INFECTION AND IMMUNITY, Jan. 1996, p. 371–374 Vol. 64, No. 1
0019-9567/96/$04.00+0

Localization of Biologically Important Regions on Toxic Shock Syndrome Toxin 1

DEBRA L. MURRAY,[1] CATHLEEN A. EARHART,[2] DAVID T. MITCHELL,[2] DOUGLAS H. OHLENDORF,[2] RICHARD P. NOVICK,[3] AND PATRICK M. SCHLIEVERT[1]*

Department of Microbiology[1] and Biochemistry,[2] University of Minnesota Medical School, Minneapolis, Minnesota 55455, and Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York, New York 10016[3]

Received 10 April 1995/Returned for modification 1 October 1995/Accepted 31 October 1995

Toxic shock syndrome toxin 1 (TSST-1) contains a long central α helix that forms the base of two grooves on opposite sides of the molecule. Previous studies indicated that residues 132, 135, and 140 along the back of the central α helix are important in the biological activities. We made mutations of additional central α-helix residues exposed along this groove on the back of TSST-1. The proteins were purified, shown not to have gross alteration in structure, and tested for both superantigenicity and ability to elicit lethal TSS, using the miniosmotic pump model of TSS. Mutations of some residues along the central α helix resulted in decreased superantigenicity, likely because of alteration in T-cell receptor binding. Mutants H135A, Q136A, and E132K/Q136K lost the ability to induce lethal TSS. The mutant Q136A was most interesting because it was superantigenic, yet nonlethal.

Staphylococcus aureus and group A streptococci produce many pyrogenic toxin superantigens (PTSAgs) which contribute to multiple illnesses including toxic shock syndrome (TSS), food poisoning, and scarlet fever. The *S. aureus* exotoxin toxic shock syndrome toxin 1 (TSST-1) is responsible for menstrual and approximately 50% of nonmenstrual cases of staphylococcal TSS (2, 29). Staphylococcal enterotoxin type B (SEB), and to a lesser extent SEC, cause the remainder of nonmenstrual staphylococcal TSS cases. The streptococcal pyrogenic exotoxins (SPEs) types A to C (13, 24) and related toxins (17, 22) have been associated with cases of streptococcal TSS.

All of these toxins are strongly mitogenic for T cells, with the mechanism of T-cell stimulation referred to as superantigenic (18) because (i) the toxins do not require proteolytic processing prior to binding with class II major histocompatibility complex molecules on antigen-presenting cells, (ii) the toxins interact with class II major histocompatibility complex molecules outside the antigenic peptide groove, and (iii) the toxins are specific for the V region of the β chain (Vβ) of the T-cell receptor. Thus, T cells with specificity for a variety of conventional antigens may be activated by a single PTSAg provided the T cell has the appropriate Vβ element. This mode of T-cell stimulation results in massive cytokine release from both T cells and macrophages. Other activities of all the toxins are pyrogenicity and enhancement of susceptibility to the lethal effects of endotoxin (6). The toxins also cause TSS-like illnesses when placed subcutaneously within miniosmotic pumps that gradually deliver toxin over a 7-day period (25).

Although these toxins have shared biological activities, their primary amino acid sequences show variable similarity. For example, SEB and SEC are approximately 70% identical (3). These enterotoxins form a subfamily with SPE A, which shares 45 to 50% homology with SEB and SEC. In contrast, TSST-1 and SPE C have little (20 to 30%) primary sequence similarity with respect to the other PTSAgs.

Comparison of the tertiary structures of TSST-1 with those of SEA, SEB, and SEC (15, 27, 28, 30) reveal that despite only 20 to 30% primary sequence identity, these toxins have nearly identical folds. TSST-1 is folded into two domains, domain A (residues 1 to 17 and residues 90 to 194) and domain B (residues 18 to 89). Domain B has five β strands folded into a barrel. Domain A contains the central α helix (residues 125 to 140) resting against a five-strand β sheet. Above this central α helix is the amino-terminal α helix, and on the sides are two loops which in TSST-1 extend just up to the axis of the central α helix. Together, these loops and the amino-terminal α helix define the walls of grooves that give access to the central α helix. The larger groove is in the back of TSST-1, as is shown in Fig. 1. For the enterotoxins, the back groove is even more open while the front groove is nearly completely closed.

Several groups have begun studies to localize biological activities on PTSAgs. Most of these studies have centered on the superantigenic activities, with the intention of defining the major histocompatibility complex class II and T-cell receptor binding regions. For example, Blanco et al. constructed point mutations at residues 135 and 141 of TSST-1 that resulted in a substantial decrease in mitogenic activity (5). When His-135 was changed to Ala (H135A), TSST-1 lost more than 90% of its mitogenic activity and was not lethal when tested in a rabbit infection model of TSS (9). Others showed that the central α-helix residues 132 and 140 of TSST-1 were important in the mitogenic activity of TSST-1 (11, 23). Finally, when the lysine residue at position 132 of the nontoxic TSST-1 variant, TSST-ovine, was changed to glutamate, as exists in TSST-1 (23), the variant became lethal, even though the superantigenic activity was increased only to a level of 10% of that of native TSST-1. It was concluded that residue 132 was critical for the lethality of TSST-1 and that lethality and superantigenicity may be separable.

The present study was undertaken to evaluate the effects on toxicity of other amino acid substitutions of central α-helix residues exposed on the back groove of native TSST-1.

Point mutations of the TSST-1 gene (*tstH*) were introduced by PCR (26). The template for PCR was chromosomal DNA isolated from *S. aureus* MN8. The upstream and downstream primers contained *Hin*dIII and *Sal*I linkers, respectively (23).

* Corresponding author. Phone: (612) 624-9471. Fax: (612) 626-0623.

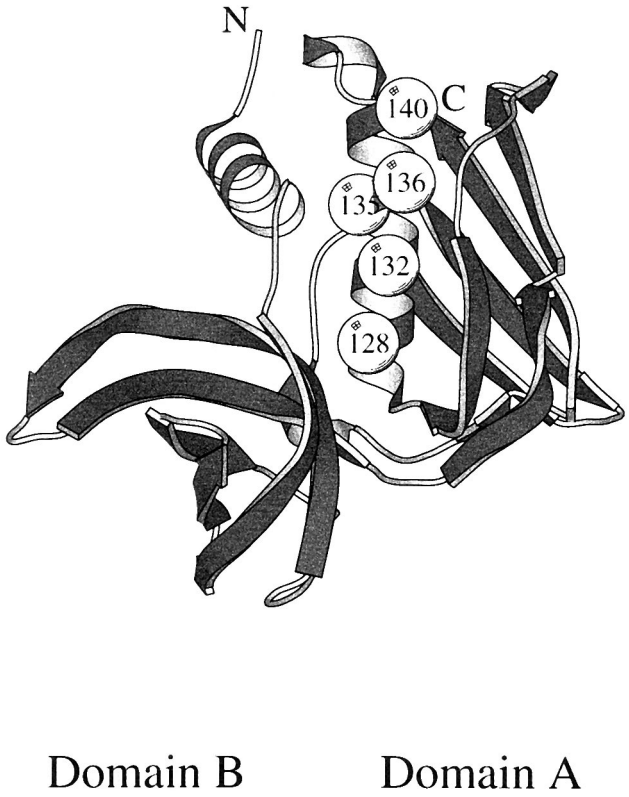

FIG. 1. Ribbon diagram representing the three-dimensional structure of TSST-1 as viewed from the back of the molecule. The amino terminus (N) and the carboxy terminus (C) are indicated. Domain A contains the N-terminal α helix, the central α helix, and the C-terminal wall of β strands. Residues on the central α helix that have been changed in this study are shown. Domain B has the five N-terminal β strands that fold into a barrel.

TABLE 1. TSST-1 mutant proteins and their physical activities compared with native TSST-1

Protein[a]	Immunoblot[b] with:		Trypsin sensitivity[c]	No. dead (n = 3)[d]
	MAb B-14	MAb 12-13		
Native TSST-1	++	++	R	3
E132K/Q136K	+	++	PS	0
Q136A	++	++	R	0[e]
I140T	++	++	R	3
T128A/E132K	++	++	PS	3
T128A	++	+	PS	3
H135A	++	+	R	0
E132K	++	++	PS	3

[a] Mutants of TSST-1 are listed by the single-letter designation for the native amino acid preceding the residue number of that amino acid followed by the single letter designation of the replacement amino acid. For example, Q136A is a TSST-1 mutant in which the glutamine (Q) at position 136 was exchanged for alanine (A).

[b] Western immunoblots were reacted with either MAb B-14 or MAb 12-13. ++, positive reactivity; +, positive but reduced reactivity.

[c] Purified proteins were subjected to trypsin treatment and then separated by SDS-PAGE. Proteins that were resistant to trypsin cleavage (R) and those that were partially sensitive to trypsin cleavage (PS) are indicated.

[d] The treatment consisted of 200 μg of the protein placed individually into miniosmotic pumps which were then singly implanted subcutaneously in rabbits. These pumps continually delivered toxin over a 7-day period. After 7 days, the total number of dead animals was determined. The mutants H135A and Q136A and the double mutant E132K/Q136K were significantly different from TSST-1 in ability to cause lethal TSS (P = 0.05, Fisher exact test).

[e] When animals were treated with 1 or 2 mg of Q136A protein no animals succumbed at either dose.

For the first round of PCR the upstream and the appropriate reverse oligonucleotides (containing the desired mutation) were combined. The following oligonucleotides were constructed for generation of mutants: H135A (histidine residue 135 replaced with alanine), 5′tagctgCGCacgaatttc; I140T, 5′gaacgatataatccatgtGtttgagtt; Q136A, 5′gagttagAGCatgacgaat; E132K, 5′tagctgatgacgaatttTaaagtctaaagt; and T128A, 5′tcaa agtctaaGGCtgatatagc. The uppercase letters designate nucleotide changes from the wild-type *tstH*. The appropriate-size product was isolated from agarose gels, and the DNA was purified by using the Geneclean kit (Bio 101, Vista, Calif.). During the second step of PCR the initial PCR product was combined with the downstream primer and a catalytic amount of template DNA. The final product (approximately 1 kb) was isolated and purified by using the Geneclean kit.

The vector, pMIN164 (23) or pCE104 (a shuttle vector containing pE194 and pUC18), and the mutated *tstH* PCR products were digested individually with *Hin*dIII and *Sal*I and then ligated with T4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and transformed into *Escherichia coli* DH5α (20). The sequence of the entire *tstH* (704 bp) gene for each mutant was determined (16). *S. aureus* RN4220 was then transformed with each mutated *tstH* gene in the plasmid pMIN164 or pCE104 by protoplast transformation (10). Transformants were selected by erythromycin resistance. Mutant toxins were purified from *S. aureus* stationary-phase cultures by precipitation with 4 volumes of ethanol followed by preparative isoelectric focusing (7) for use in structural and biological activity studies.

Seven mutant *tstH* genes were generated by using oligonucleotide site-directed mutagenesis. Because of the 3′-terminal transferase activity of *Taq* polymerase, a nucleotide was occasionally added to the 3′ end of the first-step PCR product. This resulted in an inadvertent exchange in a nucleotide, yielding the double TSST-1 mutants T128A/E132K and E132K/Q136A. The final TSST-1 mutants that were tested are listed in Table 1.

All of the mutant TSST-1 proteins reacted with polyclonal antisera raised against TSST-1 when tested in a gel double-immunodiffusion assay. The proteins were also tested by Western blotting (immunoblotting) (4) for reactivity with two monoclonal antibodies (MAbs) raised against native TSST-1. All mutants retained the ability to react with MAb B-14 (Table 1), an antibody that neutralizes the superantigenic activities of TSST-1 (6). One of the eight mutants, the double mutant E132K/Q136K, exhibited decreased reactivity with MAb B-14. Six of the eight mutants, including E132K/Q136K, reacted normally with MAb 12-13, an antibody that does not neutralize superantigenicity. However, the mutants T128A and H135A were less reactive (Table 1).

TSST-1 and the constructed mutant proteins were evaluated for resistance to trypsin cleavage to gain an indication of structural integrity. SEC1, which is readily cleaved by trypsin (8), served as a control. All proteins (100 μg) were treated with 5 μg of trypsin type XI (Sigma Chemical Company, St. Louis, Mo.) in 100 μl of buffer (0.05 M Tris [pH 8.0], 0.0115 M $CaCl_2$). After 80 min at 37°C, 10-μl samples were combined with 10 μl of sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21). Native TSST-1 is not sensitive to trypsin degradation, despite having many potential trypsin cleavage sites. The mutants H135A, Q136A, and I140T were as resistant to trypsin as TSST-1. All other mutants were partially sensitive to trypsin digestion, as determined by visual observation, in that approximately 15% of 100 μg of the protein was cleaved in 80 min.

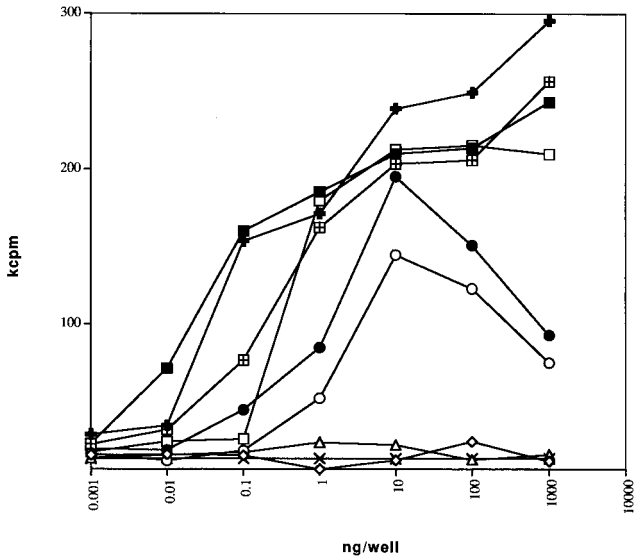

FIG. 2. Mitogenic activity of TSST-1 mutant proteins produced in *S. aureus*. Rabbit splenocytes (2×10^5 cells in 0.2 ml) were treated with 10^3 to 10^{-3} ng of purified protein in quadruplicate. Data are representative of three different rabbit splenocyte donors. The counts per minute (cpm) were plotted at each concentration of protein tested. Symbols are as follows: ✚, native TSST-1; ⊞, I140; ■, T128A; □, E132K; ●, T128A/E132K; ○, Q136A; △, H135A; ◇, E132K/Q136K; X, no protein (background cpm). kcpm, 10^3 cpm.

The positive control, SEC1, was completely digested by this trypsin treatment.

X-ray-diffraction-quality crystals were prepared of mutants T128A, H135A, and I140T. These crystals diffract to resolutions of 2.5 to 3.0 Å (0.25 to 0.30 nm) and are isomorphous to the $C222_1$ form of native TSST-1. Difference Fourier maps demonstrated that the mutations produced only small changes and those were at the site of the mutations.

Rabbit splenocytes were used to measure T-cell proliferation in a 4-day assay (1). Serial 10-fold dilutions of purified mutant and native TSST-1 were tested at a range of 10^{-3} to 10^3 ng per well. The data in Fig. 2 are representative of three separate experiments using three different rabbit donors. Two mutants, H135A and the double mutant E132K/Q136K, were devoid of superantigenic activity. These mutants consistently showed less than 7% of the activity of TSST-1. The mutant Q136A and the double mutant T128A/E132K were mitogenically similar to each other, showing a peak of activity at 10 ng per well and decreasing activity at higher concentrations. The peak responses of these two mutants corresponded to 60 and 80% of TSST-1 activity, respectively. The mutants T128A, I140T, and E132K were similar to each other in superantigenic activity, especially at concentrations greater than 1 ng per well.

Miniosmotic pumps containing 200 μg of the individual proteins (also 1 and 2 mg for Q136A) were placed subcutaneously in rabbits (25). These pumps are designed to gradually deliver toxin over a 7-day period. Animals were monitored daily for signs of TSS and for death. The TSST-1 mutants Q136A and H135A and the double mutant E132K/Q136K did not elicit TSS, and none of the three animals tested for each mutant died (Table 1). All of these animals appeared healthy and had no outward signs of TSS. Interestingly, Q136A did not cause TSS symptoms and was not lethal even at doses of 1 and 2 mg per animal. However, the spleens of these animals were enlarged between two- and threefold compared with nontoxin-treated animals, indicative of maintenance of superantigenicity. All other TSST-1 mutants as well as native TSST-1 resulted in both illness and ultimate death of all animals.

The above data are significant for several reasons. First, we have confirmed the studies of others (5, 9) who showed that residue H135 is important in both superantigenicity and lethality. Since the three-dimensional structure of the H135A mutant is essentially the same as native TSST-1, the loss of biological activity of the mutant was not the result of gross alteration of the TSST-1 structure but, rather, resulted from inability to interact with the host cell receptor(s). Second, since recent studies have localized the ability of TSST-1 to bind to class II major histocompatibility complex molecules to the N-terminal β barrel of TSST-1, interacting with the α and β chains of DR1 (19), the loss in superantigenic activity of both H135A and E132K/Q136K and the altered activity of other mutants resulted from altered interaction of TSST-1 with the T-cell receptor. Finally, the results obtained with Q136A in this study and those obtained with another mutant, TSST-O K132E, in a previous study (23) suggest that the ability of TSST-1 to induce lethality does not depend on T-cell proliferation. Mutant Q136A exhibited significant superantigenic activity, peaking at approximately 60% of that of native TSST-1, which was evidenced by development of significant splenomegaly in rabbits. Yet, Q136A was without lethal activity in the standard subcutaneous miniosmotic pump model of TSS, even at concentrations 20 times greater than necessary for TSST-1 to cause 100% lethality. In contrast, it was previously shown (23) that mutant TSST-O K132E retained only 10% of the superantigenic activity of native TSST-1, yet was completely lethal. These data suggest that other mechanisms, such as cytokine release in the absence of T-cell proliferation, endotoxin enhancement, or direct TSST-1 effects on endothelial cells, are important in lethality. Host cell receptor interactions with TSST-1 that result in lethality depend also on amino acids in the deep groove of the central α helix.

The altered activities of the Q136A mutant are of particular importance for another reason. This TSST-1 mutant has potential practical applications as a therapeutic agent for treatment of various tumors, as has been done in model systems using native PTSAgs (12, 14). The native toxins have the obvious negative side effect of being highly lethal, which would not be the case with Q136A.

This work was supported by Public Health Service grant AI22159 from the National Institute of Allergy and Infectious Diseases.

The Minnesota Supercomputer Center is acknowledged for providing computational facilities. We thank Melodie Bahan for assistance in preparing the manuscript.

REFERENCES

1. **Barsumian, E. L., P. M. Schlievert, and D. W. Watson.** 1978. Nonspecific and specific immunological mitogenicity by group A streptococcal pyrogenic exotoxins. Infect. Immun. **22:**681–688.
2. **Bergdoll, M. S., B. A. Crass, R. F. Reiser, R. N. Robbins, and J. P. Davis.** 1981. A new staphylococcal enterotoxin, enterotoxin F, associated with toxic shock syndrome *Staphylococcus aureus* isolates. Lancet **i:**1017–1021.
3. **Betley, M. J., D. W. Borst, and L. B. Regassa.** 1992. Staphylococcal enterotoxins, toxic shock syndrome toxin and streptococcal pyrogenic exotoxins: a comparative study of their molecular biology. Chem. Immunol. **55:**1–35.
4. **Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich.** 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136:**175–179.
5. **Blanco, L., E. M. Choi, K. Connolly, M. R. Thompson, and P. F. Bonventre.** 1990. Mutants of staphylococcal toxic shock syndrome toxin 1: mitogenicity and recognition by a neutralizing monoclonal antibody. Infect. Immun. **58:** 3020–3028.
6. **Blomster-Hautamaa, D. A., R. P. Novick, and P. M. Schlievert.** 1986. Localization of biologic functions of toxic shock syndrome toxin-1 by use of monoclonal antibodies and cyanogen bromide-generated toxin fragments. J. Immunol. **137:**3572–3576.
7. **Blomster-Hautamaa, D. A., and P. M. Schlievert.** 1988. Preparation of toxic

shock syndrome toxin-1. Methods Enzymol. **165:**37–43.
8. **Bohach, G. A., J. P. Handley, and P. M. Schlievert.** 1989. Biological and immunological properties of the carboxyl terminus of staphylococcal enterotoxin C1. Infect. Immun. **57:**23–28.
9. **Bonventre, P. F., H. Heeg, C. Cullen, and C. Lian.** 1993. Toxicity of recombinant toxic shock syndrome toxin 1 and mutant toxins produced by *Staphylococcus aureus* in a rabbit infection model of toxic shock syndrome. Infect. Immun. **61:**793–799.
10. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168:**111–115.
11. **Deresiewicz, R. L., J. Woo, M. Chan, R. W. Finberg, and D. L. Kasper.** 1994. Mutations affecting the activity of toxic shock syndrome toxin-1. Biochemistry **33:**12844–12851.
12. **Dohlsten, M., A. Sundstedt, M. Bjorklund, G. Hedlund, and T. Kalland.** 1993. Superantigen-induced cytokines suppress growth of human colon-carcinoma cells. Int. J. Cancer. **54:**482–488.
13. **Hauser, A. R., D. L. Stevens, E. L. Kaplan, and P. M. Schlievert.** 1991. Molecular analysis of pyrogenic exotoxins from *Streptococcus pyogenes* isolates associated with toxic shock-like syndrome. J. Clin. Microbiol. **29:**1562–1567.
14. **Hedlund, G., M. Dohlsten, C. Petersson, and T. Kalland.** 1993. Superantigen-based tumor therapy: in vivo activation of cytotoxic T cells. Cancer Immunol. Immunother. **36:**89–93.
15. **Hoffman, M. L., L. M. Jablonski, K. K. Crum, S. P. Hackett, Y. Chi, C. V. Stauffacher, D. L. Stevens, and G. A. Bohach.** 1994. Predictions of T-cell receptor- and major histocompatibility complex-binding sites on staphylococcal enterotoxin C1. Infect. Immun. **62:**3396–3407.
16. **Hsiao, K.** 1991. A fast and simple procedure for sequencing double stranded DNA with sequenase. Nucleic Acids Res. **19:**2787.
17. **Iwasaki, M., H. Igarashi, Y. Hinuma, and T. Yutsudo.** 1993. Cloning, characterization and overexpression of a *Streptococcus pyogenes* gene encoding a new type of mitogenic factor. FEBS Lett. **331:**187–192.
18. **Kappler, J. W., A. Herman, J. Clements, and P. Marrack.** 1992. Mutations defining functional regions of the superantigen staphylococcal enterotoxin B. J. Exp. Med. **175:**387–396.
19. **Kim, J., R. G. Urban, J. L. Strominger, and D. C. Wiley.** 1994. Toxic shock syndrome toxin-1 complexed with a class II major histocompatibility molecule HLA-DR1. Science **166:**1870–1874.
20. **Kushner, S. R.** 1978. An improved method for transformation of *Escherichia coli* with ColE1 derived plasmids, p. 17–24. *In* H. B. Boyer and S. Nicosia (ed.), Genetic engineering. Elsevier/North-Holland, Amsterdam.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Mollick, J. A., G. G. Miller, J. M. Musser, R. G. Cook, D. Grossman, and R. R. Rich.** 1993. A novel superantigen isolated from pathogenic strains of *Streptococcus pyogenes* with amino terminal homology to staphylococcal enterotoxins B and C. J. Clin. Invest. **92:**710–719.
23. **Murray, D. L., G. S. Prasad, C. A. Earhart, B. A. B. Leonard, B. N. Kreiswirth, R. P. Novick, D. H. Ohlendorf, and P. M. Schlievert.** 1994. Immunobiological and biochemical properties of mutants of toxic shock syndrome toxin-1. J. Immunol. **152:**87–95.
24. **Musser, J. M., A. R. Hauser, M. H. Kim, P. M. Schlievert, K. Nelson, and R. K. Selander.** 1991. *Streptococcus pyogenes* causing toxic-shock-like syndrome and other invasive diseases: clonal diversity and pyrogenic exotoxin expression. Proc. Natl. Acad. Sci. USA **88:**2668–2672.
25. **Parsonnet, J., Z. A. Gillis, A. G. Richter, and G. B. Pier.** 1987. A rabbit model of toxic shock syndrome that uses a constant, subcutaneous infusion of toxic shock syndrome toxin 1. Infect. Immun. **55:**1070–1076.
26. **Perrin, S., and G. Gilliland.** 1990. Site-specific mutagenesis using asymmetric polymerase chain reaction and a single mutant primer. Nucleic Acids Res. **18:**7433–7438.
27. **Prasad, G. S., C. A. Earhart, D. L. Murray, R. P. Novick, P. M. Schlievert, and D. H. Ohlendorf.** 1993. Structure of toxic shock syndrome toxin 1. Biochemistry **32:**13761–13766.
28. **Schad, E. M., I. Zaitxeva, V. N. Zaitsev, M. Dohlsten, T. Kalland, P. M. Schlievert, D. H. Ohlendorf, and L. A. Svensson.** 1995. Crystal structure of the superantigen staphylococcal enterotoxin type A. EMBO J. **14:**3292–3301.
29. **Schlievert, P. M., K. N. Shands, B. B. Dan, G. P. Schmid, and R. D. Nishimura.** 1981. Identification and characterization of an exotoxin from *Staphylococcus aureus* associated with toxic shock syndrome. J. Infect. Dis. **143:**509–516.
30. **Swaminathan, S., W. Furey, J. Pletcher, and M. Sax.** 1992. Crystal structure of staphylococcal enterotoxin B, a superantigen. Nature (London) **359:**801–805.

Editor: B. I. Eisenstein