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A Mutation at Histidine Residue 135 of Toxic Shock Syndrome Toxin Yields an Immunogenic Protein with Minimal Toxicity

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Structure-function studies have revealed that the region between amino acids 115 and 141 of toxic shock syndrome toxin 1 (TSST-1) constitutes a biologically active domain. A critical residue appears to be histidine 135, since a site-directed mutation that alters the histidine to alanine (H135A) results in a loss of mitogenic activity and an absence of toxicity as measured in a rabbit infection model of toxic shock syndrome. We have characterized the mutant toxin further and report here on its immunogenic activity in rabbits and on the protective ability of mutant-specific antibodies in two animal models of toxin-mediated shock. Antibodies raised in rabbits by immunization with the purified H135A are fully cross-reactive with staphylococcal TSST-1 and wild-type recombinant TSST-1 (rTSST-1) expressed in *Escherichia coli*. The H135A antibodies neutralized the mitogenic activity for murine splenic T cells equally well as did TSST-1-specific polyclonal and monoclonal antibodies. In addition, the H135A antibodies blocked the production of tumor necrosis factor by spleen cells stimulated with rTSST-1. The toxicities of rTSST-1 and H135A were compared in D-galactosamine (D-GalNH$_2$)-sensitized MRL-*lpr*/*lpr* mice. The nontoxicity of H135A was confirmed in this murine model of superantigen-induced septic shock. No toxicity of H135A was demonstrable at doses of 60 μg, while doses of rTSST-1 as low as 2 μg caused significant mortality within 24 to 72 h after challenge. Furthermore, subsequent to challenge of mice with H135A, no elevation in the serum levels of interleukin-2 or tumor necrosis factor was measurable. Passive immunization with H135A antibodies also protected MRL-*lpr*/*lpr* mice against lethal challenge with rTSST-1. Finally, rabbits actively immunized with purified H135A did not succumb to infection with a transformed strain of *Staphylococcus aureus* expressing rTSST-1. Additional animal studies will be required to confirm the immunizing potential of H135A and the efficacy of H135A antibodies as a neutralizing antitoxin.

A body of evidence suggests that toxic shock syndrome toxin 1 (TSST-1) plays a pivotal role in the pathogenesis of menstrually associated toxic shock syndrome (TSS) (2, 9, 28). TSST-1, a chromosomal gene product, is produced by virtually all *Staphylococcus aureus* strains associated with confirmed cases of menstrual TSS (8). TSST-1 belongs to a family of staphylococcal toxins now recognized as bacterial superantigens. TSST-1, like the staphylococcal enterotoxins, complexes with major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and interacts with specific Vβ families of the T-cell receptor (21). Since superantigens interact with entire families of T cells in vivo, a massive activation of the immune system occurs. An estimated 10 to 50% of the T-lymphocyte repertoire may proliferate in response to superantigen stimulation. It is believed that cytokines, particularly interleukin-1 (IL-1) and tumor necrosis factor (TNF) are prominent in the genesis of toxic shock (17, 22, 23).

To understand structure-function relationships of the toxin with the cellular receptors of the immune system, a mutational analysis of TSST-1 was performed (4). Point mutants were evaluated for the retention of biological functions and for interaction with TSST-1-specific monoclonal antibodies (MAbs) (7). Several recombinant proteins, modified at single amino acid residues, exhibited a reduced mitogenic activity for murine spleen cells and retained reactivity with TSST-1-neutralizing MAb 8-5-7 (4). One mutation, whereby histidine residue 135 was replaced by alanine, was particularly noteworthy. In addition to a total loss of mitogenic activity and retention of reactivity with MAb 8-5-7, H135A was found to lack toxicity for rabbits as tested in an infection model of TSS (5). The infection model mimics the course of events occurring in human TSS, i.e., establishment of a focal infection with *S. aureus*, dissemination of TSST-1, and development of toxic shock (5). A transformed strain of *S. aureus* encoding TSST-1, when implanted in subcutaneous chambers, led to lethal shock within several days. A transformed strain encoding H135A in the same model was nontoxic. We considered it important to characterize H135A further. As a full-length recombinant protein exhibiting loss of biological activity with no obvious loss of conformational integrity, H135A presents itself as a potential vaccine candidate. In this report, the toxin-neutralizing capacity and protective ability of H135A antibodies are demonstrated in rabbit (29) and murine (22) models of TSST-1-mediated lethal shock. Additionally, the absence of overt toxicity of H135A is confirmed with D-galactosamine (D-GalNH$_2$)-sensitized MRL-*lpr*/*lpr* mice in a murine model of superantigen-induced shock (24). Treated mice were quite sensitive to wild-type recombinant TSST-1 (rTSST-1), while H135A was nontoxic in this model.

MATERIALS AND METHODS

Toxins and nomenclature. Purified staphylococcal TSST-1 was provided by J. Parsonnet, Dartmouth Medical Center, Hanover, N.H. Details of the purification procedure have been published elsewhere (27). In previous studies (4, 5), we described unmodified (wild-type) recombinant TSST-1 as P17. In this report, the recombinant wild-type toxin is identified as rTSST-1. The mutation at histidine

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residue 135 of TSST-1 was obtained by M13 site-directed mutagenesis as described previously (3, 5). The histidine-to-alanine mutant is identified as H135A. The recombinant toxins were produced by introducing the mutated or wild-type TSST-1 gene into a pT7-7 plasmid expression vector, transforming *Escherichia coli* BL21(DE3) and selecting ampicillin-resistant colonies. The gene products were purified from the periplasmic shockates of *E. coli* cultures by a two-step high-performance liquid chromatography (HPLC) procedure described previously (4). All of the toxins used are pure as assessed by a single homogeneous 22-kDa band on Coomassie brilliant blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels.

ELISAs and immunoblotting assays. Purified rTSST-1 and H135A were measured in a direct enzyme-linked immunosorbent assay (ELISA) using purified polyclonal antibodies raised in rabbits immunized with purified staphylococcal rTSST-1 or H135A (see immunization protocol below). Wells were coated with staphylococcal TSST-1 as described elsewhere (7). The ELISA results presented are representative of several independent experiments.

Toxins were also detected by immunoblotting of SDS-polyacrylamide gels using TSST-1-specific polyclonal antibodies (Toxin Technologies, Sarasota, Fla.) and polyclonal H135A antibodies obtained from rabbits immunized with purified H135A as probes.

Mitogen assay. Purified toxins were assayed for their capacity to activate murine T cells as described previously (4). Spleens from BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.) were minced into a single-cell suspension and dispensed at 10^6 cells per well into 96-well flat-bottom tissue culture plates (Corning Glass Works, Corning, N.Y.) with or without the addition of purified toxins at the concentrations indicated. Polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.) was added to all cultures at a final concentration of 15 μg/ml to suppress potential mitogenic effects of contaminating lipopolysaccharide (LPS). Plates were incubated for 48 h (37°C, 5% CO_2) and then pulsed for 18 h with 1 μCi of [^{3}H]thymidine per well (35 Ci/mmol; ICN Radiochemicals, Irvine, Calif.). Incorporation of [^{3}H]thymidine was measured by liquid scintillation counting. Data are presented as the means of triplicate determinations ± standard deviations.

Immunization of rabbits. New Zealand White male rabbits weighing approximately 3.0 kg were subjected to an immunization schedule with injections spaced as follows: day 1, 50 μg of H135A or rTSST-1 in Titer Max (CytRx, Atlanta, Ga.) administered subcutaneously, divided in four sites; day 24, 50 μg of toxins administered as described for day 1; day 45, 100 μg of toxins in incomplete Freund's adjuvant administered subcutaneously, divided in six sites. Serum antibody titers were determined by an ELISA several weeks after the last injections. Rabbits were bled to obtain anti-H135A and anti-rTSST-1 polyclonal antibodies. Immunoglobulin was prepared from rabbit antisera by protein A column purification (Bio-Rad Laboratories, Richmond, Calif.).

Rabbit infection model of TSS. The infection model of TSS devised by Scott et al. (29) was used to test the efficacy of immunization with purified H135A. Rabbits immunized by the regimen described above were challenged with a TSST-1-producing strain of *S. aureus* which in unimmunized rabbits leads to lethal shock within 2 days (5). The model employs a sterile golf whiffle ball surgically implanted under the skin in the dorsolateral aspect. After several weeks, the ball is encapsulated and filled with a sterile transudate. The staphylococcal inoculum introduced through a hole in the plastic ball remains localized while TSST-1 is disseminated systemically. The challenge strain was obtained through transformation of the TSST-1-negative strain *S. aureus* RN4220 by electroporation with a plasmid vector encoding TSST-1. A description of the rabbit infection model and details of the electroporation procedure are published (5). Using this model of TSS, we had established that nontransformed *S. aureus* RN4220, or the same strain transformed with plasmid-encoding H135A, causes only a transient pyrexia and no mortality. *S. aureus* RN4220 expressing rTSST-1 usually caused lethal shock within 48 h (5). In this study, we wanted to determine if rabbits immunized with H135A could withstand challenge with the *S. aureus* strain producing rTSST-1.

Blood serum chemistry of rabbits. Rabbits challenged with the *S. aureus* strains producing rTSST-1 or H135A were bled prior to and after challenge. Blood urea nitrogen (BUN), serum glutamic pyruvic transaminase (SGPT), and triglycerides in serum samples were measured in a Hitachi 737 multichemistry analyzer. Parsonnet et al. (26) demonstrated that staphylococcal TSST-1 infused into rabbits leads to predictable changes in serum chemistries indicative of multiple organ system abnormalities. Thus, these measurements were used to estimate the comparative toxicities of rTSST-1 and H135A for rabbits.

Challenge of rabbits immunized with H135A. Using the rabbit model of TSS, we tested the protection afforded by immunization with H135A. Immunized and control animals were challenged with 10^9 CFU of an *S. aureus* strain producing rTSST-1. Failure to develop abnormal serum chemistry profiles and prevention of lethal shock were used as indices of protection.

Murine model of TSST-1-induced shock. Conventional mice are quite resistant to challenge with LPS (15) or staphylococcal superantigens (22, 23). The hepatotoxic compound D-GalNH_2 sensitizes mice to these toxins. Using a modification of the D-GalNH_2 model of murine septic shock, Mountz et al. (24) demonstrated that MRL-*lpr*/*lpr* mice exhibit an increased susceptibility to staphylococcal enterotoxin B (SEB)-mediated lethality not demonstrated by similarly treated conventional mice. Therefore, we utilized these mice to evaluate the potential lethality of rTSST-1 and H135A. MRL-*lpr*/*lpr* mice were obtained originally from the Jackson Laboratory (Bar Harbor, Maine) and bred in isolation facilities at the Marion Merrell Dow Research Institute (Cincinnati, Ohio) for the past 4 years. Mice received an intraperitoneal (i.p.) challenge with 20 to 40 mg of D-GalNH_2 and rTSST-1 or H135A as a mixture, and deaths were recorded over a 72-h period. In a single experiment, H135A antibodies were administered i.p. 1 h prior to challenge of mice to determine if the antibodies could passively confer immunity to rTSST-1-challenged mice. Additionally, purified H135A was administered i.p. in excess just prior to challenge to determine if H135A could act as a competitive inhibitor for TSST-1 receptors and thus provide measurable protection to MRL-*lpr*/*lpr* mice challenged with rTSST-1.

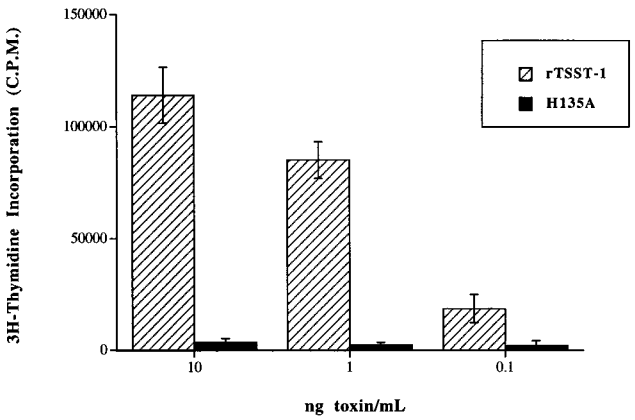

FIG. 1. Incorporation of [^{3}H]thymidine by murine spleen cells following incubation with 10, 1.0, or 0.1 ng of purified mutant H135A or wild-type toxin (rTSST-1). H135A shows loss of mitogenic activity indicated by background levels (1,706 ± 518 cpm) of [^{3}H]thymidine incorporation.

Determination of serum cytokine levels. Normal or D-GalNH_2-treated MRL-*lpr*/*lpr* mice were injected with different quantities of rTSST-1 or H135A. At designated time periods after superantigen challenge, peripheral blood was collected by orbital bleeding, and serum TNF and IL-2 levels were measured with ELISA kits (Genzyme, Cambridge, Mass.) as described in the manufacturer's procedures.

TNF bioassay. Homogenized spleens from BALB/c mice were dispensed at 10^6 cells per well into 96-well flat-bottom tissue culture plates with purified rTSST-1 or rTSST-1 plus H135A polyclonal antibodies. The toxin was preincubated with the purified antibodies (170 μg) for 30 min prior to addition to the spleen cell cultures. Cultures were incubated for 48 h (5% CO_2, 37°C), and culture supernatants were frozen at −70°C. TNF in thawed supernatants was quantitated by the L929 cytotoxicity assay (16). Culture supernatants (50 μl), or a murine TNF standard (Genzyme), along with 750 ng of actinomycin D (Sigma) per well was added to 2×10^5 L929 cells in 96-well culture dishes. Assay plates were incubated overnight (5% CO_2, 37°C), the medium was removed, and 100 μl of a 0.5% crystal violet–25% methanol mixture was added to each well. After 30 min at room temperature, the stain was decanted, the plates were rinsed with water and air dried, and the stain was eluted by adding 100 μl of 100% methanol. The optical density of the extracted dye was read at 570 nm, and quantitation of TNF in supernatants was calculated by comparison with a standard curve. Values are expressed as means of triplicate determinations ± standard deviations. As a control for the specificity of the L929 assay, the lytic activity of supernatants was neutralized with an anti-TNF antibody (Pharmingen, San Diego, Calif.). The antibody, diluted 1/2,000, was preincubated with the supernatants for 30 min prior to addition to the L929 cells. The anti-TNF antibody completely neutralized the lytic activity of the supernatants, indicating that the assay was specific for TNF.

RESULTS

Loss of mitogenic activity of H135A. A previous study by Blanco et al. (4) showed that single-amino-acid substitutions at residues 115 and 141 resulted in a significant (50%) reduction in mitogenic activity of the mutant toxins for murine T cells. In another report (5), we showed that H135A expressed in *S. aureus* had no mitogenic activity for murine spleen cells. Figure 1 shows that this mutant toxin, when expressed in *E. coli*, also has no detectable mitogenic activity. This confirms our previous observation that H135A produced by *S. aureus* was devoid of mitogenic activity and indicates no difference in the function of the recombinant toxins expressed in *E. coli*. Lack of mito-

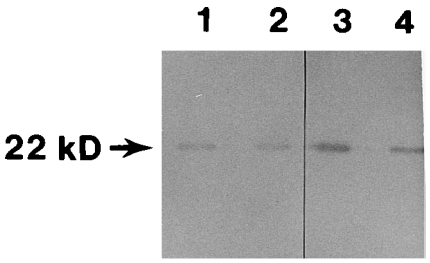

FIG. 2. Immunoblots showing cross-reactivity of rTSST-1 and H135A with polyclonal anti-TSST-1 antibodies (lanes 1 and 2) and polyclonal anti-H135A antibodies (lanes 3 and 4). Lanes 1 and 3 have 500 ng of rTSST-1; lanes 2 and 4 have 500 ng of H135A. Both polyclonal antibodies were used at a 1/200 dilution.

genic activity is not restricted to mouse cells since human peripheral blood mononuclear cells also do not respond to H135A (unpublished observations).

Cross-reactivity of rTSST-1 and H135A. Antibodies raised against rTSST-1 and H135A are similar in reactivity. In ELISA and Western blotting (immunoblotting) formats, no measurable differences were noted in the reactivities of rTSST-1 or H135A with antibodies against TSST-1 or H135A. Figure 2 shows immunoblots of SDS-polyacrylamide gels of HPLC-purified preparations of rTSST-1 and H135A probed with either H135A immunoglobulin or anti-TSST-1 immunoglobulin. The data show that rTSST-1 and H135A react equally well with anti-H135A immunoglobulin as they do with anti-TSST-1 immunoglobulin. Similarly, Fig. 3 shows comparable absorbance readings in an ELISA using comparable amounts of purified H135A and rTSST-1 antibodies. The results indicate that H135A is immunogenic and elicits antibodies which recognize rTSST-1. Our previous study showed that TSST-1 MAb 8-5-7 (7) reacted equally well with rTSST-1 and H135A (5).

Rabbit infection model of TSS to assess efficacy of immunization with H135A. A primary goal of this study was to examine the immunogenic properties of H135A and to determine if the modified toxin could be considered a potential immunizing agent. Two primary considerations were the ability of H135A antibodies to neutralize the toxicity of rTSST-1 and demonstration of the lack of toxicity of H135A for experimental animals. Rabbits immunized with H135A and unimmunized

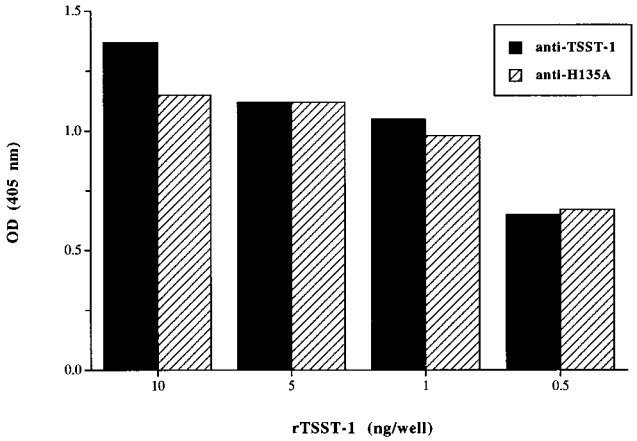

FIG. 3. Absorbance readings from an ELISA of rTSST-1 utilizing either anti-rTSST-1 or anti-H135A (purified) polyclonal antibodies. The antibodies were added to the toxin-coated wells at a 1/10,000 dilution. Similar absorbance readings indicate comparable reactivities of rTSST-1 with both antibodies. Results shown are representative of several experiments. OD, optical density.

TABLE 1. Protection provided by active immunization of rabbits with purified H135A against challenge with transformed *S. aureus* expressing rTSST-1 in the rabbit model of TSS

Rabbit group	Mortality[a]	Serum chemistry values[b]		
		BUN	SGPT	Triglycerides
Unimmunized	5/6[c]	62.2	106.2	1,916
H135A immunized[d]	0/2[e]	16.5	31	458

[a] Number of rabbits that died/total number of rabbits.
[b] Baseline values of uninfected controls: BUN, 17.9 ± 0.9 mg/dl; SGPT, 20.5 ± 2.6 U/liter; triglycerides, 81.2 ± 30 mg/dl.
[c] Surviving rabbit was moribund after 24 h but eventually recovered.
[d] H135A antibody titer of the immunized rabbits was approximately 1/100,000 by ELISA.
[e] Immunized rabbits demonstrated no evidence of illness except transient pyrexia.

control animals were tested in the rabbit infection model of TSS (29). Rabbits were inoculated with 10^9 CFU of the *S. aureus* transformant expressing rTSST-1. Predictably (5), nonimmunized control rabbits became ill, developed multiorgan system failure, and succumbed within 48 h postchallenge. In the earlier study (5), we had established that mortality in this animal model is TSST-1 mediated since infusion of toxin-neutralizing MAb 8-5-7 into the challenged rabbits prevented illness and death. Table 1 shows that rabbits immunized by several injections of purified H135A and which had developed significant antibody titers withstood challenge with the TSST-1-producing strain of *S. aureus*. The normal serum BUN and SGPT values of the immunized rabbits 24 h after challenge permit the conclusion that the H135A antibodies blunted illness and prevented lethal shock.

H135A is not toxic for MRL-*lpr*/*lpr* mice in the D-GalNH$_2$ model of TSST-1-mediated lethal shock. For H135A to be considered a potential prophylactic vaccine candidate, it was essential to show that the in vivo toxicity of unmodified TSST-1 was not demonstrable with H135A. Table 2 shows that the MRL-*lpr*/*lpr* mice sensitized with D-GalNH$_2$ succumb to extremely small doses of rTSST-1. After 72 h, 100% mortality was observed with a 10-μg challenge and significant mortality was observed with a 2-μg challenge. Similarly sensitized mice, however, are refractory to doses of H135A as high as 60 μg.

Cytokine profiles of serum samples obtained 4 to 6 h after challenge also demonstrate the loss of biological activity of H135A (Table 3). In mice challenged with rTSST-1, substantial amounts of TNF were induced at all levels of toxin challenge. In contrast, mice challenged with H135A showed no detectable TNF. In addition, the wild-type toxin induced substantial

TABLE 2. Comparative toxicities of rTSST-1 and H135A for D-GalNH$_2$-sensitized MRL-*lpr*/*lpr* mice[a]

Toxin	Dose (μg)	No. of dead mice/total no.	% Mortality
rTSST-1	10	5/5	100
	5	4/5	80
	2	2/5	40
H135A	60	0/5	0
	30	0/5	0
	10	0/5	0
D-GalNH$_2$ alone		0/2	0
rTSST-1 alone	60	0/2	0
LPS + D-GalNH$_2$	10	2/2	100

[a] D-GalNH$_2$ was administered i.p. at a dose of 40 mg per mouse as a mixture with rTSST-1 or H135A toxin.

TABLE 3. Failure of H135A to induce TNF and IL-2 in D-GalNH_2-sensitized MRL-*lpr*/*lpr* mice

Toxin	Dose (μg)	Cytokine levels (pg/ml)[a]	
		TNF	IL-2
rTSST-1	10	10,726 ± 6,230	3,096 ± 900
	5	4,566 ± 3,520	526 ± 1,342
	2	61.7 ± 8.8	0.0
H135A	60	17.5 ± 8.2	0.0
	30	9.3 ± 13.1	0.0
	10	10.8 ± 11.3	0.0

[a] Baseline values for control mice: TNF, 41.4 ± 12 pg/ml; IL-2, 0.0 pg/ml. Values are from serum samples of mice obtained 4 to 6 h after challenge. TNF and IL-2 were measured by commercial kits (Genzyme).

amounts of IL-2 at 10 and 5 μg but not at 2 μg. However, H135A was inactive at all concentrations.

Passive protection of D-GalNH_2-sensitized MRL-*lpr*/*lpr* mice by H135A antibodies. As another way to test the protection provided by H135A antibodies, the D-GalNH_2-sensitized mice challenged with rTSST-1 were pretreated with two preparations of H135A antibodies. Figure 4 shows that the mortality of the toxin-challenged MRL-*lpr*/*lpr* mice was reduced by pretreatment with either polyclonal H135A rabbit serum or purified immunoglobulin from immune serum purified by means of a protein A column. The data suggest that H135A antibodies administered passively conferred protection against challenge with rTSST-1. A quantitative estimate of protection afforded by H135A antibodies as compared with that of homologous (i.e., rTSST-1) antibodies will necessitate additional in vivo experiments.

Use of H135A as a competitive inhibitor of rTSST-1 in vivo. The activation of T cells by superantigens requires the dual interaction of toxin with MHC class II antigens and the Vβ elements of the T-cell receptor (21). Cullen et al. (11) have shown that H135A is able to bind as efficiently to MHC class II-expressing cells as does rTSST-1. Thus, it was of interest to ascertain if H135A administered in molar excess to D-GalNH_2-sensitized MRL-*lpr*/*lpr* mice could provide protection against a challenge with rTSST-1. The rationale for the experiment was the possible saturation of MHC class II binding sites on target cells by the biologically inactive mutant. Several mice were inoculated i.p. with 60 μg of H135A 1.0 h prior to challenge with 5 μg of rTSST-1. Mice challenged with 5 μg of rTSST-1 without H135A resulted in 80% mortality (four of five mice died). However, only 40% (two of five) of mice pretreated with H135A succumbed to the rTSST-1 challenge. Additional experiments will be required to determine if this therapeutic approach might be feasible.

H135A antibodies neutralize the mitogenic activity of rTSST-1. The activation of T cells and the expansion of specific Vβ families of the T-cell receptor is a hallmark of superantigen-mediated illness (21). Thus, prevention of significant T-cell activation is essential in providing protective immunity. Figure 5 shows that preincubation of mouse spleen cells with H135A immunoglobulin reduces the mitogenic activity of rTSST-1 to extremely low levels but does not suppress T-cell proliferation completely. The data show that the efficacy of neutralization by H135A antibodies is comparable to that achieved with antibodies directed against unmodified TSST-1.

H135A antibodies inhibit the production of TNF by mouse spleen cells. TNF production is a prominent feature of superantigen-induced illness (22, 23, 25). To test whether H135A antibodies could inhibit expression of this cytokine in vitro,

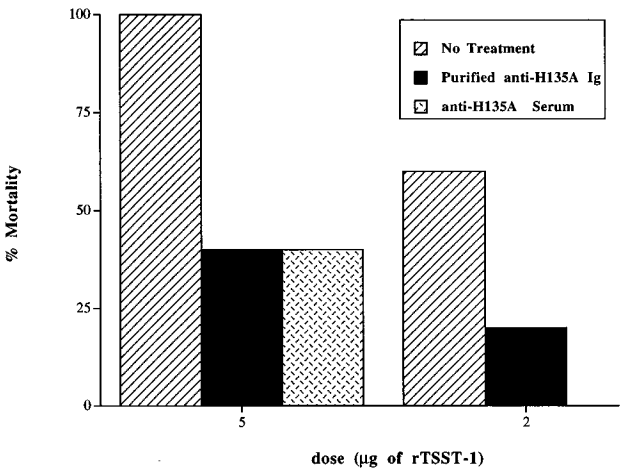

FIG. 4. Protection of D-GalNH_2-sensitized MRL-*lpr*/*lpr* mice against rTSST-1-induced lethality. Purified polyclonal anti-H135A antibody (1 mg per mouse) or anti-H135A serum (1 ml, undiluted) was administered 1 h prior to challenge. Ig, immunoglobulin.

TNF production by murine spleen cells exposed to rTSST-1 was measured in the absence or presence of H135A antibodies. Figure 6 shows that preincubation with antibodies raised against mutant toxin markedly suppressed rTSST-1-induced TNF expression by murine splenocytes. However, at the highest toxin concentration tested (100 ng/ml), TNF production was not inhibited by H135A antibodies. It should be pointed out that MAb 8-5-7 also did not inhibit TNF production by 100 ng of rTSST-1 per ml nor did it neutralize the mitogenic activity of toxin at that concentration (unpublished observations).

DISCUSSION

Several years ago, we initiated a structure-function study of staphylococcal TSST-1 using a mutational analysis (4). Based on evidence obtained with several recombinant mutant toxins, a region between amino acids 115 and 141 of the 194-amino-acid toxin was tentatively identified as a biologically active domain. Single-amino-acid substitutions at positions 115 and 141 yielded recombinant toxins of reduced mitogenic activity for murine T cells. In later studies, we observed that a single-amino-acid substitution of the histidine residue at position 135 resulted in a total loss of mitogenic activity (3, 5).

The relationship between T-cell proliferation in response to TSST-1 and the onset of lethal shock in TSS is uncertain. Using a rabbit infection model of TSS (5), we found that infection with *S. aureus* transformants expressing H135A did not cause lethal shock, whereas transformants expressing rTSST-1 or mutants of reduced mitogenic activity (mutations at residue 115 or 141) all precipitated septic shock. Thus, we speculated that TSST-1-mediated illness was causally related to the massive T-cell proliferation induced by this staphylococcal superantigen. Furthermore, it appeared that the mutation at histidine residue 135 abolished the biological activity of TSST-1 without altering the conformational properties of the protein significantly. The present study was intended to examine some additional characteristics of H135A. We compared the immunogenic properties of purified rTSST-1 and H135A as well as their relative toxicities in animal models of TSST-1-mediated lethal shock. The data show that histidine residue 135 of TSST-1 is of critical importance in the expression of the toxin's in vivo toxicity as well as for the mitogenic activation of murine

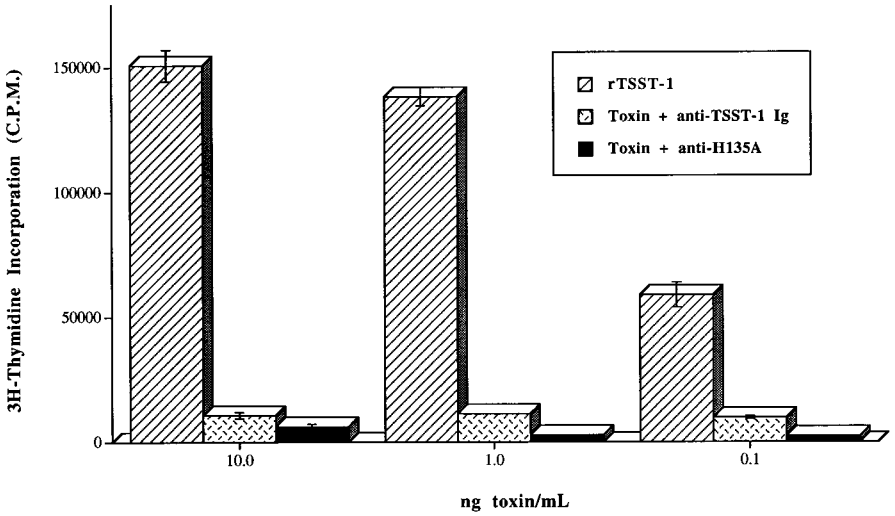

FIG. 5. Inhibition of rTSST-1-induced murine spleen cell proliferation by either anti-H135A or anti-rTSST-1 antibodies. Antibodies (55 μg of immunoglobulin purified from the sera of immunized animals) were preincubated with the toxin for 1 h prior to adding to the cells. Background levels of [^{3}H]thymidine incorporation (10,902 ± 1,542 cpm) indicate complete neutralization of mitogenic activity by both antibodies at the toxin doses tested. Neither of the antibodies neutralized the mitogenic activity of rTSST-1 at a toxin concentration of 100 ng/ml.

T cells. One of the goals of our investigation was to ascertain if H135A could be considered a potential vaccine candidate. The two major prerequisites of any candidate are immunogenicity and nontoxicity. Admittedly, the use of a vaccine to protect against staphylococcal TSS would not be widespread but might be useful for prophylactic immunization of selected populations. For example, it might be considered for immunization of chronic vaginal carriers of *S. aureus* (20), individuals prone to recurrent staphylococcal infections, or those who have experienced clinical TSS without developing an adequate antibody response to TSST-1 (6).

We have shown here and elsewhere (5) that H135A is devoid of mitogenic activity. Nevertheless, the modified toxin is immunogenic, eliciting antibody titers in rabbits comparable to those achieved with rTSST-1. The H135A antibodies are fully cross-reactive with rTSST-1 and staphylococcal TSST-1 as measured by ELISA and Western blotting assays. Purified H135A behaves identically to rTSST-1 in migration as a 22-kDa protein on SDS-polyacrylamide gels. Furthermore, H135A antibodies neutralize rTSST-1-induced mitogenic activity and TNF production by murine spleen cells in vitro. These observations, which suggest that H135A is conformationally similar to rTSST-1, are corroborated by the in vivo experiments described here. Active immunization of rabbits with purified H135A provides protection of rabbits against challenge with *S. aureus* producing rTSST-1. Unimmunized rabbits succumb to lethal shock, while immunized animals suffer no serious illness. The indicators of renal and hepatic dysfunction suggested by the abnormally elevated BUN and SPGT levels of blood samples taken from the unprotected rabbits prior to death were not observed in the challenged rabbits immunized previously with H135A. These observations suggest that H135A antibodies can protect against the lethal effects of TSST-1.

Normal inbred mice are quite resistant to the lethal effects of LPS and enterotoxin superantigens. However, treatment with D-GalNH_2 markedly increases the susceptibility of mice to LPS (15) and SEB (22, 23). Miethke et al. (23) adapted the D-GalNH_2 sensitization model to study SEB-mediated shock in BALB/c mice. In that study, the 50% lethal dose of SEB for D-GalNH_2-treated mice was reduced by nearly 2 orders of magnitude. In a subsequent study, these investigators showed that microgram quantities of TSST-1 also caused lethal shock in BALB/c mice sensitized with D-GalNH_2 (22). Our attempts to adapt the D-GalNH_2 model (24) by using BALB/c mice and TSST-1 were inconclusive since lethal toxicity was not consistently demonstrable. Thus, we were obliged to develop the shock model with an unconventional strain of mice.

The extreme susceptibility of the MRL-*lpr*/*lpr* mice to the lethal effects of SEB in the D-GalNH_2 model prompted us to determine if MRL-*lpr*/*lpr* mice were suitable for our study with rTSST-1 and H135A. Using MRL-*lpr*/*lpr* mice, we were able to demonstrate two important characteristics of H135A. First, we confirmed earlier observations with rabbits (5) that H135A lacked the toxic properties of native TSST-1. MRL-*lpr*/*lpr* mice sensitized with D-GalNH_2 succumbed to doses of rTSST-1 as low as 2.0 μg, while similarly treated mice withstood challenge doses of H135A as high as 60 μg. Lack of toxicity of H135A was also reflected by the failure of the mutant to elicit signif-

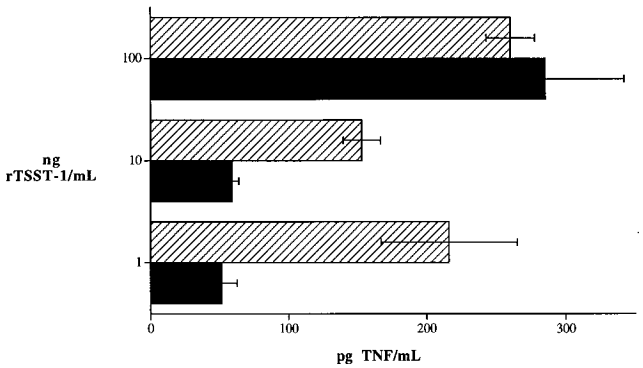

FIG. 6. Inhibition by anti-H135A antibodies of rTSST-1-induced TNF expression by murine spleen cells. Symbols: striped bars, TNF expression in the absence of anti-H135A antibody; solid bars, TNF expression after preincubation of rTSST-1 with anti-H135A antibody. TNF expression induced by the medium control was <6.24 pg/ml. As seen for mitogenicity (Fig. 5), TNF production is not neutralized at the 100-ng/ml toxin level (see text for interpretation).

icant levels of IL-2 and TNF in the MRL-*lpr*/*lpr* mice. High levels of TNF in vivo are a useful indicator of staphylococcal toxin-mediated shock (23). Second, we show that D-GalNH_2-sensitized MRL-*lpr*/*lpr* mice passively immunized with rabbit H135A antibodies were measurably more resistant to challenge with rTSST-1.

Last, we made the observation that relatively large quantities of H135A administered parenterally to MRL-*lpr*/*lpr* mice can inhibit the lethal effects of rTSST-1. Although the small number of animals tested does not permit evaluation of statistical significance, the data show that approximately 50% of the H135A-treated mice were protected against rTSST-1-mediated lethal shock. This suggests that the biologically inactive mutant toxin competes successfully for TSST-1 receptor sites on antigen-presenting cells and thus may prevent the activation of T cells by the toxin. This hypothesis is supported by the observations that H135A retains normal binding characteristics with MHC class II-positive B cells (11) and yet fails to elicit TNF and IL-2 in the MRL-*lpr*/*lpr* mice (Table 3).

Our data provide evidence that H135A is not significantly altered from its native configuration. Conservative changes of histidine 135 to glutamine or asparagine, rather than alanine, yielded mutant toxins as inactive in the mitogen assay as the alanine mutant (unpublished observations). Furthermore, we demonstrated that rTSST-1 and H135A exhibit comparable circular dichroism spectra (unpublished observations) and that the mutant in excess competitively inhibits the in vitro mitogenic activity of TSST-1 (3). Additional evidence that H135A is structurally similar to native toxin includes retention of reactivity with TSST-1-neutralizing MAb 8-5-7 (5), comparable behaviors of rTSST-1 and H135A in Western blotting assays and ELISAs, and in the normal binding characteristic of H135A to MHC class II molecules (11). H135A most likely undergoes a localized change in structure which leads to the observed loss of biologic activity (1).

A complete characterization of H135A and perhaps additional TSST-1 mutants is warranted. Although many of the properties of TSST-1 have been defined, the precise pathway leading to toxic shock is not known. We have argued that the retention of T-cell mitogenicity is a critical factor in the expression of toxicity in vivo (4, 5). Effective interactions of TSST-1 with the T-cell receptor via specific Vβ elements stimulates large numbers of T cells with concomitant overproduction of cytokines. This may be sufficient to cause many of the clinical features of TSS. Other activities of TSST-1 not yet defined may also be contributory. Lee et al. (18) speculate that TSST-1-induced vascular leakage rather than T-cell proliferation is responsible for TSS. The authors conclude that the superantigen exerts a direct cytotoxic effect upon endothelial cells which is independent of the immunomodulating properties of TSST-1 (19). Deresiewicz et al. (13), however, in attempts to identify an occult cytotoxic (enzymatic) activity of TSST-1, resorted to a yeast expression system similar to that used successfully for the study of shiga-like toxin (12) and ricin mutants (14). Intracellular expression of these cytotoxins with known enzymatic functions reduced the viability of *Saccharomyces cerevisiae* by several orders of magnitude. An engineered strain of *S. cerevisiae* expressing intracellular TSST-1, however, had no effect on growth kinetics or yeast morphology. The authors concluded that TSST-1 likely does not possess enzymatic activity cytotoxic for eukaryotic cells (13). They point out that leakage of porcine endothelial cells observed by Lee et al. (19) required a concentration of TSST-1 in the microgram-per-milliliter range, while T-cell activation is demonstrable in the picogram-per-milliliter range. Thus, the apparent cytotoxicity of TSST-1 may be artifactual. Our data presented here and elsewhere (5) suggest that TSST-1 exerts its toxicity in vivo only when total or partial mitogenicity of the superantigen is demonstrable. Whether or not massive T-cell activation and high levels of cytokines produced in vivo can account for all of the biological consequences of TSST-1 remains to be proven. In support of this notion, however, is our recent observation that a synthetic transcriptional inhibitor of TNF (10) protects MRL-*lpr*/*lpr* mice against lethal challenge with rTSST-1 (unpublished observations).

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