Characterization and Biological Properties of a New Staphylococcal Exotoxin

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Summary

Staphylococcus aureus strain D4508 is a toxic shock syndrome toxin 1–negative clinical isolate from a nonmenstrual case of toxic shock syndrome (TSS). In the present study, we have purified and characterized a new exotoxin from the extracellular products of this strain. This toxin was found to have a molecular mass of 25.14 kD by mass spectrometry and an isoelectric point of 5.65 by isoelectric focusing. We have also cloned and sequenced its corresponding genomic determinant. The DNA sequence encoding the mature protein was found to be 654 base pairs and is predicted to encode a polypeptide of 218 amino acids. The deduced protein contains an NH_2-terminal sequence identical to that of the native protein. The calculated molecular weight (25.21 kD) of the recombinant mature protein is also consistent with that of the native molecules. When injected intravenously into rabbits, both the native and recombinant toxins induce an acute TSS-like illness characterized by high fever, hypotension, diarrhea, shock, and in some cases death, with classical histological findings of TSS. Furthermore, the activity of the toxin is specifically enhanced by low quantities of endotoxins. The toxicity can be blocked by rabbit immunoglobulin G antibody specific for the toxin. Western blotting and DNA sequencing data confirm that the protein is a unique staphylococcal exotoxin, yet shares significant sequence homology with known staphylococcal enterotoxins, especially the SEA, SED, and SEE toxins. We conclude therefore that this 25-kD protein belongs to the staphylococcal enterotoxin gene family that is capable of inducing a TSS-like illness in rabbits.

The staphylococcal enterotoxins are extracellular products secreted by a variety of *Staphylococcus aureus* strains (1). In addition to being responsible for food poisoning in humans, increasing evidence suggests that enterotoxins contribute significantly to staphylococcus-associated toxic shock syndrome (TSS)[1], an acute illness characterized by fever, hypotension, dysfunction of multiple organ systems, and desquamation of the skin during recovery (2–5). There are five known members of the staphylococcal enterotoxin family, designated as staphylococcal enterotoxins A–E (SEA–E). Each member has its unique biochemical and serological properties, but shares significant homology in sequence, structure, and biological functions with other family members (1, 6). Staphylococcal enterotoxins are also characterized as "superantigens," with all members activating large numbers of T cells bearing particular TCR Vβ elements (7–9). As a result of T cell activa-

tion, T cell proliferation and cytokine expression are profoundly elevated. The superantigen properties of these toxins have led to the concept of their use as potential therapeutic agents in the treatment of carcinoma and autoimmune disorders (10, 11).

In a previous study, we reported the identification of a new exotoxin from staphylococcal strain D4508, a TSS toxin 1 (TSST-1)–negative clinical isolate from a patient with nonmenstrual TSS (12). This toxin, called BR4, was shown to be related to but different from other known enterotoxins and TSST-1. When injected into rabbits, partially purified BR4 induced a TSS-like disease and death in the animals. The toxicity was completely blocked by polyclonal antibodies against the BR4 material. However, since the preparation was only partially purified from the staphylococcal extracellular products, the issue as to the true nature of the BR4 protein, its properties, and its correlation with other known staphylococcal toxins, was not completely resolved (4, 12).

In this report, we purified the BR4 protein from the extracellular products of strain D4508 to homogeneity and studied its biochemical and biological properties. This toxin was found to have a molecular mass of 25 kD, an isoelectric

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[1] *Abbreviations used in this paper:* FPLC, fast protein liquid chromatography; LB, Luria-Bertani medium; pI, isoelectric point; SEA, SEB, SEC1, SEE, staphylococcal enterotoxins A, B, C1, D, and E, respectively; TSS, toxic shock syndrome; TSST-1, TSS toxin 1.

point (pI) of 5.65 and to be capable of inducing acute TSS-like illness in rabbits. To further study the structural characteristics of this 25-kD protein and its relationship with other staphylococcal toxins, the toxin was cloned and its DNA sequence analyzed. By virtue of shared biological properties and considerable sequence homology at both the nucleic acid and protein levels with staphylococcal enterotoxins A through E, this toxin is suggested to be a member of the staphylococcal enterotoxin gene family and has been provisionally named SEH.

Materials and Methods

Strains and Vector. S. aureus strain D4508 was obtained from the Centers for Disease Control (Atlanta, GA) (12). *Escherichia coli* strains Y1088, Y1089, Y1090, and K-12 XL1-Blue were purchased from Stratagene (La Jolla, CA). *E. coli* INVαF' and BL21(DE3)pLysS were obtained from Invitrogen (San Diego, CA) and Novagen (Madison, WI), respectively. The bacteriophage λgt11 vector was purchased from Stratagene. Plasmid pHSG399 was a gift from the Japanese Cancer Research Resources bank (Tokyo, Japan) (13). Plasmid pCRII and pET-17b were purchased from Invitrogen and Novagen, respectively. *S. aureus* cultures were grown in chemically defined medium (12). *E. coli* strains were grown in Luria Bertani medium (LB) or NZCYM medium as required (14).

Enzymes and Chemicals. Restriction and modifying enzymes were purchased from Stratagene and Promega (Madison, WI) or Boehringer Mannheim (Indianapolis, IN) and used according to the recommendations of the manufacturers. Sequetide Nucleotide Premix was purchased from DuPont NEN Research Products (Boston, MA). Reagents for syntheses of oligonucleotide primers were purchased from Pharmacia P-L Biochemicals (Milwaukee, WI). Antibiotics and general reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Monitoring the Purification of SEH. A partially purified BR4 preparation was kindly provided by W. W. Hall (The Rockefeller University, New York) (12). BR4, present in this sample and electroeluted from preparative SDS-PAGE gel slices, was then used as a standard to isolate additional SEH for antibody preparation. Both the electroeluted SEH and affinity-purified anti-SEH F(ab')₂ antibody were then used to monitor chromatographic purification of SEH.

Gel Electrophoretic Analysis. All analysis for polypeptides was carried out using 12.5% SDS-PAGE gels (15). Silver staining was performed by using a Gelcode System (Color Silver Stain Kit; Pierce, Rockford, IL). Preparative SDS-PAGE was performed on a vertical gel (16 × 10 × 0.5 cm) in a SE 400 system using a 2-well comb (Hoefer Scientific Instruments, San Francisco, CA). All analysis for DNA was performed with 0.8% agarose gels according to standard procedures (14).

Preparation of Anti-SEH F(ab')₂ Fragments. Anti-SEH sera were prepared by immunizing rabbits with gel slices excised from a preparative SDS-PAGE (16). Primary and booster injections consisted of ∼50–70 μg of protein. Rabbits were boosted twice at monthly intervals and bled 10 d thereafter. The titer was monitored by Western blotting.

Rabbit anti-SEH IgG, isolated from hyperimmune serum using immobilized protein A–Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ), was then affinity purified (17) and pepsin digested (Pierce) as outlined by the company. The purity of the F(ab')₂ was evaluated by SDS-PAGE.

Immunoblotting Analyses. Phage plaques developed on LB plates or polypeptides separated on SDS-PAGE were transferred to nitrocel-

lulose membranes (Schleicher & Schuell, Inc., Keene, NH) according to the procedure of Towbin et al. (18). After overnight blocking, the first antibody was applied and allowed to react for 1 h with shaking at room temperature. Alkaline phosphatase–conjugated secondary antibody (1:1,000) (Sigma Chemical Co.) was added and reactive bands were visualized as described by Blake et al. (19). SEA-E and TSST-1 toxins were obtained from Toxin Technology, Ltd. (Madison, WI). Mouse monoclonal IgG specific for SEA-E and TSST-1 were obtained from IGEN Inc. (Rockville, MD) and diluted 1:500 in blocking buffer before use.

Purification of SEH. Extracellular products were isolated from strain D4508, precipitated stepwise with 55 and 75% saturation of ammonium sulfate (4°C, overnight), and partially purified with DEAE-cellulose (Sigma Chemical Co.) columns (6 × 2.5 cm) as previously described (12). The DEAE fractions containing partially purified SEH were concentrated further with a Diaflo concentrator fitted with a PM10 filter (Amicon Corp., Danvers, MA), dialyzed against 25 mM Tris-HCl (pH 8.1), and applied to a fast-protein liquid chromatography (FPLC) column (Mono Q HR5/5; Pharmacia Fine Chemicals), equilibrated in the same buffer. The column was washed and protein eluted with a linear gradient from 0 to 500 mM NaCl. Fractions of 1 ml each were collected and analyzed by SDS-PAGE and Western blot. Fractions containing SEH were dialyzed against 50 mM ammonium bicarbonate, and lyophilized. After resuspension in 500 μl of this same buffer, the sample was applied to an FPLC Superose 12 column (Pharmacia Fine Chemicals) and fractions (1 ml each) were analyzed as described above for SEH. The purity of the protein was evaluated by SDS-PAGE followed by Coomassie blue and silver staining. The apparent molecular size of SEH was determined by SDS-PAGE with Rainbow Protein Molecular Markers (Amersham, Arlington Heights, IL). Protein concentration was determined by a microtiter BCA assay (Pierce). The purified SEH was stored at −20°C.

IEF. IEF was performed with a Rotorfor cell (Bio-Rad Laboratories, Richmond, CA). Briefly, ∼100 μg of the purified SEH protein was dialyzed against 5 mM NaCl and concentrated to 2 ml with a Diaflo concentrator fitted with a PM10 filter. This was added to 52 ml of 2% Bio-lyte ampholyte, pH 3–10 (Bio-Rad Laboratories) containing 20% glycerol, and applied to the Rotofor cell. Initial focusing was carried out at 12 W constant power for 4 h at 4°C. The pH values and the absorbance at 280 nm of each 2.5-ml fraction were determined. Aliquots of each fraction were analyzed on SDS-PAGE and Western blot. Fractions 7–9, having a pH range of 5.55 to 6.00, and containing the majority of SEH, were pooled, diluted up to 52 ml of double distilled H₂O containing 20% glycerol, and refocused for an additional 4 h.

Mass Spectrometry. The matrix-assisted laser desorption mass spectrometric analysis was performed at The Rockefeller University (Laboratory of Mass Spectrometry and Gas Phase Ion Chemistry) on a time-of-flight mass spectrometer (20).

NH₂-terminal Sequence. The purified SEH was subjected to automated Edman degradation on a sequenator (model 470 A; Applied Biosystems, Inc., Foster City, CA). All analytic procedures were performed by the Protein Sequence/Biopolymer Facility of The Rockefeller University.

Genomic DNA Cloning Procedures. Chromosomal DNA was isolated from strain D4508 as described (21) and a genomic DNA library was constructed in λgt11 (14). The unamplified library was plated with *E. coli* Y1090 cells and screened using immunoblots reacted with the affinity-purified anti-SEH F(ab')₂ which was absorbed with *E. coli*/phage lysate (Promega) before use.

Subcloning Procedure. The method of Struhl (22) was used to subclone DNA fragments from agarose gels. To clone the insert

into pHSG 399, the ligation products were transformed into strain XL1-Blue and selected on LB agar plates containing 30 μg/ml chloramphenicol. For TA cloning using vector pCR II, ligation products were transformed into strain INVαF' and selected by blue/white color on ampicillin-resistant LB plates containing X-Gal (1 mg/plate) (Boehringer Mannheim).

Oligonucleotide Synthesis and PCR Procedures. The oligonucleotide primers were synthesized on a DNA synthesizer (model 380B; Pharmacia Fine Chemicals). The PCR primers used for direct TA cloning were K3 (GTAATAATGCTAAAAACGAAAATAA) along with two primers of gt11 sequences. The PCR primers used to clone the complete coding region for the mature SEH were K4 (CCCTCGAGTTATACTTTTTTCTTAGTATATAG) and R3 (TCAAGCTTGGAAGATTTACACGATAAAAGT). 25 cycles of amplification (1 min at 94°C, 2 min at 50°C, and 1 min at 72°C) were performed in a thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT).

DNA Sequence Analysis. The recombinants pSEH1 and pSEH2 were used to determine the *seh* sequence. The DNA sequence was obtained using the method of Sanger et al. (23) with Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH). The *seh* sequence was submitted to EMBL/GenBank/DDBJ and has been assigned the accession number U11702. Hydropathy analysis of the deduced amino acid of SEH was performed using the DNA strider™ computer program employing the method of Hopp and Woods (24). Predictions of signal sequence and its cleavage sites as well as Shine-Dalgarno site were performed as described previously (25–27). The deduced amino acid sequence of SEH was used to search the translated GenBank database for greatest homology using the program Fasta (28). The alignment of SEH with related exotoxins was created using the Pileup program (GCG, Madison, WI).

Expression of SEH in T7 Promoter System. PCR was used to amplify *seh* from chromosomal DNA of strain D4508. The PCR product was then cloned into pET-17b creating pKR100 and transformed into strain BL21 (DE3) cells. Transformants were selected on carbenicillin plates. IPTG (Isopropylthio-β-D-galactoside)-induced cell lysates of positive clones were then analyzed for the expression of SEH by SDS-PAGE and Western blotting. A cell lysate containing the pET vector alone was used as a control.

Purification of the rSEH. BL21(DE3)(pLysS)(pKY100) cells were grown in LB broth at 32°C to an OD_{600} of 0.6 and T7 RNA polymerase expression was then induced by adding IPTG to a final concentration of 0.4 mM. After 30 min incubation, rifampicin (200 μg/ml) was added to the culture. 2 h later, cells were collected and lysed by freeze-thaw and briefly by sonication in the presence of 0.1% Triton X-100. Cell suspension was then centrifuged and supernatant was collected and used for further purification as described above for the purification of wild-type toxin.

Biologic Analysis of SEH in Rabbits. The toxic effect of the native or rSEH was evaluated in rabbits (29). New Zealand White rabbits >1 yr old were obtained from Hazleton Dutchland Labs, Inc. (Denver, PA). Purified SEH, suspended in pyrogen-free saline, was applied to Endotoxin Removing Gel (Pierce) before use. The SEH was delivered intravenously into the rabbits through the marginal ear vein at doses ranging from 40 to 150 μg/kg. Rabbits were monitored closely for clinical symptoms and death for 5 d. To evaluate the pyrogenic effects, rectal temperatures were measured and the course of temperature changes (temperature of injected rabbit minus that of normal control) was monitored at appropriate time points. Age- and sex-matched rabbits receiving isotonic saline served as normal controls. Student's *t*-test was used for statistical analyses and values of $p < 0.05$ were considered significant.

To study the biological effects of SEH in the presence of sublethal

doses of endotoxin, a method used for studying TSST-1 was adapted (30). Briefly, a dose of 5 μg/kg LPS (from *E. coli*; Difco Laboratories, Inc., Detroit, MI) was injected intravenously into rabbits 4 h after SEH injection. Rabbits receiving 5 μg/kg LPS alone served as the controls.

For protection studies, SEH was mixed with anti-SEH IgG at a 1.5:1 molar ratio, incubated at 37°C for 2 h, and centrifuged at 12,000 *g* for 5 min. The supernatant fluid was then used for i.v. injection. The mixture of SEH and normal rabbit IgG, processed the same way, was used as the control. The rabbits were observed over a 72-h period for clinical signs of illness and death.

Histopathologic Studies. Necropsies were performed on selected animals that died after injection of SEH. Tissues were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned at 8–10 μm, stained with hematoxylin and eosin, and examined via light microscopy. Photomicrographs were taken at magnifications of 10 and 25. All procedures were performed in the Laboratory Animal Research Center at The Rockefeller University.

Results

Purification of SEH Protein. SEH could be concentrated from the total extracellular products of strain D4508 by ammonium sulfate precipitation of between 55 and 75% saturation. The SEH protein in this concentrate could be eluted with 100 mM NaCl from a column of DEAE cellulose. Further purification on a MonoQ ion exchange and a Superose 12 molecular sieve resulted in a single homogenous protein migrating just below 30 kD on a SDS-PAGE gel (Fig. 1, *a–c*). A similar migration pattern was seen under reducing and nonreducing conditions.

Biochemical Properties. The apparent molecular mass of SEH could be readily distinguished from that of other staphylococcal exotoxins (Fig. 2 *A*). Analysis of the purified SEH by laser desorption mass spectrometry revealed a monomer of 25.145 kD. Isoelectric focusing of purified SEH indicated of pI value of 5.65. NH_2-terminal amino acid sequence analysis of the purified SEH revealed a single amino acid at all 26 positions with the following sequence: E D L H D K S E L T D L A L A N A Y G Q Y N H P F I. The result further verified the homogeneity of the preparation.

Immunological Properties. As shown in Fig. 2 *B*, anti-SEH F(ab')₂ antibody did not show any detectable reactivity with SEA, B, C, D, E or TSST-1 (lanes *b–g*). Furthermore, mAbs specific for enterotoxins A, B, C, and TSST-1 showed no
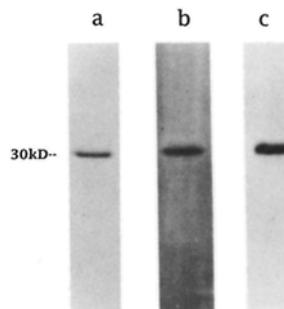

Figure 1. SDS-PAGE and Western blot analysis of the purified SEH protein after FPLC-Superose chromatography. (Lane *a*) Gel stained with Coomassie blue. (Lane *b*) A positive image of a photographed silver stained gel; after silver stain, a negative image is seen in the position of SEH, indicating that the protein does not bind to silver. (Lane *c*) A Western blot of SEH reacted with rabbit anti-SEH IgG. The mass of carbonic anhydrase (*30 kD*) molecular weight standard is indicated.
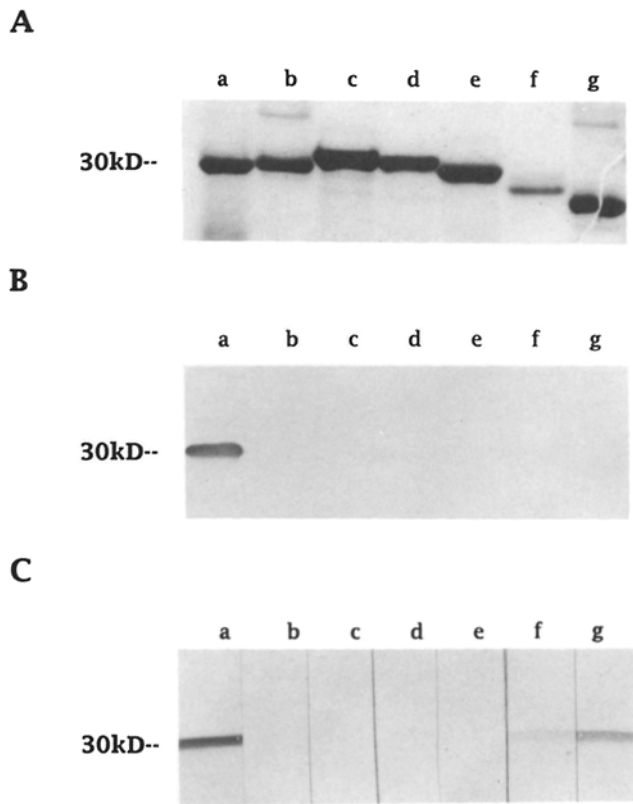
A

B

C

Figure 2. SDS-PAGE and Western blot analysis of SEH and other known staphylococcal toxins. (*A*) SDS-PAGE stained with Coomassie blue: lanes *a–g* are SEH, A, B, C, D, E, and TSST-1, respectively. (*B*) Western blot of toxins SEH, A, B, C, D, E, and TSST-1 (lanes *a–g*, respectively) reacted with rabbit anti-SEH IgG. (*C*) SEH toxin, separated on a toothless gel, was transferred to nitrocellulose and reacted with rabbit anti-SEH IgG (lane *a*), mouse mAb specific for SEA, B, C, TSST-1, SEE, and SED (lanes *b–g*), respectively.

cross-reactivity with SEH (Fig. 2 *C*, lanes *b–e*). However, mAbs to SED and SEE did exhibit binding to SEH (Fig. 2, lanes *f* and *g*).

Cloning and Expression of the Gene Encoding SEH. Genetic determinants coding for the mature SEH were isolated from a genomic library of strain D4508 using antibodies specific for wild-type SEH. Of 3 × 10⁶ independent gt11 phage clones screened, one positive clone was isolated and called gSEH.1. Immunoblot analysis of the crude phage lysates of this clone showed that it encoded a 25-kD protein corresponding in size and antibody reactivity to the wild-type SEH (Fig. 3, lanes *a* and *c*). This protein is not present in phage lysates prepared from the vector of λgt11 alone (Fig. 3, lane *b*).

The Structure of the seh Gene. The genomic library was constructed using the EcoRI cloning sites of λgt11. However, digestion of gSEH.1 phage DNA with EcoRI revealed that one EcoRI site had been lost during the cloning process. Accordingly, SacI, which cleaves λgt11 1 kb away from the cloning site, was used in subcloning procedures. A 1.7 kb SacI-EcoRI fragment was subcloned into plasmid pHSG399, and the resulting plasmid was named pSEH1. DNA sequencing of pSEH1 indicated the 0.7-kb insert contained

nearly the entire structural gene for SEH, except for a small portion at the 5′ end (Fig. 4 *A*).

To obtain the 5′ end of *seh*, a HpaI-EcoRI fragment from pSEH1 was used as a probe to screen the original λgt11 library. 21 positive phage clones were isolated. PCR was used to amplify phage DNA from each clone using primers of both K3 and λgt11 sequence. PCR products from amplification of positive clones were subcloned into the pCR II vector. One recombinant plasmid, pSEH2, contained a 1.4-kb insert. DNA sequencing of pSEH2 revealed the complete 5′ end of *seh*. A restriction map of the complete *seh* gene is shown in Fig. 4 *A*.

The complete nucleotide sequence of *seh* and its predicted amino acid sequence are shown in Fig. 5. The *seh* gene contains a long open reading frame of 726 nucleotides starting from the initiation codon ATG at nucleotide positions 208–210 and ending at the termination codon TAA at positions 931–933. This open reading frame predicts a protein of 241 amino acids in length. The signal peptide is 24 amino acids in length, consisting of 21 nonpolar hydrophobic and 3 basic amino acids, and shares significant similarities with known signal peptide sequences (25). The hydropathy profile of the deduced amino acid sequence revealed the hydrophilic nature of the protein and also indicated the presence of a hydrophobic NH₂ terminus consistent with a signal sequence (Fig. 4 *B*). The proposed processing site for the mature protein is located before the glutamic acid encoded at nucleotide position 280 (Fig. 5). An NH₂-terminal amino acid sequence obtained from purified SEH matched the amino acid sequence deduced from the *seh* nucleotide sequence beginning at position 280 (Fig. 5). This suggests that the first 24 amino acids of SEH are a signal sequence which is removed to produce a mature protein of 25.21 kD, and is consistent with that obtained by mass spectrometry of purified SEH (25.145 kD). Proximal to the 5′ end of the open reading frame is a sequence of GAGGAG, which resembles the consensus Shine-Delgarno ribosome binding site (27).

Western blot analysis of crude cell lysate from *E. coli* BL21(DE3)(pLYS)(pSEH1), revealed a single protein band with identical molecular weight and immunogenicity to the native SEH (Fig. 6). Comparison of the amino acid composition of SEH with those of other staphylococcal toxins re-
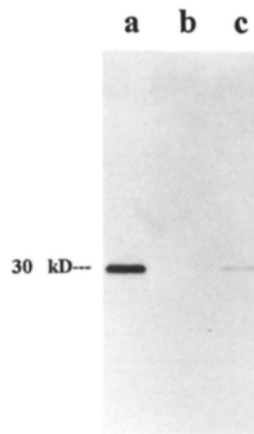

Figure 3. Western blotting of native and recombinant SEH. (Lane *a*) SEH purified from *S. aureus* strain D4508; (lane *b*) *E. coli* strain Y1089 phage lysate containing λgt11; and (lane *c*) *E. coli* strain Y1089 phage lysate containing λgt11 with an *seh* insert (gSEH.1). Proteins transferred to nitrocellulose were reacted with antibody specific for wild-type SEH.
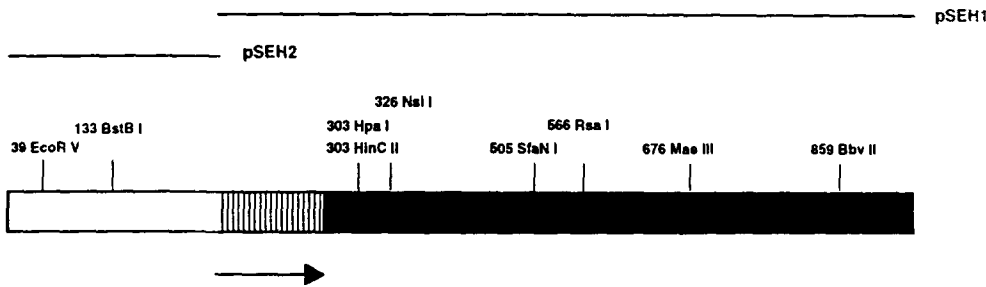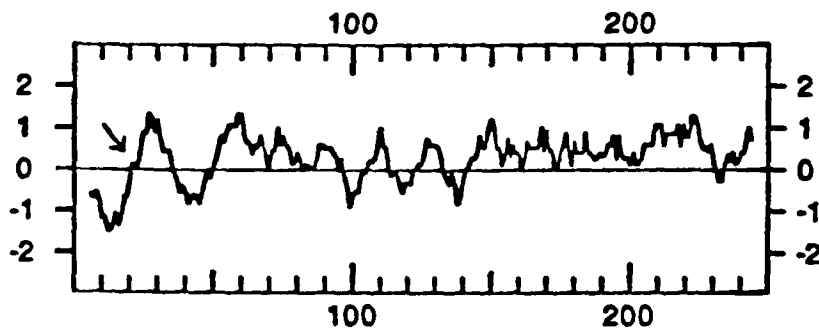
A

pSEH1

pSEH2

326 Nsi I
303 Hpa I
133 BstB I
39 EcoR V
303 HinC II
505 SfaN I
666 Rsa I
676 Mae III
859 Bbv II

B

100 200

100 200

Figure 4. (A) Schematic representations and a restriction map of lambda clones encoding portions of SEH. Two phage DNA clones, gSEH.1, and gSEH.2, encoding different regions of *seh*, are shown. To unique restriction enzyme cleavage sites of these clones are also indicated. (*Dark solid box*) The SEH open reading frame. (*Striped box*) The signal sequence. (*Arrow*) The orientation of the *seh* reading frame. (B) Hydropathy profile of the SEH amino acid sequence. The hydropathy plot was obtained using the DNA Strider™ computer program employing the method of Hopp and Woods (24). The numbers on the horizontal axes indicate the positions of amino acid residues. (*Arrow*) The NH₂-terminal end of SEH.

vealed significant similarities, particularly with those of the enterotoxin family (1).

Sequence Homology of SEH with Known Proteins. Using the deduced amino acid sequences of the proposed mature SEH, sequence homology with other known proteins was examined using the translated GenBank database. The proteins with the highest similarity scores of SEH were within the staphylococcal enterotoxin family, including SEA through SEE and the *Streptococcus pyogenes* pyrogenic exotoxins A and C. SEH exhibited 38% amino acid identity with SEE, 37% with SEA, 37% with SED, 33% with SEB, and 27% with SEC. Thus, SEH appears to belong to the staphylococcal enterotoxin family and is most closely related to the enterotoxins SEA, SED, and SEE (Fig. 7). Furthermore, if conservation of the chemical characteristics of amino acids is taken into account, an even closer relationship is observed between SEH and these enterotoxins.

Lethal Toxicity of Wild-type SEH in Rabbits. The toxic effect of SEH was evaluated in rabbits given a single i.v. dose varying from 40 to 150 µg/kg. All rabbits receiving 150 µg/kg of the purified protein developed acute symptoms that were generally characterized by high fever, hyperemia, lethargy, conjunctival suffusion, and diarrhea (Table 1). Two of three receiving this dose succumbed to lethal toxic shock, one within 24 h and the other within 48 h. The third rabbit suffered severe shock but recovered in 72 h. Rabbits given either 40, 60, or 80 µg/kg of SEH showed acute illness with high fever

and hyperemia, with two of the three having diarrhea. All rabbits receiving these doses recovered after 48–72 h (Table 1).

High fever was a consistent and significant feature of the illness in all animals receiving SEH. The first sign of fever occurred as early as 1 h after the injection, and temperature increased rapidly to a mean value of 3.0°F above normal (p <0.05) within 4 h (Fig. 8). The maximum increase of 3.5°F was observed between 18 and 24 h. Up to 36 h after injection, the temperature remained elevated above control and there was no significant difference in the kinetics of the fever induced by any given dose of the SEH. The temperature of the rabbits receiving 40–80 µg/kg decreased rapidly between 36 and 48 h. Only one rabbit receiving 150 µg/kg was still alive at 48 h and its temperature remained high (Fig. 8). The temperature changes were significantly different between the two dosage groups at 48 h (p <0.05).

The ability of SEH to induce a TSS-like illness in rabbits was confirmed by protection studies. When SEH was allowed to react with its specific antibody before injection, the toxicity of the protein was inhibited. All rabbits challenged in this way were protected from symptoms, including fever, although a small elevation of temperature was observed in these rabbits in the first few hours after injection (solid squares in Fig. 8).

Histopathological examination of tissue samples from two rabbits injected with 150 µg/kg of purified SEH revealed marked pyloric muscular hypertrophy, multisystemic visceral

Figure 5. The complete annotated nucleotide sequence of *seh*, upstream DNA and the deduced amino acid sequence of SEH. Numbers above the sequences refer to the nucleotide positions. The potential ribosome binding site (*RBS*) is identified at nucleotides 195–200. NH₂-terminal amino acid sequences identical to those of the native protein are underlined twice. The proposed signal sequence processing site is underlined once. These sequence data are available from EMBL/GenBank/DDBJ under accession number U11702.

Figure 6. Analysis of SEH expression in a T7 promoter system. SEH preparations separated by SDS-PAGE were either stained with Coomassie blue (lanes *a*, *c*, and *e*) or Western blotted with antibody specific for wild-type SEH (lanes *b*, *d*, and *f*). (Lanes *a* and *b*) Lysates of *E. coli* strain BL-21(DE3) carrying pET-17b alone; (lanes *c* and *d*) lysates of *E. coli* strain BL-21(DE3) carrying pET-17b with a *seh* insert. (Lanes *e* and *f*) rSEH purified from a lysate of *E. coli* strain BL-21(DE3) carrying pET-17b with a *seh* insert.
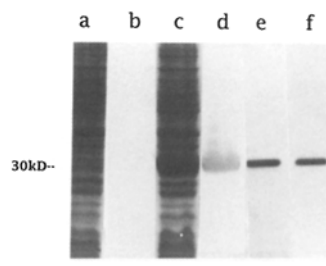
congestion, marked hyperemia, and mild multifocal fibrinoid degeneration of small vessel walls. Perivascular hemorrhage, especially in the adrenal gland, lungs, meninges, and cardiac papillary muscles, was also noticed (data not shown). Multifocal coalescing edema or atelectasis and leukocyte sequestration were observed in the lung, interstitial nephritis and tubular degeneration and regeneration was seen in the kidney, and centrilobular vacuolation was visualized in liver tissue. These histopathological features are consistent with those found in septic shock (31).

Enhancement of Toxicity with LPS. Whereas the amount of SEH alone required to induce a TSS-like disease in rabbits was quite high (see Table 1), numerous reports (for a review see reference 30) indicated that staphylococcal toxins like TSST-1 markedly enhanced LPS-induced toxic shock and death in rabbits. Accordingly, when injection with SEH was followed 4 h later by i.v. administration of LPS (5 μg/kg), the dose of SEH needed to induce toxic shock and death was markedly reduced (Table 1). LPS alone at 5 μg/kg did not result in any observable symptoms in the rabbits. When the injection of a mixture of SEH and anti-SEH rabbit IgG was followed by LPS injection, the capacity of LPS to enhance the activity of SEH was blocked. In contrast, two rabbits receiving the mixture of SEH plus normal rabbit IgG and LPS succumbed to lethal shock within 48 h (Table 1). These observations indicate that a relatively low dose of LPS is sufficient to increase the activity of SEH leading to death.

Toxicity of the rSEH in Rabbits. To determine if rSEH retained the toxic characteristics of the native molecule, purified rSEH was injected into rabbits. As shown in Table 2, purified

Figure 7. Comparison of the primary amino acid sequence of SEH and staphylococcal enterotoxins SEA, SEE, and SED. The alignment was created using the Pileup program (GCG). Residues that are identical with SEH are boxed.

Table 1. *Biological Effects of the Purified Wild-type SEH on Rabbits*

SEH*	LPS‡	Number of Rabbits			
		Fever§	Hyperemia	Diarrhea	Dead
μg/kg	μg/kg				
40–80‖	0	3/3	3/3	2/3	0/3
150	0	3/3	3/3	3/3	2/3
40–80‖	5	3/3	3/3	2/3	3/3
150	5	2/2	2/2	1/2	2/2
50 + NR IgG¶	5	2/2	2/2	2/2	2/2
50 + anti-SEH¶	5	0/3**	0/3	0/3	0/3
0	5	0/2	0/2	0/2	0/2

* Rabbits were injected intravenously with SEH at doses ranging from 40 to 150 μg/kg body weight.

‡ In some experiments, endotoxin (LPS) was given intravenously at a dose of 5 μg/kg body weight 4 h after injections of SEH.

§ Temperatures were determined within 4 h after SEH administration.

‖ Each of three animals was injected with either 40, 60, or 80 μg/kg SEH.

¶ Either normal rabbit IgG (NR IgG) or anti-SEH IgG was incubated with SEH at a 1.5:1 molar ratio at 37°C for 2 h prior to injection of rabbits.

** Temperature elevation was observed in all three rabbits injected but the mean value was <1°F.

rSEH induced a TSS-like illness in rabbits when delivered intravenously. Like the native SEH, LPS also enhanced the activity of SEH. Toxicity of rSEH was blocked by antibody specific for SEH but not by normal rabbit antiserum.

Table 2. *Biological Effects of the Purified Recombinant SEH on Rabbits*

rSEH*	LPS‡	Number of Rabbits			
		Fever§	Hyperemia	Diarrhea	Dead
μg/kg	μg/kg				
50	0	2/2	2/2	0/2	0/2
50	5	2/2	2/2	0/2	2/2
50 + NR IgG‖	5	1/1	1/1	0/1	1/1
50 + anti-SEH‖	5	0/1¶	1/1	0/1	0/1
0	5	ND	0/2	0/2	0/2

* Rabbits were injected intravenously with rSEH at a dose of 50 μg/kg body weight.

‡ In some experiments, endotoxin (LPS) was given intravenously at a dose of 5 μg/kg body weight 4 h after injections of SEH.

§ Temperatures were determined within 4 h after SEH administration.

‖ Either normal rabbit IgG (NR IgG) or anti-SEH IgG was incubated with recombinant SEH at a 1.5:1 molar ratio at 37°C for 2 h before injection of rabbits.

¶ Temperature elevation was observed but the mean value was <1°F.

Discussion

It is now clear that staphylococcal infections can cause TSS in a clinical setting in which women, children, and men are equally involved. The proportion of nonmenstrual to menstrual cases, however, is increasing. Mortality with nonmenstrually related TSS is approximately three times higher than menstrually related cases (31). The increased mortality has
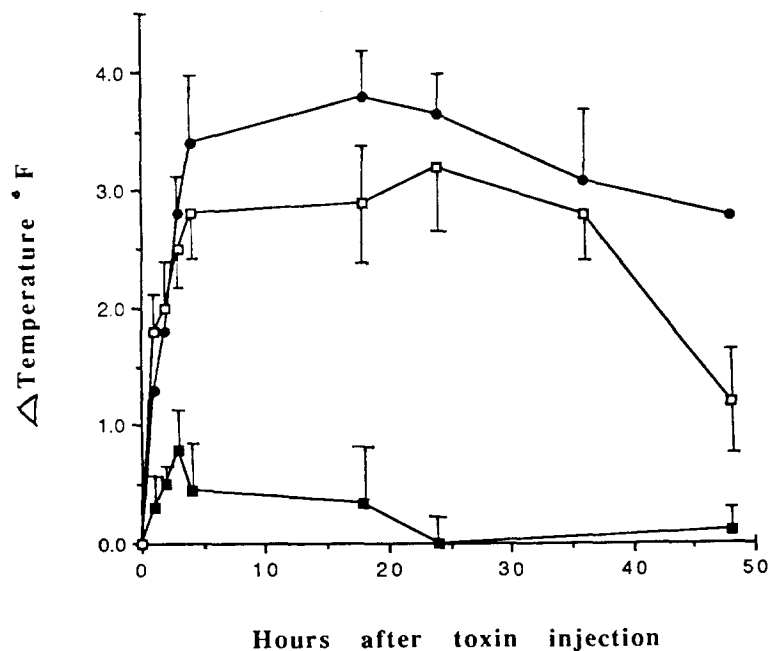
Figure 8. Pyrogenic effects of SEH in rabbits. Mean changes in rectal temperatures among three groups of rabbits injected with different amounts of SEH. Group 1: each of three rabbits receiving either 40, 60, or 80 μg/kg toxin (□). Group 2: each of three rabbits receiving 150 μg/kg toxin (●). Group 3: each of three rabbits receiving a mixture of 50 μg/kg toxin and anti-SEH IgG (■). Two of the group 2 rabbits died, one before 24 h, and the other between 24 and 48 h, after i.v. injection of SEH. The means and standard errors of deviation for each group are indicated at each time point.

been attributed to either the production of TSST-1 or other toxins or to delayed diagnosis (31). Observations that staphylococcal strains isolated from nonmenstrual TSS often do not secrete or even contain the gene for TSST-1 (4) prompted searches for other putative toxins. Controversial results, however, have been reported. Scott et al. (32), for example, noted that a TSS-like syndrome in rabbits was associated with a toxin called TSST-2 from staphylococcal strain D4508, the same nonmenstrual TSS clinical isolate used in our studies. McCollister et al. (4) demonstrated that enterotoxin A is the major TSS-associated toxin produced by the D4508 strain and that TSST-2 was not a required factor. Our earlier report suggesting a new exotoxin from D4508 capable of causing a TSS-like syndrome in rabbits, raised questions as to the true nature of this toxin (4, 12). The present work was therefore undertaken in an attempt to resolve this issue.

By using a multistep procedure including anion exchange and molecular sieve chromatography on FPLC, SEH was purified to homogeneity. Laser desorption mass spectrometry analysis of the purified SEH revealed a monomer with a molecular mass of 25.145 kD. Isoelectric focusing revealed that this protein has a relatively acidic pI of 5.65. These biochemical properties clearly distinguish SEH from other known staphylococcal exotoxins (1). Immunoblotting analyses revealed that the antibody specific for SEH reacted only with its corresponding antigen whereas SEH did exhibit binding to mAbs specific for SEE and SED, indicating that SEH contains unique as well as common antigenic epitopes shared by some other enterotoxins (Fig. 2).

By cloning and sequencing the genetic determinant encoding SEH and by comparing the deduced amino acid sequence with that in the translated GenBank, SEH was shown to have significant similarity with staphylococcal enterotoxin SEA-E and was more closely related to the SEA, SED, and SEE group of enterotoxins. We thus believe that SEH might be a new member of the enterotoxin gene family.

SEH also shares many biological activities with the other known staphylococcal exotoxins. When administered intravenously into rabbits, highly purified native as well as rSEH induced an acute clinical illness characterized by high fever, diarrhea, and histopathological changes in multiorgan systems. These symptoms reflected the pyrogenic and toxic properties of SEH toxin, many of which are salient biological features of the staphylococcal family of enterotoxins (30). The histopathologic changes are consistent with TSS observed in humans and are similar to those seen in animal studies using TSST-1 (33). Since infection of patients with both gram-positive and gram-negative bacteria commonly occurs, and since endotoxemia has been seen in some patients with TSS (31), we evaluated the effects of SEH on enhancing the susceptibility of rabbits to lethal endotoxic shock in a synergistic manner. As noted in Tables 1 and 2, the result was quite striking and the amount of SEH toxin required to induce lethal toxic shock in the presence of LPS was markedly diminished when compared to administration of toxin alone.

Whereas the close correlation between the introduction of a TSS-like syndrome in rabbit with SEH did suggest the possibility of a cause-and-effect relation, the mechanism by which the toxicity occurs is not clear. Preliminary experiments (30, 34, 35) indicate that SEH stimulates the proliferation of human peripheral mononuclear cells and highly purified T cells in a manner similar to that observed for enterotoxins and TSST-1, suggesting that SEH, like other staphylococcal enterotoxins, might also be a superantigen (data not shown). Comparison of the SEH amino acid sequence with other enterotoxins revealed that SEH contains many identical and chemically conserved amino acid residues (Fig. 7).

The availability of the recombinant *seh* clone will enable us to construct isogeneic mutants that are deficient in the production of this toxin to examine its role in the pathogenesis of the disease (36), which might have value for the early diagnosis and management of human TSS in which SEH plays a role. In addition, the ability to completely block the localized effects of SEH with anti-SEH antibody may provide a new therapeutic method for blocking some of the effects of the toxin in a clinical setting.

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References

1. Bergdoll, M.S. 1983. Enterotoxins. *In* Staphylococci and Staphylococcal Infections. vol 2: The Organism in Vivo and In Vitro. C.S.F. Easmon and C. Adlam, editors. Academic Press, London. 559–598.

2. Todd, J., M. Fishaut, F. Kapral, and T. Welch. 1978. Toxic-shock syndrome associated with phage-group-1 staphylococci. *Lancet.* 2:1116.

3. Jacobson, J.A., E. Kasworm, and J.A. Daly. 1989. Risk of

developing toxic shock syndrome associated with toxic shock syndrome toxin 1 following nongenital Staphylococcal infection. *Rev. Infect. Dis.* 11(Suppl. 1):S8.

4. McCollister, B.D., B.M. Kreiswirth, R.P. Novick, and P.M. Schlievert. 1990. Production of toxic shock syndrome-like illness in rabbits by *Staphylococcus aureus* D4508: association with enterotoxin A. *Infect. Immun.* 58:2067.

5. Schlievert, P.M. 1986. Staphylococcal enterotoxin B and toxic shock syndrome toxin 1 are significantly associated with non-menstrual TSS [letter]. *Lancet.* 1:1149.

6. Marrack, P., and J. Kappler. 1990. The staphylococcal enterotoxins and their relatives. *Science (Wash. DC).* 248:705.

7. Karp, D.R., R.N. Jenkins, and E.O. Long. 1992. Distinct binding sites on HLA-DR for invariant chain and staphylococcal enterotoxins. *Proc. Natl. Acad. Sci. USA.* 89:9657.

8. Herman, A., N. Labrecque, J. Thibodeau, P. Marrack, J.W. Kappler, and R.-P. Sekaly. 1991. Identification of the staphylococcal enterotoxin A superantigen binding site in the $\beta1$ domain of the human histocompatibility antigen HLA-DR. *Proc. Natl. Acad. Sci. USA.* 88:9954.

9. White, J., A. Herman, A.M. Pullen, R. Kubo, J.W. Kappler, and P. Marrack. 1989. The Vβ-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell.* 56:27.

10. Newell K.A., J.D.I. Ellenhorn, D.S. Bruce, and J.A. Bluestone. 1991. *In vivo* T-cell activation by staphylococcal enterotoxin B prevents outgrowth of a malignant tumor. *Proc. Natl. Acad. Sci. USA.* 88:1074.

11. Johnson, H.M., J.K. Russel, and C.H. Pontzer. 1992. Superantigens in human disease. *Sci. Am.* 266:92.

12. Hall, W.W., S. Blander, R. Kakani, V.A. Fischetti, and J.B. Zabriskie. 1989. Identification of a new toxin from a strain of *Staphylococcus aureus* isolated from a patient with non-menstrual toxic shock syndrome. *Rev. Infect. Dis.* 11(Suppl. 1):S130.

13. Takeshita, S., M. Sato, M. Toba, W. Masahashi, and T. Hashimoto-Gotoh. 1987. High-copy-number and low-copy-number plasmid vectors for *lacZ*-complementation and chloramphenicol- or kanamycin-resistance selection. *Gene.* 61:63.

14. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

15. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.

16. Harlow, E., and D. Lane. 1988. Antibodies, A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 726 pp.

17. Olmsted, J.B. 1981. Affinity purification of antibodies from Diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* 256:11955.

18. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350.

19. 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.

20. Beavis, R.C., and B.T. Chait. 1990. Rapid, sensitive analysis of protein mixtures by mass spectrometry. *Proc. Natl. Acad. Sci. USA.* 87:6873.

21. Cheung, A.L., J.M. Koomey, C.A. Butler, S.J. Projan, and V.A. Fischetti. 1992. Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr. Proc. Natl. Acad. Sci. USA.* 89:6462.

22. Struhl, K. 1985. A rapid method for creating recombinant DNA molecules. *BioTechniques.* 3:452.

23. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463.

24. Hopp, T.P., and K.R. Woods. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA.* 78:3824.

25. Oliver, D. 1985. Protein secretion in *Escherichia coli. Annu. Rev. Microbiol.* 39:615.

26. von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14:4683.

27. Shine, J., and L. Dalgarno. 1975. Determinant of cistron specificity in bacterial ribosomes. *Nature (Lond.).* 254:34.

28. Pearson, W.R., and D.J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA.* 85:2444.

29. 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.

30. 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.

31. Bone, R.C., and M.J. Barkoviak. 1992. Update on toxic shock: new causes, novel clinical settings. *Journal of Critical Illness.* 7:1032.

32. Scott, D.F., J.M. Kling, and G.K. Best. 1986. Immunological protection of rabbits infected with *Staphylococcus aureus* isolates from patients with toxic shock syndrome. *Infect. Immun.* 53:441.

33. de Azavedo, J.C.S. 1989. Animal models for toxic shock syndrome: overview. *Rev. Infect. Dis.* 11(Suppl. 1):S205.

34. Langford, M.P., G.J. Stanton, and H.M. Johnson. 1987. Biological effects of staphylococcal enterotoxin A on human peripheral lymphocytes. *Infect. Immun.* 22:62.

35. 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.

36. 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.