Characterization of a Novel Staphylococcal Enterotoxin-like Superantigen, a Member of the Group V Subfamily of Pyrogenic Toxins†

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ABSTRACT: *Staphylococcus aureus* is an important human pathogen, causing a variety of diseases. Major virulence factors of this organism include staphylococcal enterotoxins (SEs) that cause food poisoning and toxic shock syndrome. Our study identified a novel enterotoxin-like protein that is a member of the new subfamily (group V) of pyrogenic toxin superantigens (PTSAgs) and examined its biochemical and immunobiological properties. The gene encoding the SE-like protein is directly 5′ of another recently identified PTSAg, SEK. The SE-like protein had a molecular weight of 26000 and an experimentally determined isoelectric point between 7.5 and 8.0. We demonstrated that the PTSAg had many of the biological activities associated with SEs, including superantigenicity, pyrogenicity, and ability to enhance endotoxin shock, but lacked both lethality in rabbits when administered in subcutaneous miniosmotic pumps and emetic activity in monkeys. Recombinant protein stimulated human CD4 and CD8 T cells in a T cell receptor variable region, β chain (TCRVβ) specific manner. T cells bearing TCRVβ 2, 5.1, and 21.3 were significantly stimulated.

Staphylococcus aureus is an important human pathogen in part because of production of exotoxins (*1*−*3*). The organism is a significant cause of both hospital and community acquired infections. The spectrum of staphylococcal illnesses ranges from relatively benign furuncles to life-threatening toxic shock syndrome (TSS).[1]

The major exotoxins secreted by *S. aureus* include several hemolysins (α, β, γ, δ), leukocidin, exfoliative toxins A and B, and the large family of pyrogenic toxin superantigens (PTSAgs) (*1*−*3*). These latter toxins include toxic shock syndrome toxin 1 (TSST-1) (*4*, *5*), staphylococcal enterotoxins (SEs) A−P excluding F (*1*−*3*), and streptococcal pyrogenic exotoxins (SPEs A−C, F−J, streptococcal mitogenic exotoxin Z, and streptococcal superantigen) (*2*, *3*, *6*−*9*). Recent reports have described enterotoxin genes contained on the termini of pathogenicity islands (*2*, *10*), including our description of a new enterotoxin-like gene *sek* (*11*).

Numerous studies have shown that PTSAgs are important determinants for TSS and food poisoning (*2*, *12*). Todd and co-workers first recognized *S. aureus* as the etiologic agent of TSS (*13*). Subsequent work by Schlievert et al. (*4*) and Bergdoll and colleagues (*5*) identified TSST-1 as the major toxin associated with this illness whether menstrual or nonmenstrual. Later work by Schlievert and others established that SEs are also important causes of nonmenstrual TSS (*14*). The SEs, particularly SEA and SED and to a lesser extent SEB and SEC, are also common causes of staphylococcal food poisoning (*12*, *15*, *16*).

Crystallographic studies of the PTSAgs have shown that these molecules share highly similar three-dimensional structures (*1*, *2*). The toxins have a short N-terminal α helix that leads into a β barrel structure (also known as domain B or OB fold). The OB fold is connected to a C-terminal wall of β strands with a central diagonal α helix, forming domain A (β grasp fold). All PTSAgs have these features in common, but some differ in the size of loops connecting the secondary structural elements. The most notable of these is a disulfide bond found in the β4−β5 loop, a feature found in classical

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[1] Abbreviations: SEs, staphylococcal enterotoxins; PTSAgs, pyrogenic toxin superantigens; TCRVβ, T cell receptor variable region β chain; TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin 1; SPEs, streptococcal pyrogenic exotoxins; MHC, major histocompatibility complex; rSEQ, recombinant staphylococcal enterotoxin Q; PBS, phosphate-buffered saline; HBSS, Hank's balanced salt solution; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; APC, allophycocyanin; PE, phycoerythrin; PerCP, streptavidin−perdinin−chlorophyll; LPS, lipopolysaccharide; EU, emetic units; SaPI3, *Staphylococcus aureus* pathogenicity island 3; PBMC, peripheral blood mononuclear cell.

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SEs (SEs A−E) and SPE A (*12*). This loop is thought to be important for emetic activity in SEs, based on studies of mutants (*11*, *17*). Recently, SEs I and K have been identified which lack the disulfide bond in the $\beta4-\beta5$ loop; these toxins were shown to be superantigenic, but the emetic activity of SEI was significantly reduced in magnitude (*11*, *18*). SEK was not tested for emetic activity.

Overall, PTSAgs share numerous biological activities, including superantigenicity, pyrogenicity, capacity to enhance endotoxin shock, and lethality when administered in subcutaneous miniosmotic pumps (*1*, *2*, *19*). PTSAgs bind to the variable region of the β chain of certain T cell receptors (TCRVβ) and to relatively invariant regions of major histocompatibility complex (MHC) class II molecules (*20*, *21*). This mode of binding the TCR is much less specific than the typical TCR−peptide−MHC complex that is required for T cell activation by antigenic peptides. Depending on the PTSAg, up to 50% of the host T cells may be activated, resulting in massive cytokine release, with subsequent induction of capillary leak, leading to hypotension (*22*, *23*). The other biological activities, pyrogenicity, endotoxin enhancement, and lethality when administered in miniosmotic pumps, also depend on cytokine release (*1*). Among PTSAgs only SEs have emetic activity, and this activity has been clearly separated from superantigenicity (*17*, *24*, *25*).

This study was undertaken to purify and characterize a new toxin as a member of the group V subfamily of PTSAgs. We showed that a functional toxin was encoded by the identified open reading frame. We demonstrated that the recombinant protein functioned as a superantigen and was lethal in the endotoxin enhancement model of TSS but was not emetic and not lethal in the miniosmotic pump model of TSS.

MATERIALS AND METHODS

Cloning and Sequencing. The gene (*seq)* for the enterotoxin-like protein, which we have provisionally designated SEQ, was cloned from *S. aureus* nonmenstrual TSS isolate strain MN NJ. (Upon examination of biological activities of SEQ in this work, the protein was shown to be nonemetic and, thus, lacking the defining property of SEs. It may, therefore, be inappropriate to refer to the protein as an SE. However, the standard convention thus far has been to refer to proteins with sequence similarity to SEs as enterotoxins. We recognize that in the future it may be necessary to change the provisional name of SEQ.) PCR primers including several hundred additional nucleotides at both ends of the structural gene were included in the original clone. The primers used for this cloning have the following sequence: 5′GTGATGGGAAAAGTTGTATTG, 3′AGGGTAGGCGGGC. The PCR product was electrophoresed in 1% agarose, and the appropriate size band was excised from the gel. DNA was purified from the agarose using the Geneclean II kit (Bio101, La Jolla, CA) and cloned into the TA vector pGEM T-easy (Promega, Madison, WI), resulting in the plasmid pPMO021. This clone was transformed into *Escherichia coli* XL-1 blue, and the insertion of the proper length restriction fragment was verified by plasmid purification with use of the Qiagen spin miniprep kit (Qiagen, Valencia, CA) and digestion with *Eco*RI, which cuts on either side of the TA insertion site. The DNA sequence of *seq* was determined by automated sequencing (Advanced Genetic Analysis Center, St. Paul, MN). Further constructs were made using this plasmid as a template. *seq* was excised from pPMO021 after digestion with *Eco*RI and subcloned into pCE104 (*26*), forming pPMO022. To examine the biochemical and biological properties of SEQ, a signal peptide deletion mutant of *seq* was cloned into pET28b by PCR amplification of the pCE104 (pPMO022) insert. *Nco*I and *Bam*HI restriction sites were included in the primers for in-frame insertion of the signal peptide deletion mutant into the pET28b multiple cloning site. The sequence of the primers was as follows: 5′ *Nco*I, CCCCCATGGATGTAGGGGTAATCAACCTTAG; 3′ *Bam*HI, GGGGGATCCTTATTCAGTCTTCTCATATGAAATCTC. Transformation of the resultant plasmid pPMO023 into *E. coli* XL-1 blue was followed by purification of the plasmid and verification of the construct by restriction digestion with *Nco*I and *Bam*HI. After verification, the plasmid was introduced into *E. coli* BL-21 DE3 for expression using the p_{tac} system. The deleted signal peptide of SEQ was replaced with an N-terminal methionine. The determined N-terminal sequence of recombinant SEQ (rSEQ) was MDVGVINLRN.

Expression and Purification. The pET28b clone containing *seq* (pPMO023) in BL-21 DE3 was grown to early logarithmic phase in LB medium containing kanamycin (50 μg/mL), at 37 °C with shaking, and then induced with 200 mM IPTG. (At the same time, 1 mL amounts of early log phase culture in 15% glycerol were stored at −70 °C for use in subsequent experiments.) rSEQ was contained in inclusion bodies within the BL-21 DE3 cells. To detect the protein in small samples, the cultures were centrifuged at 10000*g* for 10 min and pellets resuspended to $^1/_{40}$th initial volume in 6 M urea. The urea suspensions were then placed in a boiling water bath for 5 min to lyse the cells and then centrifuged to remove cell debris (10000*g*, 10 min). The extracted proteins were examined by SDS−PAGE (15% gels) using the method of Laemmli (*27*). Proteins were stained with Coomassie brilliant blue R250. Induction of the expression system was verified by the presence of a prominent band of the expected size of rSEQ in the extract. Subsequent growth, expression, and purification of rSEQ were done using the stored frozen aliquots of the original culture.

Large-scale production of rSEQ was performed analogously to the above protocol. Beef heart medium (1200 mL flasks) containing 1% glucose phosphate buffer and 50 μg/mL kanamycin was inoculated with 200 μL of the frozen expression clone (*11*). The cultures were grown to early logarithmic phase and induced with 200 mM IPTG as before. Cells were centrifuged at 10000*g* for 10 min and then resuspended in 30 mL of 6 M urea per flask. A total culture volume of 2.4 L was combined in a single toxin preparation. The urea concentrate was boiled for 5 min to lyse the cells and centrifuged as before. The toxin-containing supernate was dialyzed overnight in 12000−14000 molecular weight exclusion dialysis tubing (Sigma Chemical Co., St. Louis, MO) against 4 L of distilled water at 4 °C. The dialyzed supernate was then subjected to preparative thin-layer isoelectric focusing. Successive gradients of pH 3.5−10 and 6−8 were used to isolate rSEQ. Final purification of rSEQ was accomplished using a gel filtration column (Bio-Rad Laboratories, Hercules, CA) containing Sephadex G-75

(Sigma). Purity was verified by SDS−PAGE. Purified protein concentration was assessed with the Bradford protein assay (Bio-Rad Laboratories), in which known amounts of TSST-1 were used to create a standard curve, and protein was stored in a lyophilized state until used in biological and biochemical assays.

Hyperimmune Serum. A Dutch belted rabbit (Birchwood Farms, Red Wing, MN) was immunized with 50 μg of purified rSEQ in phosphate-buffered saline (PBS, 0.005 M $NaPO_4$, pH 7.2, 0.15 M NaCl) after being emulsified in Freund's incomplete adjuvant. This mixture was injected subcutaneously into the rabbit three times at 2 week intervals. One week after the last injection, blood was drawn from the hyperimmune animal. The blood was allowed to clot overnight and then centrifuged to separate the serum fraction. The serum was stored at 4 °C and preserved with a drop of liquefied phenol. This antiserum was used to locate rSEQ fractions during subsequent purification procedures.

Biochemical Assays. The size and homogeneity of purified rSEQ were determined by SDS−PAGE (*27*). Isoelectric point determination was accomplished by measuring the pH of the rSEQ fraction obtained from isoelectric focusing plates.

Superantigenicity Assay. Rabbit splenocytes were seeded into the wells of a 96-well microtiter plate at a concentration of 2×10^5 cells per well (*28*). Tenfold dilutions of toxin were added to wells in quadruplicate, starting with 1.0 μg per well down to 10^{-8} μg per well. These dilutions were compared to cells incubated in the presence of PBS alone as a negative control and serial dilutions of TSST-1 as positive controls. The splenocytes were grown at 37 °C for 3 days and pulsed with 1 μCi of [^{3}H]thymidine overnight (*28*). The cells were harvested the next day, and cell proliferation, as determined by [^{3}H]thymidine incorporation into DNA, was measured in a scintillation counter (Beckman Instruments, Fullerton, CA).

Flow Cytometric Analysis of T Cell Repertoire. Peripheral blood mononuclear cells (PBMC) obtained from three normal human donors were isolated from heparinized venous blood by density gradient sedimentation over Ficoll-Hypaque (Histopaque, Sigma) (*29*−*31*). Cells were then washed three times in Hank's balanced salt solution (HBSS) (Mediatech Cellgro, Herndon, VA) and resuspended in medium for cell culture. PBMC (at 1×10^6 cells/mL) were cultured in RPMI 1640 (Mediatech Cellgro) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gemini Bioproducts, Woodland, CA), 20 mM HEPES buffer (Mediatech Cellgro), 100 μg/mL penicillin (Mediatech Cellgro), 100 μg/mL streptomycin (Mediatech Cellgro), and 2 mM L-glutamine (Mediatech Cellgro). Cells were cultured in the presence of either anti-CD3 (20 ng/mL) or SEQ (100 ng/mL) for 3 days, washed, and allowed to grow for an additional day in the presence of interleukin 2 (50 units/mL) before being washed and stained for immunofluorescence analysis of T cell repertoire as previous described (*29*−*31*).

For flow cytometry studies, PBMC were washed in HBSS and resuspended at 10×10^6 cells/mL in a staining solution [PBS with 5% FCS (Gemini Bioproducts), 1% immunoglobulin (Alpha Therapeutic Corp., Los Angeles, CA), 0.02% sodium azide (Sigma)]. Cells were stained in 96-well, round-bottomed plates with a panel of biotinylated monoclonal antibodies against human TCRVβ 2, 3, 5.1, 5.2, 7, 8, 11, 12, 13.1, 13.2, 14, 16, 17, 20, 21.3, 22 (Immunotech, Westbrook, ME), TCRVβ 9, 23 (Pharmingen, San Diego, CA), and TCRVβ 6.7 fluorescein isothiocyanate (FITC) (Endogen, Woburn, MA) and then incubated for 30 min at 37 °C in the dark. After the incubation period, cells were washed twice with washing buffer [PBS, 2% FCS (Gemini Bioproducts), 0.02% sodium azide (Sigma)] by centrifugation at 300*g* for 5 min at 4 °C. Cell pellets were resuspended in staining solution and incubated with anti-CD3 allophycocyanin (APC), anti-CD4 phycoerythrin (PE) (Becton Dickinson, San Jose, CA), anti-CD8 (FITC) (Becton Dickinson), and a streptavidin−peridinin−chlorophyll protein (PerCP) conjugate (Becton Dickinson) for 30 min at 4 °C. Stained cells were again washed twice in washing buffer and once in 0.02% sodium azide (Sigma) in PBS by centrifugation at 300*g* for 5 min at 4 °C. Finally, the cells were fixed in 200 μL of 1% (v/v) formaldehyde (Polysciences, Warrington, PA) in PBS. Analysis was performed using four-color flow cytometry (FACS Calibur, Becton Dickinson) as described previously (*32*). Methods of cytometer set up and data acquisition have also been described previously (*32*). List mode multiparameter data files (each file with forward scatter, side scatter, and four fluorescent parameters) were analyzed using the Cellquest program (Becton Dickinson). Analysis of activated populations was performed with the light scatter gate set on the T cell blast population. Negative control reagents were used to verify the staining specificity of experimental antibodies.

Pyrogenicity and Endotoxin Enhancement. Dutch belted rabbits were injected with toxin in PBS at doses of 4.0, 0.4, and 0.04 μg/kg intravenously. Three rabbits were injected with each dose. Each rabbit's temperature was measured at 0 and 4 h. After 4 h each rabbit was injected intravenously with 10 μg/kg lipopolysaccharide (LPS) from *Salmonella typhimurium* ($^1/_{50}$th of the LD_{50} of endotoxin alone). Lethality of this toxin regimen over a 48 h period was assessed (*33*).

Miniosmotic Pumps. Nine American Dutch belted rabbits in groups of three were implanted with subcutaneous miniosmotic pumps on the left flanks, each containing 200 μL (200 or 500 μg) of rSEQ or 200 μg of TSST-1. Lethality of the toxins was assessed over a period of 15 days as described previously (*19*).

Emetic Activity in Monkeys. The emetic capacity of rSEQ and positive control SEC1 was compared using a standard monkey feeding assay (*34*), modified as described previously (*17*, *25*). Each toxin was dissolved in flavored fruit punch (Lyons-Magnus, Clovis, CA) and fed to young adult pigtail monkeys (*Macaca nemestrina*) with the assistance of a sterile syringe. Prior to use in experiments, the animals were trained to willingly accept fluids from a syringe so that neither physical restraint nor anesthesia was needed. The minimal emetic dose for SEC1 in this assay has been determined previously to be 0.1−1.0 μg/kg.

RESULTS

Sequence of SEQ. seq was cloned and sequenced from the TSS clinical isolate MN NJ. The open reading frame encoded a polypeptide of 242 amino acids in length (Figure 1). Analysis of the sequence 5′ of the putative translational start site (Figure 1) revealed a Shine Dalgarno sequence typical of *S. aureus* genes previously described (*35*).

Using the online signal peptide prediction program SignalP v.1.1 (www.expasy.ch), we predicted the N-terminal se-

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ttcaataaatacaagaattctatggataaaacattaaagtgatgggaaaagttgtattgt
gattaaaaatgcctatatggcgttgtaatataaaaaaaaggaggcatcaaaatgaataaa
                                                  M  N  K
atatttagaatactcactgttagcttgtttttcttcacatttttaataaaaaacaatcta
I  F  R  I  L  T  V  S  L  F  F  F  T  F  L  I  K  N  N  L
gcatatgctgatgtaggggtaatcaaccttagaaacttttatgctaattatgaacctgaa
A  Y  A *D  V  G  V  I  N  L  R  N  F  Y  A  N  Y  E  P  E
aagcttcaaggagttagttctggaaatttttctacttctcatcaattagagtatattgat
K  L  Q  G  V  S  S  G  N  F  S  T  S  H  Q  L  E  Y  I  D
ggaaaatacactttatattcacagtttcataatgaatatgaagcgaagagattaaaagat
G  K  Y  T  L  Y  S  Q  F  H  N  E  Y  E  A  K  R  L  K  D
cataaagtagatatctttggaataagttactcaggtctttgtaatacaaaatatatgtat
H  K  V  D  I  F  G  I  S  Y  S  G  L  C  N  T  K  Y  M  Y
ggtggaattacgttggcgaatcaaaatttagataaacctagaaatatacctattaatctc
G  G  I  T  L  A  N  Q  N  L  D  K  P  R  N  I  P  I  N  L
tgggtcaatggtaagcaaaatactatatctacagacaaagtttctactcaaaaaaaagag
W  V  N  G  K  Q  N  T  I  S  T  D  K  V  S  T  Q  K  K  E
gtaactgctcaagagattgatattaagttacgaaaatacctacaaaacgaatacaatata
V  T  A  Q  E  I  D  I  K  L  R  K  Y  L  Q  N  E  Y  N  I
tatggttttaataaaacaaaaaaaggtcaggaatatggatatcagtcaaaatttaattct
Y  G  F  N  K  T  K  K  G  Q  E  Y  G  Y  Q  S  K  F  N  S
gggtttaacaaaggaaaaattactttccatttaaataatgaaccctcttttacatacgat
G  F  N  K  G  K  I  T  F  H  L  N  N  E  P  S  F  T  Y  D
ttgttttacaccggaactggtcaagcagagagtttttaaaaatttacgataataataaa
L  F  Y  T  G  T  G  Q  A  E  S  F  L  K  I  Y  D  N  N  K
actatagatacagagaattttcatttggatgtagagatttcatatgagaagactgaataa
T  I  D  T  E  N  F  H  L  D  V  E  I  S  Y  E  K  T  E  *
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FIGURE 1: Nucleotide and inferred amino acid sequence of *seq* and SEQ, respectively, cloned from nonmenstrual staphylococcal TSS isolate MN NJ. Putative ribosome binding site is highlighted. * residue marks the predicted N terminus of the mature protein (with residues from protein sequencing underlined) after removal of the signal peptide.

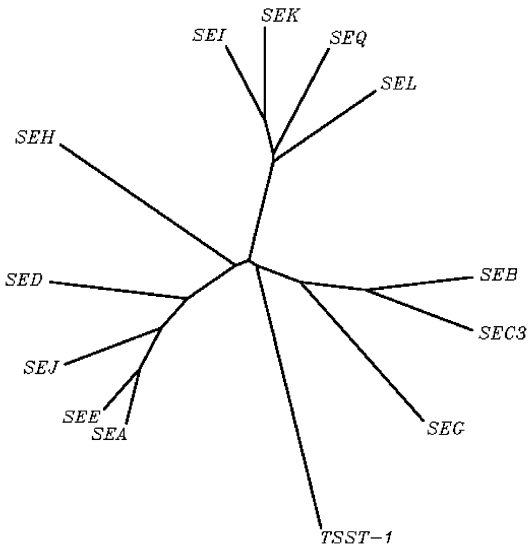


FIGURE 2: Phylogenetic tree diagram of the family of PTSAgs. The five distinct subfamilies can be observed. Groupings are an indication of relatedness, but distances are not quantitatively related to evolutionary distance.

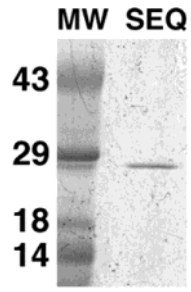


FIGURE 3: SDS−PAGE analysis (15% gel) of rSEQ. The gel was stained with Coomassie brilliant blue R250. Molecular weight (MW) standard with sizes given to the left in thousands; lane Q, 10 μg of rSEQ. The apparent size of rSEQ was 28000.

quence of the secreted form of SEQ to be DVGVINLRNF. The experimentally determined N-terminal sequence of rSEQ through 10 residues was MDVGVINLRN after cloning in the pET system (methionine was added to the N terminus of rSEQ to facilitate expression). Amino acid sequence similarity of the inferred SEQ to other PTSAgs was also examined using the predicted mature SEQ protein sequence. We observed that SEQ fit into a new subfamily of PTSAgs together with SEI and another very recently identified SE-like protein designated SEK (Figure 2) (*11*). These toxins were distinct from the other subfamilies of SEs in that they have a short β4−β5 loop devoid of the disulfide bond found in many of the SEs and which is hypothesized to be important in emetic activity (*17*). Like the SEA/D/E/J subfamily (*2*), SEQ contained a putative zinc coordination site, important for the high-affinity MHC II binding site on these toxins (includes residues His17, His209, and Asp211 with the fourth coordination residue provided by MHC II). *sek* and *seq* were adjacent genes in the *S. aureus* MN NJ chromosome, separated by only 15 nucleotides, and also encoded similar predicted amino acid sequences. These two genes were likely to have arisen through a gene duplication event.

Biochemical Properties of rSEQ. To study the *seq* gene product, a recombinant construct was made using the predicted N-terminal sequence, with a methionine added to the end for the purposes of expression in the pET system. rSEQ was experimentally determined to have a p*I* of approximately 7.5. The p*I* predicted from computer analysis (www.expasy.ch) of the primary sequence was 7.0. The predicted molecular weight of this polypeptide was 25207. When purified and evaluated by SDS−PAGE, the protein had an apparent molecular weight of 28000 (Figure 3). It is typical of the PTSAgs to appear of higher molecular weight

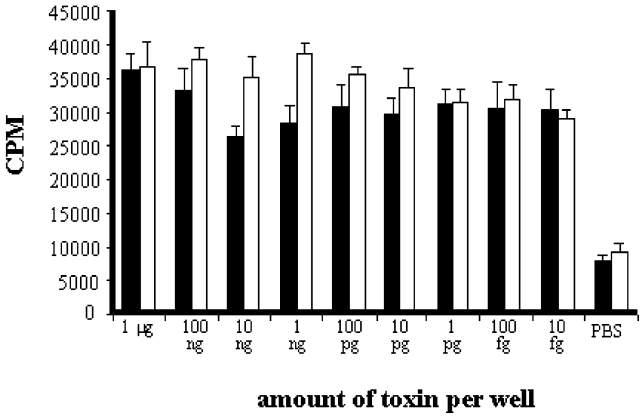
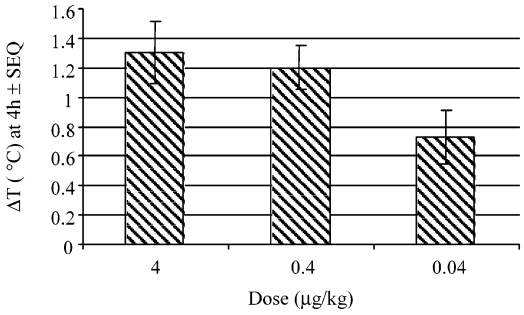


FIGURE 4: Superantigenicity assay of rSEQ (□) compared to TSST-1 (■) as assessed by measuring proliferation of rabbit splenocytes (2×10^5 per well per 200 μL). Splenocytes in complete RPMI were incubated for 4 days in quadruplicate samples in 96-well microtiter plates in the presence of rSEQ or TSST-1 used as a control at the designated concentrations added in 20 μL volumes. Negative control wells contained 20 μL of PBS rather than toxin. [^{3}H]Thymidine (1 μCi/well) was added to all wells after 3 days, and splenocyte proliferation was measured by determining counts per minute (CPM) of radiolabel incorporation into DNA. Results are the average CPM of quadruplicate experiments. Values represent mean ± standard error of the mean (SEM).



Dose rSEQ (µg/kg)	Lethality after 48 h
4	3/3
0.4	3/3
0.04	3/3

FIGURE 5: Pyrogenicity of rSEQ in rabbits and the ability of the toxin to enhance host susceptibility to lethal endotoxin shock. rSEQ was injected intravenously at doses of 4.0, 0.4, and 0.04 μg kg^{-1} mL^{-1} in PBS. Fever development was assessed at 0 and 4 h using rectal thermometers. Values presented represent the mean ± SEM. At the 4 h time point, all rabbits were injected with 10 μg/kg lipopolysaccharide from *S. typhimurium*. Lethality was assessed during the first 48 h postinjection.

by SDS−PAGE than the predicted amino acid sequence would suggest.

Biological Activity of rSEQ. The ability of rSEQ to stimulate rabbit splenocytes was assessed in a standard superantigenicity assay (Figure 4). rSEQ stimulated splenocyte proliferation comparably to TSST-1, with activity even at 10 fg per well. The lack of drop in activity with dilution of both SEQ and TSST-1 likely resulted from the high sensitivity of these particular splenocytes to the toxins. We have occasionally observed this phenomenon in other rabbit splenocyte superantigen assays. The pyrogenic activity of rSEQ and its ability to enhance endotoxin shock were also examined (Figure 5). rSEQ caused fever in rabbits at doses as low as 0.04 μg/kg. The minimum pyrogenic dose of rSEQ at 4 h (defined as the dose required to give a 0.5 °C average rise in body temperature) was extrapolated to be 0.015 μg/kg. The same toxin doses that caused fever also enhanced the lethality of endotoxin (Figure 5). Finally, the lethality of rSEQ when infused into rabbits from subcutaneous miniosmotic pumps was assessed. All six rabbits infused with 200 or 500 μg of rSEQ survived (3/3 and 3/3, respectively) when tested over a 15 day period. TSST-1 was lethal (3/3 succumbed) in this model at a dose of 200 μg.

The emetic activity of rSEQ was assessed by oral administration of the toxin to young adult pigtail monkeys. We determined that a dose of rSEQ (50 μg/kg), corresponding to 100 emetic units (EU) of SEC1, did not cause emesis in either of the test subjects (0/2). By comparison, administration of a similar dose of SEC1 caused emesis in both monkeys tested (2/2). As mentioned in Materials and Methods, it may be inappropriate to designate SEQ as an enterotoxin because of its lack of emetic activity. Thus, we use SEQ provisionally because of its sequence similarity to SEs. In the future, it is likely to be necessary to arrive at a consensus for naming the various subfamilies of PTSAgs.

The TCRVβ stimulation profile of human T cells incubated with rSEQ was examined using flow cytometry with monoclonal antibodies directed against the different human TCRVβ subsets. It was observed that T cells bearing TCRVβs 2, 5.1, 5.2, 6.7, and 21.3 were preferentially activated by the toxin (Figure 6). The expansion of TCRVβ 5.1, 6.7, and 21.3 T cells was statistically significantly stimulated ($p < 0.05$). T cells with TCRVβ 6.7 responded with the highest level of expansion (31% of T cells in one patient), which is likely to be biological significant. We observed that primarily CD4 T cells but also CD8 T cell populations were expanded. As expected, some T cells bearing certain TCRVβ subsets were reduced in relative population, consistent with the effect of a PTSAg. Examples of this effect included TCRVβ 3, 8, 13.1, and 14.

DISCUSSION

The data presented in this work indicate that a novel PTSAg gene, *seq*, is present in SaPI3 together with *seb* (*8*) and the recently described *sek* (*11*). The encoded novel toxin shares many biological activities with previously described enterotoxins (*1*), except rSEQ is neither emetic nor lethal when administered to rabbits in subcutaneous miniosmotic pumps. The presence of this third toxin on SaPI3 further validates that SaPI3 is a pathogenicity island.

Analysis of the inferred protein sequence of *seq* shows that SEQ fits into a new subfamily of SEs, together with the recently described SEI (*18*) and SEK (*11*) (Figure 2) and two uncharacterized toxins. SEQ is more closely related to the SEA/D/E/J (*2*) subfamily in that it has the zinc-dependent MHC II site than to the SEB/C/G subfamily, although its gene is genetically linked to *seb* and there is slightly more sequence alignment with the SEB/C/G subfamily (Figure 2).

rSEQ is superantigenic, pyrogenic, and enhances the toxic effects of endotoxin in a rabbit model of TSS, typical of PTSAgs. It is, however, not lethal in a miniosmotic pump model of TSS in rabbits. This lack of lethality in the miniosmotic pump model is likely to be the result of either intrinsic lack of function or instability of the purified protein

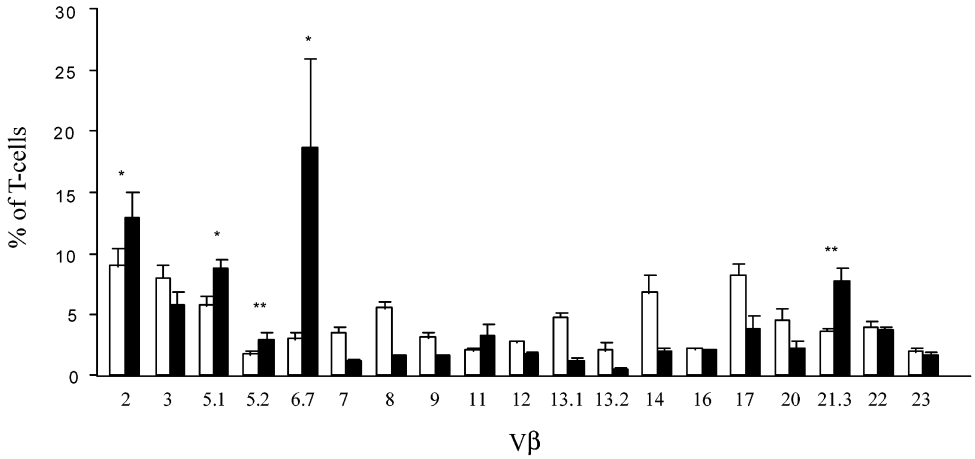


FIGURE 6: TCRVβ profile of human T cells stimulated with rSEQ. Three patients' peripheral blood mononuclear cells were stimulated with either anti-CD3 (white bars), which stimulates all T cells, or rSEQ (black bars), which selectively stimulates T cells dependent on TCRVβ composition. Cells were stained with monoclonal antibodies against listed TCRVβ chains, and the results were evaluated by flow cytometry. The percentages of T cells expressing the listed TCRVβs are shown. *p* values were determined using the paired Student's *t*-test (*, $p = 0.079$; **, $p < 0.05$). Error bars represent SEM for each data set.

in vivo. The toxin is stable enough in vivo to enhance susceptibility to endotoxin in rabbits in a 4 h assay, and we did not observe rSEQ to be unstable in vitro. However, the host may inactivate the molecule through proteolysis or precipitation within the pumps. It should be noted that rSEQ used in these studies has been cloned without its putative N-terminal signal sequence. In addition, to express the protein in the pET system, a methionine is present at the N terminus of the recombinant protein. Thus, the N-terminal sequence of rSEQ is MDVGVINLRN, while the putative N-terminal sequence of SEQ is DVGVINLRNF. This alteration does not appear to have abrogated the biological activity of the toxin, although it might hypothetically contribute to the inactivity of rSEQ in the miniosmotic pump model.

rSEQ is a potent T cell superantigen capable of stimulating proliferation of both CD4 and to a lesser extent CD8 T cells, even at femtogram quantities. The activity is comparable to that of TSST-1 and other PTSAgs in this regard (*1*, *4*). rSEQ is also a potent inducer of fever in rabbits, mediated by cytokine release from macrophages, and the protein enhances endotoxin shock to a similar extent as TSST-1, also previously shown to depend on release of cytokines (*36*, *37*). At a dose of 100 EU, however, rSEQ does not cause emetic responses in monkeys. Since SEs are defined by their abilities to cause emesis after oral administration (*3*, *34*), it may be inappropriate to refer to SEQ as an enterotoxin, despite sequence similarity. TSST-1 and SPEs also lack emetic activity and, thus, their different names. This has led to our designation of SEQ, I, and K as members of a new subfamily of PTSAgs we refer to as group V, based on their both having a zinc-dependent MHC II site and lacking classic enterotoxin emetic activity (*2*).

The study of SEQ as a member of the group V subfamily should increase our understanding of the overall structure−function relationships among PTSAgs. The structures of TSST-1 (group I) (*38*, *39*), SEB (*40*, *41*) and C (*42*, *43*), SPE A (*44*, *45*), and streptococcal superantigen (*46*) (group II), SEA (*47*), D (*48*), and H (*49*) (group III) (*47*), and SPE C (*50*) and SMEZ (*51*) (group IV) have been solved. In addition, SEB and C have been crystallized in complex with the T cell receptor (*52*, *53*), as well as SEB and H, TSST-1, and SPE C in complex with the MHC II (*41*, *54*−*56*). These studies have allowed a detailed model of superantigen activation of T cells to be developed. However, no structures of group V toxins alone or complexed with immune cell receptor molecules have been determined. Because SEQ has an apparent zinc coordination site in domain A, it is likely the toxin will have both low affinity (in OB fold) (*41*, *55*) and high affinity (in β grasp) (*54*, *56*) MHC II sites, but this remains to be determined. In addition, we now know that there are multiple mechanisms of interaction of PTSAgs with TCRVβs. For example, in the standard view SEs B and C interact with TCRVβ on the top front of the toxins in a groove between the two domains (*22*, *52*, *53*). In contrast, TSST-1 interacts with TCRVβ in a groove on the top back of the toxin (*57*). How group V PTSAgs interact with TCR is also unknown.

There are other facets of PTSAg function that have not been explained in terms of structural difference. A prime example of this is emetic activity. The classical SEs are characterized by an ability to cause an emetic response when administered orally to monkeys (*1*, *12*, *34*), but we still do not completely understand what parts of the molecules are required for this function. It has been proposed that the disulfide-containing $\beta4-\beta5$ loop is important (*17*, *25*). Studies of rSEQ indicate that the toxin is not able to induce emesis even at 100 EU, or 100 times the minimal emetic dose of SEC1. Structural comparison of SEQ with emetic toxins such as SEC1 may more clearly define essential toxin elements required for that activity.

SEQ stimulates T cells in a manner similar to that of previously described superantigens (*20*), but it has a distinct TCRVβ profile. We have shown that TCRVβ 2, 5.1, 5.2, 6.7, and 21.3 are significantly stimulated in a population of human PBMCs by rSEQ in vitro. Only two other characterized toxins stimulate TCRVβ 5.1 (SEE, SEK), and only SEK has been observed to stimulate 5.2 or 6.7 (*11*). TCRVβ 5.1 in particular has been observed to be overrepresented in several diseases of unknown etiology, in particular Crohn's disease, a severe small bowel inflammatory disorder (*58*). T cells from the TCRVβ 5 family have also been implicated in juvenile rheumatoid arthritis and periodontitis (*59*, *60*).

In the latter case, Vβ 6.7 has also been observed to be overrepresented (*59*). Future studies should examine the role, if any, of these PTSAgs in such illnesses. Such studies will first depend on defining the full extent of PTSAg production by *S. aureus* strains.

The recently identified toxin gene *sek* was identified adjacent to *seq* in SaPI3. SEK was expressed in *E. coli* in a manner similar to the experiments in this work, and similar activities were observed for both SEQ and K (*11*). The only difference between the two toxins in terms of biological activity was that rSEK was lethal in rabbits in the miniosmotic pump model, whereas rSEQ was not. The two toxin genes have been localized near the 3′ end of SaPI3. Other studies have also noted that PTSAgs in other pathogenicity islands are also localized near the termini. An interesting evolutionary origin may be hypothesized for the presence of these two toxin genes (*sek* and *seq*) adjacent to one another at the same end of these SaPIs. It appears that SEQ is less stable in vivo than SEK, as suggested by our miniosmotic pump model studies, and that gene duplication and subsequent evolution led to the maintenance of the more stable SEK.

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