toxin

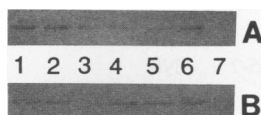


FIG. 2. Immunoblots of culture supernatant fluids of transformed *S. aureus* 4220 strains with polyclonal and monoclonal (MAb 8-5-7) anti-TSST-1 serum. (A) Polyclonal anti-toxin; (B) MAb 8-5-7. Lanes: 1, 4220-135; 2, 4220-141; 3, 4220-115; 4, TSST-1 standard (0.25 µg); 5, 4220-80.82; 6, 4220-p17; 7, 4220.

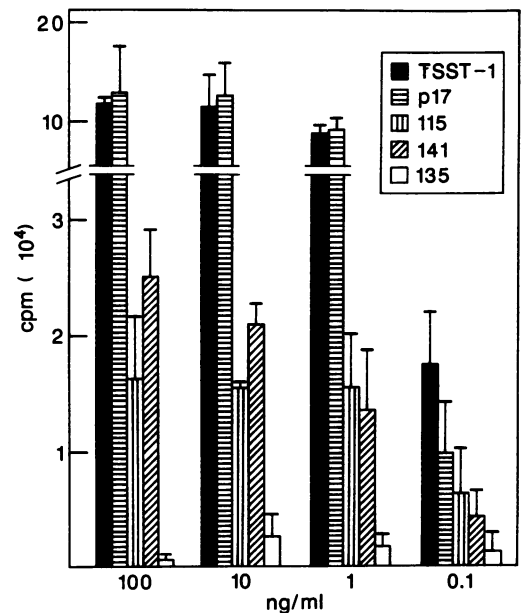


FIG. 3. Mitogenic activity of FPLC-purified toxins from transformed *S. aureus* 4220 strains in a lymphocyte proliferation assay with mouse splenocytes.

135, however, retained virtually no mitogenic activity. Thus, we considered mutant toxin 135 to represent the best candidate for determining whether the capacity to activate T cells was essential for expression of toxicity in vivo.

Toxicity of recombinant toxins for rabbits. A primary objective of this study was to evaluate the toxicity of wild-type and mutant TSST-1 toxins in a rabbit infection model of TSS. The hypothesis to be tested was that the mitogenic potential of TSST-1 may be linked to its in vivo toxicity. To that end we evaluated the in vivo toxicity of the transformed *S. aureus* RN4220 strains for rabbits. Table 1 shows composite mortality data from rabbits with staphylococcal infections localized to the polyethylene chambers. Death of rabbits suffering fatal infection occurred within 24 to 48 h after challenge. The *S. aureus* RN4220 host strain, which produces no toxin, did not cause significant illness. On the other hand, a TSST-1-positive strain originally isolated from a patient with TSS (*S. aureus* 587) and the host strain *S. aureus* RN4220 transformed with the intact TSST-1 gene (p17) uniformly led to a toxemic death. Blood cultures from these rabbits were negative, showing that the staphylococcal infection was restricted to the polyethylene chambers and had not disseminated. Mortality attendant with infections by *S. aureus* 587 and *S. aureus* 4220-p17, both of which express intact TSST-1, was prevented by parenteral administration of MAb 8-5-7 (Table 1). Infection with mutant toxin 80.82, which retains full mitogenic activity, resulted in fatal infection. Mutant toxins 115 and 141, both of which possess approximately 50% of maximum mitogenic activity, caused death of rabbits within the same time frame as that of the *S. aureus* RN4220 carrying the unmodified toxin gene (p17). In contrast, mutant toxins 135 and 141.144, both of which possess negligible mitogenic activity, were nontoxic for rabbits. *S. aureus* RN4220 transformed with plasmids bearing mutations at residue 135 or at residues 141 and 144 did not cause significant illness or death in the rabbit infection model of TSS.

TABLE 1. Toxicity of *S. aureus* RN4220 transformants producing intact or mutant recombinant TSST-1 evaluated in the rabbit infection model of TSS

<i>S. aureus</i> strain	No. challenged	No. of survivors	Mortality (%)
587 ^a	3	0	100
4220 ^b	2	2	0
4220-p17	4	0	100
4220-80.82	2	0	100
4220-115	2	0	100
4220-141	1	0	100
4220-135 ^c	4	4	0
4220-141.144	2	2	0
587 + MAb 8-5-7 ^d	4	4	0
4220-p17 + 8-5-7 ^e	2	2	0

^a *S. aureus* 587 is a TSST-1⁺ isolate originally from a patient with TSS.

^b *S. aureus* RN4220 is the host strain used for transformation with the plasmids encoding either unmodified TSST-1 (p17) or the five mutant forms of TSST-1 listed. The markers of each mutant designate the tyrosine and/or histidine residue(s) modified to alanine by site-directed mutagenesis.

^c $P < 0.05$ using a two-tailed Fisher exact test when data are compared with those for 4220-p17.

^d MAb 8-5-7 is a TSST-1-neutralizing antibody (5) that was administered intravenously as described in Materials and Methods.

^e $P = 0.067$ using a two-tailed Fisher exact test when data are compared with those for 4220-p17.

Table 2 reveals a correlation between toxicity for rabbits and mitogenicity for murine T cells of the recombinant toxins. *S. aureus* RN4220 strains producing recombinant TSST-1 with a mitogenic capacity equal to that of wild-type staphylococcal TSST-1 caused a fatal infection of rabbits. Mutant toxins 115 and 141, which are not maximally mitogenic, apparently retain sufficient toxicity to precipitate lethal shock in rabbits. The two mutant toxins devoid of mitogenicity for T cells did not express significant toxicity in the rabbit infection model of TSS.

In an effort to show that the transformed strains of *S. aureus* RN4220 were unmodified by in vivo passage in rabbits and that the inocula retained the capacity to produce intact or mutant TSST-1, the following experiment was performed. Culture supernatants of *S. aureus* RN4220-p17 and *S. aureus* RN4220-135 grown by the membrane-over-agar method were subjected to SDS-PAGE (Fig. 4). The bacteria were then grown overnight, and inocula were prepared for injection into rabbits. The strains expressing either

TABLE 2. In vivo toxicity and mitogenicity of recombinant TSST-1 and mutant toxins produced by transformed strains of *S. aureus* RN4220

<i>S. aureus</i> strain	In vivo toxicity ^a	Mitogenicity ^b
587	+	++
4220	-	-
4220-p17	+	++
4220-80.82	+	++
4220-115	+	+
4220-141	+	+
4220-135	-	-
4220-141.144	-	-

^a Evaluated by using the rabbit infection model of TSS (see Materials and Methods): +, causes fatal infection; -, causes transient illness.

^b Evaluated by using a mouse splenocyte assay (3): ++, mitogenic responses equal to those of wild-type TSST-1; +, mitogenic responses approximately 50% of those of wild-type TSST-1; -, mitogenic responses less than 10% of those of wild-type TSST-1.

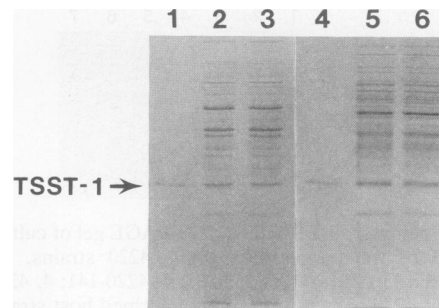


FIG. 4. Coomassie blue-stained SDS-PAGE gel of culture supernatant fluids of transformed *S. aureus* 4220 strains. Lanes: 2 and 3, strains before challenge; 5 and 6, strains recovered from whiffle balls of infected rabbits; 1 and 4, TSST-1 standard (0.25 µg); 2 and 5, 4220-p17; 3 and 6, 4220-135.

p17 or 135 were inoculated into the implanted chambers as described previously. After 24 h, samples were aspirated from the encapsulated whiffle balls with 20-gauge needles and again grown in the membrane-over-agar system. The presence of protein bands corresponding to TSST-1 was again confirmed (Fig. 4). This simple experiment shows that the ability of the transformed strains of *S. aureus* to produce the recombinant toxin was retained during in vivo residence. We interpret these results to mean that the plasmids are stable in vivo and that the encoded toxins are produced and rapidly disseminated.

Changes in blood chemistries of infected rabbits. Parsonnet et al. (21) showed that parenteral infusion of TSST-1 into rabbits leads to predictable changes in serum chemistries indicative of multiple organ system abnormalities. Marked elevation of blood urea nitrogen and serum glutamic pyruvic transaminase were taken as indicators of renal and hepatic dysfunction. We monitored serum chemistries of rabbits infected with the transformed *S. aureus* RN4220 strains to discern the multiple organ system involvement of the illness induced in the infection model of TSS. In addition to BUN and SGPT, triglyceride levels and body temperature were measured. Table 3 provides a semiquantitative estimate of these changes after infection with the untransformed host strain and the transformed strains expressing recombinant toxins. By using the values obtained with the blood sera of normal uninfected rabbits as baselines and comparing these with the values of sera from infected animals, several correlations can be made. Infection with *S. aureus* RN4220-p17, which leads to fatal toxemia, causes severe elevations

TABLE 3. Abnormalities in blood chemistry values of rabbits challenged with transformed strains of *S. aureus* RN4220

<i>S. aureus</i> strain	Relative increase above baseline values ^a			
	BUN	SGPT	Triglycerides	Fever
4220	NS	NS	+	+
4220-p17	+	+	+	+
4220-p17 + MAb 8-5-7	NS	-	+	+
4220-80.82	+	+	+	+
4220-135	NS	NS	NS	+

^a Baseline values \pm standard errors of the means of uninfected controls: BUN, 17.9 \pm 0.9 mg/dl; SGPT, 20.5 \pm 2.6 U/liter; triglycerides, 81.2 \pm 6.1 mg/dl; body temperature, 102.6 \pm 0.2°F (ca. 39.2 \pm 0.1°C). NS, not statistically different from normal value ($P > 0.05$); +, statistically different by t test ($P < 0.05$); -, sample size too small to calculate statistical significance.

of BUN and SGPT as well as sharp increases in triglyceride levels and body temperature. Similar changes occur in rabbits challenged with the TSS isolate *S. aureus* 587 (data not shown). These abnormalities are consistent with those seen in rabbits infused with purified staphylococcal TSST-1 (5, 21) and are indicative of renal and hepatic dysfunction. MAb 8-5-7, a neutralizing antibody that prevents lethal shock (5), also prevented the elevation in BUN and blunted the increase in SGPT. Triglycerides, however, remained elevated in the antibody-treated rabbits. Since triglycerides are sharply increased in rabbits infected with the nontoxic *S. aureus* RN4220 host strain, we conclude that infection per se and not TSST-1 is responsible for the abnormality in lipid metabolism. Infection with untransformed *S. aureus* RN4220, however, does not elevate BUN and SGPT compared with infection with *S. aureus* RN4220-p17. Since the only difference between these isogenic strains is the presence of the TSST-1-encoding plasmid, the abnormalities in kidney and liver function can be ascribed to TSST-1. Mutant toxin 135, which is nonmitogenic (Fig. 3) and nontoxic for rabbits (Table 1), also does not raise BUN or SGPT levels. We cannot draw a conclusion concerning the determinant of pyrogenic activity of TSST-1, since infection with the nontoxic host strain (RN4220) elicited high fever comparable to that caused by infection with TSST-1⁺ strains. In addition, mutant toxins 135 and 141.144, which in the rabbit model are nontoxic, also cause a high fever.

DISCUSSION

In this study, we corroborated and extended the results of previous studies describing the toxicity of TSST-1, the neutralizing characteristics of MAb 8-5-7 (5), and a structure-function analysis of TSST-1 with a mutational strategy (3). Evidence that a region spanning amino acids 115 through 144 of TSST-1 constituted a biologically active site (i.e., specific mutations in the region resulted in partial or total loss of mitogenicity) was presented. The strategy of this study was to establish a potential correlation between in vitro activity of recombinant TSST-1 and several toxin mutants with their in vivo toxicity for rabbits. We provide evidence that replacement by alanine of histidine 135 of the 194-amino-acid toxin not only ablates mitogenic activity but also destroys the lethal potential of the toxin for rabbits.

The rabbit infection model of TSS is based on the surgical insertion of a polyethylene whiffle ball under the skin and the creation of a diffusible chamber after encapsulation and equilibration with a fluid transudate. Inoculation of *S. aureus* into the chamber contains the infection locally but permits systemic dissemination of TSST-1. Scott et al. (24) established the validity of the model by showing that local infection in the chamber with a TSST-1 strain of *S. aureus* resulted in a TSS-like illness and death of rabbits, whereas a non-toxin-producing strain of *S. aureus* caused only transient systemic symptoms. Best et al. (1) showed further that in this model of TSS, rabbits succumbed to the effects of TSST-1, since parenterally administered toxin-neutralizing MAb 8-5-7 provided full protection. We used this rabbit infection model in this study to evaluate the effects of recombinant TSST-1 and TSST-1 mutants expressed by transformed *S. aureus* strains inoculated into subcutaneously implanted chambers. It was considered of particular interest to determine whether loss of mitogenic activity as a result of a mutation resulted in loss of the capacity to induce lethal shock in rabbits.

The data show that recombinant intact TSST-1 produced

by *S. aureus* RN4220-p17 during growth within the encapsulated chamber was fully toxic and that its effects were indistinguishable from those of TSST-1 made by a wild-type TSST-1 strain (*S. aureus* 587). Intact recombinant toxin causes blood chemistry changes indicative of hepatic and renal dysfunction, with death of rabbits occurring within 48 h. Administration of MAb 8-5-7 intravenously was protective and confirmed the cause of death in unprotected rabbits as being TSST-1 mediated. Intact recombinant TSST-1 (p17) made by *S. aureus* RN4220 is maximally mitogenic and reacts normally with monoclonal and polyclonal antibodies in immunoblotting assays and ELISAs. Mutation 80.82, which involves replacement of a tyrosine residue and a histidine residue with alanines, yields a toxin retaining full mitogenicity and reactivity with TSST-1 antibodies (Table 2). In the rabbit infection model of TSS, the 80.82 mutant was lethal within 24 to 48 h and thus behaved identically to *S. aureus* RN4220-p17. Two mutations (135 and 141.144) in the TSST-1 gene, however, encode mutant toxins possessing minimal mitogenicity for murine T cells and for human peripheral blood mononuclear cells (data not shown). Mutant toxin 141.144 is no longer recognized by MAb 8-5-7 but reacts weakly with polyclonal antibodies. Mutant toxin 135 also is virtually devoid of mitogenic activity but, in contrast to mutant toxin 141.144, is recognized normally by polyclonal antibodies and MAb 8-5-7. When tested in the rabbit infection model, strains of *S. aureus* RN4220 encoding mutant toxin 135 or 141.144 were found to be nontoxic. Rabbits infected with either mutant did not develop the TSS-like illness that results with the transformed strain of *S. aureus* producing intact TSST-1. In rabbits challenged with *S. aureus* RN4220-135, the BUN and SGPT levels remained normal, whereas the temperature elevation and slightly elevated triglyceride levels indicated a mild, transient illness not mediated by TSST-1. Therefore, the results show that alanine substitution of histidine 135 or histidine 141 and tyrosine 144 of TSST-1 not only resulted in loss of mitogenic activity but also eliminated the lethal potential of the toxin for rabbits.

Of the two nontoxic mutants, 135 is the more interesting. Mutant toxin 141.144 is a double mutant in which histidine 141 and tyrosine 144 are replaced by alanines. These changes result in a protein no longer recognized by the neutralizing MAb 8-5-7 and may be indicative of conformational changes. On the other hand, mutant toxin 135 constitutes a toxin with a single substitution of a histidine residue by alanine at position 135. This mutant toxin is recognized normally by MAb 8-5-7. Additionally, wild-type TSST-1 and mutant toxin 135 exhibit comparable intrinsic fluorescence emission spectra, and mutant toxin 135 competitively inhibits intact TSST-1 in a lymphoproliferative assay suggesting that mutant toxin 135 is conformationally intact (2). Thus mutant toxin 135 constitutes a structurally intact protein possessing minimal mitogenicity, minimal toxicity for rabbits, and unchanged reactivity with a TSST-1-specific neutralizing monoclonal antibody. We recently constructed two additional mutants with mutations at residue 135 in which the more conservative substitutions of glutamine or asparagine (rather than alanine) were made. The conservative substitutions resulted in mutant proteins that were devoid of mitogenic activity (2). Thus it appears that histidine 135 constitutes a critical amino acid for retention of biological activity of TSST-1.

The structure-function correlations we have made (2, 3, 5) suggest that the ability of TSST-1 to induce T-cell proliferation is linked to its lethal toxicity for rabbits. Only mutant

toxins devoid of mitogenic activity for murine T cells fail to demonstrate toxicity in rabbits. In a collaborative study with J. Parsonnet, we are currently conducting an evaluation of our TSST-1 mutants with the Parsonnet toxin infusion model of TSS (21). In that rabbit model, purified toxin is infused slowly, with subsequent development of symptoms. These experiments in progress should provide definitive evidence corroborating or negating our hypothesis that mitogenicity of TSST-1 is linked to its *in vivo* toxicity. Lee et al. (15) concluded that T-cell proliferation and toxicity for rabbits are unlinked processes. Their conclusion was based on the failure of unrelated T-cell mitogens (e.g., concanavalin A) to cause death in the rabbit toxin model of TSS. Although polyclonal activators cause considerable T-cell proliferation, they do not select specific T-cell populations like TSST-1 and other staphylococcal superantigens do (12). Additionally, the cytokine profiles elicited by concanavalin A and TSST-1 are undoubtedly different in composition and magnitude. T-cell proliferation by TSST-1 involves the participation of mononuclear phagocytes (macrophages) to present toxin to the T-cell receptor in association with MHC class II molecules. Thus, monokines such as interleukin 1 and tumor necrosis factor probably play significant roles in pathogenesis. Miethke et al. (18) have recently developed a mouse model of lethal shock in which staphylococcal enterotoxin B (SEB), also a superantigen, causes death of D-galactosamine-sensitized mice. Miethke et al. present evidence that T-cell-dependent tumor necrosis factor plays a major role in pathogenesis. We are attempting to adapt this mouse model for our structure-function studies of TSST-1 as another means to assess the role of T-cell proliferation in the TSST-1-induced shock syndrome. Marrack et al. (16) concluded that the pathogenic effects of SEB in mice are due to T cells. They used nude mice or mice treated with cyclosporin A. In both cases, mice that lacked T-cell function did not develop disease when given SEB, whereas control animals did. Another study by Kappler et al. (11) identified mutant SEBs that had lost the ability to interact with the T-cell receptor while maintaining normal MHC class II binding. When administered to mice, these mutant toxins were incapable of causing the pathogenic effects of the wild-type toxin. These studies suggest the critical role of T-cell proliferation and concomitant cytokine release in the development of disease to be a result of exposure to superantigens, in this case SEB.

The data we present also suggest that TSST-1 acting as a superantigen causes massive activation of T cells and induction of lethal shock. This interpretation of the data remains equivocal, however, since the amino acid substitution at histidine 135 may have pleiotropic effects; i.e., the mutation may result not only in loss of mitogenic activity but also in ablation of a still-undefined effect of TSST-1 causally linked to *in vivo* toxicity. Vascular leakage due to a previously described permeability effect of TSST-1 on capillary endothelium is a possibility suggested by Lee et al. (15). It would be instructive to test mutant toxin 135 in this context. In the context of T-cell proliferation, however, we are conducting further studies to determine whether mutant toxin 135 is defective in its interaction with MHC or, alternatively, whether the modified TSST-1 fails to bind with the β subunit of the T-cell receptor. In either case, effects on cytokine profiles would be significant. We have preliminary evidence that the mitogen-deficient mutants bind with MHC class II on antigen-presenting cells and that the defect in the mutant probably resides in its interactions with the T-cell receptor (8).

Our observations suggest two other possibilities. High fever is a prominent feature of TSS in humans and in rabbits (20). The fever induced by the nontoxic host strain *S. aureus* RN4220 used in this study is clearly mediated by mechanisms unrelated to TSST-1. The pleiotropic activities of TSST-1, which include mitogenicity and pyrogenicity, may be governed by different sites on the TSST-1 protein. The nonspecific pyrexia exhibited by rabbits in the infection model of TSS may preclude its use for discerning such sites. But the toxin model of TSS (21), in which purified TSST-1 is introduced parenterally via an osmotic pump, appears to be well suited to comparing the effects of recombinant TSST-1 and mutant toxins. Comparison of purified recombinant (wild-type) TSST-1, purified 135 toxin, and other mutant toxins will permit us to determine whether the mitogenic site of TSST-1 is distinct from the site responsible for fever induction. A final speculation based on these data is the potential use of nontoxic mutant TSST-1 proteins as immunogens. If a nontoxic mutant elicits TSST-1-neutralizing antibodies, the recombinant protein would constitute a potential vaccine candidate.

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