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Biochemical and Biological Properties of Staphylococcal Enterotoxin K

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Staphylococcus aureus **is an important human pathogen which is implicated in a wide variety of diseases. Major determinants of the virulence of this organism include extracellular virulence factors. Staphylococcal enterotoxins (SEs) are important causative agents in staphylococcal toxic shock syndrome and food poisoning. Our study identified a novel enterotoxin, SEK, and examined its biochemical and biological properties. SEK had a molecular weight of 26,000 and an experimentally determined pI of between 7.0 and 7.5. SEK was secreted by clinical isolates of *S. aureus*. We demonstrated that SEK had many of the biological activities associated with the SEs, including superantigenicity, pyrogenicity, the ability to enhance the lethal effect of endotoxin, and lethality in a rabbit model when administered by subcutaneous miniosmotic pump. Recombinant SEK was shown to stimulate human $CD4^+$ and $CD8^+$ T cells in a Vβ-specific manner; T-cells bearing Vβ 5.1, 5.2, and 6.7 were significantly stimulated to proliferate.**

Staphylococcus aureus is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance (5, 7, 9, 12, 14, 31, 35). The organism is a significant cause of nosocomial infections, as well as community-acquired disease. The spectrum of staphylococcal infection ranges from pimples and furuncles to toxic shock syndrome (TSS) and sepsis (24, 26, 35).

The virulence factors secreted by *S. aureus* are major determinants of both disease causation and severity during the course of infection. These factors include several hemolysins (α, β, γ, and δ), leukocidin, exfoliative toxins A and B, and the large family of pyrogenic toxin superantigens (PTSAgs) (reviewed in references 5, 11, and 20). The latter toxins include toxic shock syndrome toxin 1 (TSST-1) and the staphylococcal enterotoxins (SEs) A to J, excluding F (11). All of the staphylococcal PTSAgs are encoded on variable genetic elements, with TSST-1 and enterotoxins B and C, among others, being present on pathogenicity islands (SaPIs) (29).

Numerous studies have shown that PTSAgs are important determinants for TSS (11) and food poisoning (reviewed in reference 2). Todd and coworkers were the first to recognize *S. aureus* as the etiologic agent of TSS (56). Subsequent work by Schlievert et al. (51) and Bergdoll and colleagues (3) identified TSST-1 as the major toxin associated with this illness, whether menstrual or nonmenstrual associated; TSST-1 accounts for 75% of all TSS cases. Later work by Schlievert and others established that SEs, notably SEB and -C, were important causes of nonmenstrual-associated TSS (3, 48). The SEs, particularly SEA and -D, and, to a lesser extent, SEB and -C, are also common causes of staphylococcal food poisoning (11, 54).

Crystallographic studies of the PTSAgs have shown that these molecules share the same basic three-dimensional structure (11, 50). The toxins begin with a short N-terminal α helix that leads into a β barrel structure, also known as the B domain or oligonucleotide binding (OB). The OB fold is connected to a C-terminal wall of β strands by a central diagonal α helix, forming domain A. All PTSAgs have these features in common, but some differ in that they have a small number of additional loops. The most notable of these is a cystine loop structure present in the SEs (15). This cystine loop is thought to be important for emetic activity, based on studies of mutants (11, 15). Recently, however, SEI has been identified, which lacks the cystine loop structure; this toxin is both superantigenic and emetic, although the emetic activity is significantly reduced (34).

Overall, the PTSAgs share numerous biological activities, including superantigenicity, pyrogenicity, the capacity to enhance endotoxin shock, and lethality when administered in subcutaneous miniosmotic pumps (11, 50). For superantigenicity (18, 30, 31), PTSAgs bind to the variable region of the β chain (Vβ) of certain T-cell receptors (TCRs). This mode of binding to the TCR is much less specific than the typical TCR-peptide-major histocompatibility complex (MHC) II trimolecular complex that is required for T-cell activation. Depending on the Vβ specificity of the PTSAg, up to 50% of the host T cells may be activated, resulting in massive cytokine release, with concomitant induction of capillary leak (hypotension) (8, 11, 30, 33). The other biological activities, pyrogenicity, endotoxin enhancement, and lethality when given in miniosmotic pumps, are also dependent on cytokine release (11). Among PTSAgs, only SEs have emetic activity, and this activity has been separated from superantigenicity (11, 15).

This study was undertaken to purify and characterize a new SE, designated SEK, and to determine whether a functional PTSAg is encoded by its gene. In this report, we demonstrate that recombinant SEK (rSEK) functions as a superantigen and is lethal in rabbit models of TSS, and the toxin is expressed by clinical isolates.

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MATERIALS AND METHODS

Cloning and sequencing. The gene *sek* was cloned from *S. aureus* TSS isolate MN NJ. This isolate produces SEB as well as SEK. PCR primers were chosen based on the *sek* sequence of SaPI1 (29), as well as comparison with the unfinished genome sequence of methicillin-resistant *S. aureus* strain COL, available online at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). PCR primers including several hundred nucleotides at either end of the gene were included in the original clone. The sequences of the *sek* primers were 5′ GAATTACGTTGGCGAATC and 3′ AGGGTAGGCGGGC. The PCR product was electrophoresed in 1% agarose, and the 1.4-kb band containing the entire *sek* gene was cut out of the gel. The DNA was purified from the agarose by using the Geneclean II kit (Bio101, La Jolla, Calif.) and cloned into the TA vector pGEM T-easy (Promega, Madison, Wis.), resulting in the plasmid pPMO001. The vector plasmid contains TT overhangs that allow for cloning of PCR products directly without restriction digestion. Subsequently, the gene was excised from this vector with *Eco*RI, using restriction sites on either side of the insertion site in the vector, and ligated with *Eco*RI-digested plasmid vector pCE104 (44). The resultant plasmid was transformed into *Escherichia coli* XL-1 Blue (Stratagene, La Jolla, Calif.), and a DNA insert with a restriction fragment of the proper length was verified by plasmid purification with the Qiagen Spin Miniprep kit (Qiagen, Valencia, Calif.), digestion with *Eco*RI, and separation in 1% agarose gels. This plasmid was referred to as pPMO002. The DNA sequence of *sek* was determined by automated sequencing with fluorescent labeled deoxynucleoside triphosphates (dNTPs) (Advanced Genetic Analysis Center, St. Paul, Minn.). Further constructs were made with this plasmid as a template. In order to examine the biochemical and biological properties of SEK, a signal sequence deletion mutant was cloned into pET28b by PCR amplification of the pCE104 (pPMO002) *sek* insert, resulting in pPMO003. Restriction sites were encoded in the primers for translation of the gene from the pET28b ribosome binding site (RBS). The primer sequence were 5′ GGGGGATCCTTATATCG TTTCTTTATAAGAAATATCGAC and 3′ CCCCCATGGGCCAAGGTGAT ATAGGAATTGATAAT. Transformation of pPMO003 into *E. coli* XL-1 Blue (Stratagene) was followed by purification of the plasmid and verification of the desired construct by restriction digestion with *Nco*I and *Bam*HI. Subsequent to verification, the plasmid was introduced into *E. coli* BL-21 DE3 for expression with the p_{tac} system. The deleted signal peptide of SEK was replaced with an N-terminal methionine. In addition, for the purposes of expression in pET28b, the N-terminal glutamine was replaced with glycine. The N-terminal sequence of rSEK was MGGDIGIDNLR.

Expression and purification. The pET28b clone containing *sek* (pPMO003) in BL-21 DE3 was grown to early log phase in Luria-Bertani medium supplemented with kanamycin (50 μg/ml) at 37°C with shaking (approximately 100 rpm; Gyrotary Shaker; New Brunswick Scientific, New Brunswick, N.J.) and then induced with 200 mM isopropyl-β-D-thiogalactopyranoside (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. After growth overnight under the same conditions, the cultures were treated with 4 volumes of absolute ethanol for 48 h to precipitate toxins. The precipitated extracellular and released periplasmic proteins were resuspended at a concentration 20 times that of the original culture volume and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (21). Proteins were stained with Coomassie blue R250. Induction of the expression system was ascertained by the presence of a prominent band of the expected size of rSEK in the extract. Subsequent growth, expression, and purification of SEK were done through use of the stored frozen aliquots of the original culture.

Large-scale production of rSEK was performed analogously to the above protocol. Beef heart medium (1,200 ml in Feherenbach flasks), containing 1% glucose-phosphate buffer (51) and 50 μg of kanamycin per ml, was inoculated with 200 μl of the frozen expression clone. The culture was grown to early log phase and induced with 200 mM IPTG as before. Proteins were precipitated in the presence of 4 volumes of ethanol for 48 h, followed by decantation and collection of the precipitate by centrifugation (500 × *g*, 10 min). Five liters of total culture volume was combined in a single toxin preparation. The precipitate was air dried after centrifugation and then resuspended in 100 to 150 ml of pyrogen-free water. The resuspended precipitate was centrifuged at 10,000 × *g* for 30 min, and the concentrated supernatant was removed, placed in molecular weight 12,000 to 14,000 exclusion dialysis tubing (Spectrum Laboratories, Inc., Miami, Fla.), and dialyzed overnight against 4 liters of distilled water. The dialyzed supernate was then subjected to preparative isoelectric focusing. Successive gradients of pH 3.5 to 10 and 6 to 8 were used to isolate highly pure rSEK. Final purification of rSEK was accomplished with a gel filtration column (Bio-Rad Laboratories, Hercules, Calif.) containing Sephadex G-75 (Sigma). Purity was verified by SDS-PAGE in which 10 μg gave a homogeneous band of the appropriate molecular weight. The purified protein concentration was assessed with the Bradford protein assay (Bio-Rad), and protein was stored in the lyophilized state until used in biological and biochemical assays. A standard curve for the Bradford assay was developed by using purified TSST-1 diluted from 1 mg/ml as determined by Ouchterlony double immunodiffusion (39).

Hyperimmune serum. An American Dutch Belted rabbit (Birchwood Farms, Grantsburg, Wis.) was immunized with 50 μg of purified rSEK 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 hyperimmunized animal. The blood was allowed to clot overnight and then centrifuged (500 × *g*, 10 min) to separate the serum fraction. The serum was stored at 4°C and preserved with a drop of liquified phenol.

Biochemical assays. The size and homogeneity of purified rSEK were determined by SDS-PAGE and Western immunoblotting (4) with a rabbit polyclonal antiserum to rSEK used as the probe.

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. Serial 10-fold dilutions of rSEK or TSST-1 were added to each well in quadruplicate, starting with 1 μg/well and with dilution to 10^{-8} μg/well. These dilutions were compared to cells incubated in the presence of PBS alone. The splenocytes were grown at 37°C for 3 days and pulsed with 1 μCi of [^{3}H]thymidine overnight (51). The cells were harvested the next day, and cell proliferation (incorporation of ^{3}H into DNA) was measured in a scintillation counter (Beckman Instruments, Fullerton, Calif.).

Pyrogenicity and endotoxin enhancement. American Dutch Belted rabbits were injected with rSEK at doses of 4.5, 0.45, and 0.045 μg/kg of body weight per ml intravenously. Three rabbits were injected with each dose. Each rabbit's temperature was measured with rectal thermometers at 0 and 4 h. After 4 h, each rabbit was injected intravenously with 10 μg of lipopolysaccharide (LPS) from *Salmonella enterica* serovar typhimurium (1/50 of the 50% lethal dose of endotoxin alone). The lethality of this toxin regimen over a 48-h period was assessed (47, 52).

Miniosmotic pump lethality studies. Six American Dutch Belted rabbits had miniosmotic pumps, containing 200 μl (200 μg) of either TSST-1 or rSEK, implanted subcutaneously on the left flank (41). Lethality of the toxins was assessed over a period of 15 days.

Expression of SEK by clinical isolates. Clinical TSS isolates (36) that tested positive for TSST-1, SEB, or SEC were also evaluated for production of SEK. Crude supernatants (10 ml) of these isolates were collected by centrifugation, and toxin was precipitated with ethanol as described above. The precipitates were resuspended at a 100× concentration in sterile pyrogen-free water. Toxin production was detected by use of Ouchterlony double immunodiffusion (39) and by Western immunoblotting (4) with antibody raised against rSEK as the probe.

Flow cytometric analysis of T-cell repertoire. Peripheral blood mononuclear cells (PBMCs) obtained from three normal human donors were isolated from heparinized venous blood by density gradient sedimentation over Ficoll-Hypaque (Histopaque; Sigma Chemical Co., St. Louis, Mo.). Cells were then washed three times in Hanks balanced salt solution (HBSS; Mediatech Cellgro, Herndon, Va.) and resuspended in medium for cell culture. PBMCs (at 10^6 cells/ml) were cultured in RPMI 1640 (Mediatech Cellgro) supplemented with 10% heat-inactivated fetal calf serum (Gemini Bioproducts, Woodland, Calif.), 20 mM HEPES buffer (Mediatech Cellgro), 100 U of penicillin per ml (Mediatech Cellgro), 100 μg of streptomycin per ml (Mediatech Cellgro), and 2 mM L-glutamine (Mediatech Cellgro). Cells were cultured in the presence of either anti-CD3 (20 ng/ml) or SEK (100 ng/ml) for 3 days, washed, and allowed to grow for an additional day in the presence of interleukin 2 (50 U/ml) before being washed and stained for immunofluoresence analysis of the T-cell repertoire as previous described (23, 25, 53). For flow cytometry studies, PBMCs were washed in HBSS and resuspended at 10×10^6 cells/ml in a staining solution (PBS with 5% fetal calf serum [FCS; Gemini Bioproducts], 1% immunoglobulin [Alpha Therapeutic Corp., Los Angeles, Calif.], 0.02% sodium azide [Sigma]). Cells were stained in 96-well, round-bottomed plates with a panel of biotinylated monoclonal antibodies against human Vβ 2, 3, 5.1, 5.2, 7, 8, 11, 12, 13.1, 13.2, 14, 16, 17, 20, 21.3, and 22 (Immunotech, Westbrook, Maine); Vβ 9 and 23 (Pharmingen, San Diego, Calif.); and Vβ 6.7-fluorescein isothiocyanate (FITC) (Endogen, Woburn, Mass.) 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, anti-CD4 phycoerythrin (Becton Dickinson, San

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10020   AACTATAGATACAGAGAATTTTCATTTGGATGTAGAGATTTCATATGAGAAGACTGAATA
        AATATAATTTAAGGAGAAAAGGCAATGAAAAAATTAATAAGCATCTTATTAATAAATATA
                                M  K  K  L  I  S  I  L  L  I  N  I
        ATAATTTTAGGTGTCTCTAATAGTGCCAGCGCTCAAGGTGATATAGGAATTGATAATCTC
        I  I  L  G  V  S  N  S  A  S  A  *Q  G  D  I  G  I  D  N  L
        AGGAATTTTTATACAAAAAAAGACTTCGTAGATTTAAAAGATGTAAAAGACAATGATACT
        R  N  F  Y  T  K  K  D  F  V  D  L  K  D  V  K  D  N  D  T
        CCTATAGCTAATCAACTACAATTTTCAAATGAATCTTATGATTTAATTTCAGAATCAAAA
        P  I  A  N  Q  L  Q  F  S  N  E  S  Y  D  L  I  S  E  S  K
        GATTTTAATAAATTTAGTAATTTCAAGGGAAAAAAACTTGATGTTTTTGGTATTAGTTAT
        D  F  N  K  F  S  N  F  K  G  K  K  L  D  V  F  G  I  S  Y
        AATGGCCAGTGTAACACTAAATACATATATGGCGGAGTCACAGCTACTAACGAATATCTA
        N  G  Q  C  N  T  K  Y  I  Y  G  G  V  T  A  T  N  E  Y  L
        GATAAATCTAGAAATATACCTATAAATATATGGATTAATGGAAATCACAAAACTATTTCT
        D  K  S  R  N  I  P  I  N  I  W  I  N  G  N  H  K  T  I  S
        ACCAATAAAGTTTCGACAAATAAAAAATTTGTTACCGCTCAAGAGATTGATGTCAAATTA
        T  N  K  V  S  T  N  K  K  F  V  T  A  Q  E  I  D  V  K  L
        AGAAAGTACCTTCAAGAAGAATACAACATTTATGGACATAACGGCACTAAAAAAGGAGAA
        R  K  Y  L  Q  E  E  Y  N  I  Y  G  H  N  G  T  K  K  G  E
        GAATATGGTCATAAATCAAAATTTTATTCTGGATTTAATATTGGTAAAGTAACGTTCCAT
        E  Y  G  H  K  S  K  F  Y  S  G  F  N  I  G  K  V  T  F  H
        TTAAATAATAATGACACTTTTTCATATGATTTATTCTACACAGGAGATGATGGGTTACCA
        L  N  N  N  D  T  F  S  Y  D  L  F  Y  T  G  D  D  G  L  P
        AAAAGTTTTTTAAAAATTTACGAAGACAATAAAACTGTAGAGTCTGAGAAATTCCATTTG
        K  S  F  L  K  I  Y  E  D  N  K  T  V  E  S  E  K  F  H  L
10800   GATGTCGATATTTCTTATAAAGAAACGATATAAATCTATTAATTATATTATAATCATTAT
        D  V  D  I  S  Y  K  E  T  I
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FIG. 1. Nucleotide and inferred amino acid sequences of *sek* and SEK, respectively, cloned from staphylococcal TSS isolate MN NJ. The putative RBS is in boldface, and putative −10 and −35 promoter sequences are underlined. An asterisk marks the predicted amino terminus of the mature protein after removal of the signal peptide.

Jose, Calif.), 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% (vol/vol) formaldehyde (Polysciences, Warrington, Pa.) in PBS. Analysis was performed by four-color flow cytometry (FACSCalibur; Becton Dickinson) as described previously (53). Methods of cytometer setup and data acquisition have also been described previously (53). List mode multiparameter data files (each file with forward scatter, side scatter, and 4 fluorescent parameters) were analyzed with 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.

RESULTS

Sequence of *sek*. *sek* was cloned and sequenced from the clinical isolate MN NJ. The open reading frame encoded a polypeptide 242 amino acids in length (Fig. 1). This polypeptide was nearly identical to an open reading frame present in SaPI1 (29). The reading frame presented here was named *sek*, and we suggest renaming *ent* from SaPI1 to *sek*2. In the putative regulatory region of *sek*, we identified a potential RBS as well as possible −35 and −10 promoter sequences (Fig. 1). The two putative SEK polypeptides (from MN NJ and RN 4282 containing SaPI1) differed by two amino acids, one of which was in the likely signal peptide. Using the online signal peptide prediction program SignalP v.1.1 (Center for Biological Sequence Analysis; http://www.cbs.dtu.dk/services/SignalP), we predicted the N-terminal sequence of the secreted form of SEK to be QGDIGIGNLR. Amino acid homology to the other PTSAgs was also examined by using the predicted mature SEK protein. We observed that SEK fit into a subfamily of PTSAgs together with SEI and another recently identified enterotoxin (unpublished data), designated SEL (Fig. 2). These three relatively new toxins (SEI, SEK, and SEL) comprised a new subfamily of SEs. These toxins were distinct from the other subfamilies of SEs in that they lacked the cystine loop hypothesized to be important in emetic activity. This family was related to the SEA subgroup more closely than the SEB-SEC subgroup, despite linkage on a pathogenicity island to the latter toxin subfamily (Fig. 2). Some regions of the toxins retain significant homology across all three toxin subfamilies (Fig. 3).

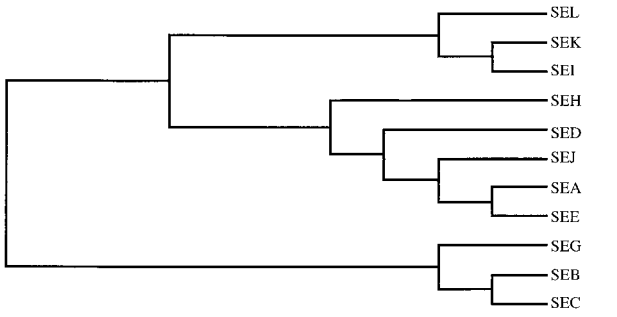

FIG. 2. Phylogenetic tree diagram of the family of SEs of serotypes A to L. Three distinct subfamilies can be observed. Groupings are an indication of relatedness, but distances are not quantitatively related to evolutionary distance.

Biochemical properties of recombinant SEK. In order to work with the gene product, a recombinant construct was made with the predicted N-terminal sequence, with a methionine added to the amino terminus for expression in the pET system. This recombinant protein, rSEK, was observed to have a pI of between 7.0 and 7.5. The pI predicted from computer primary sequence analysis was 6.5. The predicted molecular weight of this polypeptide was 25,539. Both of these values were produced with the online Compute pI/MW tool (http://expasy.cbr.nrc.ca/tools/pi tool.html) with the predicted mature SEK sequence. When purified and evaluated by SDS-PAGE, the recombinant protein had an apparent molecular weight of about 30,000 (Fig. 4). It is not uncommon for PTSAgs to have a higher apparent molecular weight by SDS-PAGE than the predicted amino acid sequence would suggest.

Detection of SEK in clinical isolates. A set of 36 clinical isolates was examined for the ability to produce detectable

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SEK  ----------QGDIGIGNLRNFYTKKFVDLKDVKDNDTPIANQLQFS----- 38
SEI  ----------QGDIGVGNLRNFYTKHIYIDLKGVTLKNLPIANQLEFS----- 38
SEA  SEKSEEINEKDLRKKSELQGTAIGNLKQIYYYNEKANTENKESHEQFVQHTLLFKGFFTD 60
SEB  ---ESQPDPKPDELHKSSKFTGLMENMKVLYDDNHVSATLVKSIEQFLVFLNYSIKDTK 57

SEK  -NESYDLISESKDFNKFSNFKGKKLDVFGISYNGQCN----------IKYIYGG 81
SEI  -TGTNDLISESNNWDEISKFKGKKLDIFGIDYNGFGK----------SKYMYGG 81
SEA  HSWYNDLLVDFDSKDIVDKYKGKKVDLYGAYYYQCAGGTPN----------KTACMYGG 110
SEB  LGNYDNVRVDFKNKDLADKYKDKYVDVFGANYYYQCYFSKKTNDINSHQTDKRKTCMYGG 117

SEK  VTATN-EYLDKSRNIPINIWINGNHKTISTNKYSINKKLVTAQELDVKLRKYLQEEYNIY 140
SEI  ATLSG-QYLNSARKIPINLWVNGKHKTISTDKTATNKKLVTAQFIDVKLRRYLQEEYNIY 140
SEA  VTLHDNNRLTEEKKVPINLWLDGKQNTVPLETVKTNKKNVTVQELDLQARRYLQEKYNLY 170
SEB  VTEHNGNQLDRYRSITVRVFEDGKN--ELSFDVQTNKKKVTAQELDYLTRHYLVKNKKLY 175

SEK  GFNGIKKGEEYGHKSKFYSGFNTGKVTFHLNNNETFSYDLFYTGDDGLLEKS-FLKIYEDN 199
SEI  GHNGIKKGKEYGYKSKFYSGFNNGKVLFHLNNEKSFSYDLFYTGDGLLEVG-FLKIYEDN 198
SEA  NS------------DVFDGKVQRSLTVSHTSTEPSVNYDLFGAQ-GQYSNT-LLRIYRDN 216
SEB  EFN-------------NSPYEIGYIKQIEN-ENSFWYDMMPAPGDKFDQSKYLMMYNDN 220

SEK  KTVESEKFHLDVDISYKETI 219
SEI  KTIESEKFHLDVEISYVDSN 218
SEA  KTINSENMHIDIYLYTS--- 233
SEB  KMVDSKDVKIEVYLTTKK-- 238
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FIG. 3. Alignment of the mature form of SEK with representative toxins from the major enterotoxin subfamilies (SEA representing the SEA, -D, -E, -H, and -J subfamily; SEB representing the SEB, -C, and -G subfamily; and SEI representing the SEI, -K, and -L subfamily). Residues that are homologous to residues in SEK are highlighted in black. Dashes represent gaps in the aligned sequences.

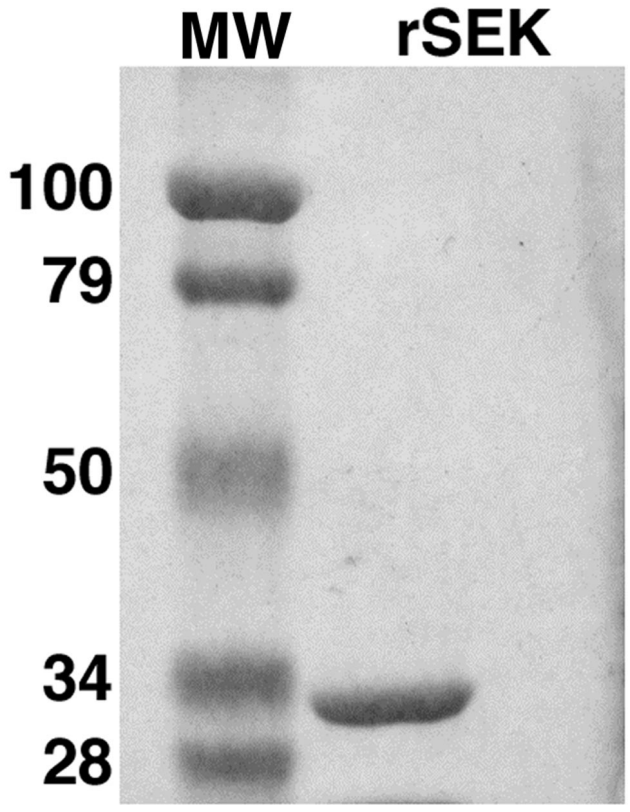

FIG. 4. SDS-PAGE analysis (15% polyacrylamide gel) of rSEK. The gel was stained with Coomassie brilliant blue R250. Lane 1, molecular weight (MW) standards with sizes given to the left in thousands; lane 2, 10 μg of rSEK. The apparent size of rSEK was 30,000.

levels of SEK. We were able to detect toxin production by Ouchterlony double immunodiffusion in one isolate (MN JA) and were able to see a reaction with a polyclonal antiserum on a Western blot (Fig. 5). Fourteen of the 36 isolates, including MN NJ, contained bands of the correct size which were reactive with the polyclonal antiserum to rSEK (data not shown).

Biological activity of rSEK. The ability of rSEK to stimulate rabbit splenocytes was assessed in a standard superantigenicity assay (Fig. 6). We observed that rSEK was able to stimulate splenocyte proliferation comparable to that of TSST-1. The pyrogenic activity of rSEK and its ability to enhance host susceptibility to endotoxin shock were also examined (Fig. 7). It was observed that rSEK caused fever in rabbits at doses of 4.5 and 0.45 μg/kg, but not at 0.045 μg/kg. The minimum pyrogenic dose of rSEK at 4 h (defined as the dose required to give a 0.5°C average rise in body temperature) was 0.2 μg/kg. This was consistent with minimum doses of other PTSAgs to cause fever. The same doses that were able to cause fever enhanced the lethality of endotoxin, while the nonpyrogenic dose was not active. Finally, the lethality of rSEK when infused into rabbits from subcutaneous miniosmotic pumps was assessed. All three rabbits infused with 200 μg of rSEK died within a 15-day period. The same dose of TSST-1 was also lethal in this model.

The TCR Vβ stimulation profile of rSEK was assessed for human T cells from three volunteers by use of flow cytometry. Antibodies were directed against the different TCR Vβ subsets in human PBMCs. It was observed that TCR Vβs 5.1, 5.2, and

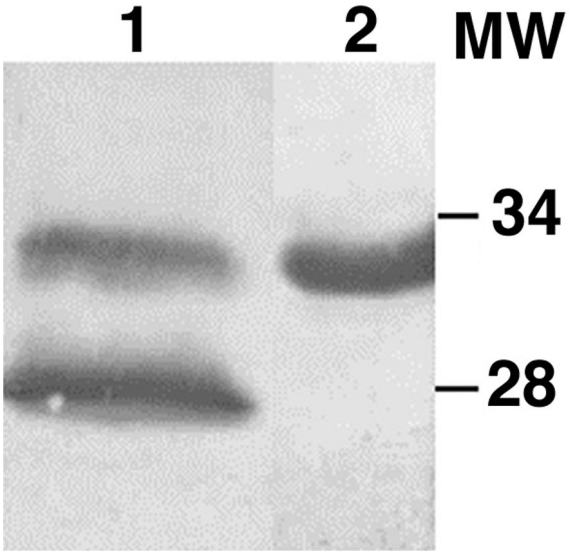

FIG. 5. Western immunoblot of concentrated supernatant fluid from TSS isolate MN JA compared to purified rSEK. Samples were electrophoresed by SDS-PAGE (15% polyacrylamide) and transferred to a nitrocellulose membrane. The blot was developed with hyperimmune rabbit polyclonal antiserum to rSEK. Bound antibody was detected with a secondary antibody to rabbit immunoglobulin G conjugated to alkaline phosphatase followed by substrate (4). Lane 1, 10 μl of crude extract of MN JA supernatant; lane 2, 10 μg of rSEK. MW, sizes in thousands as determined by SDS-PAGE.

6.7 were preferentially activated by the toxin (Fig. 8). The most prominent subset from all three subjects used was Vβ 5.1, reaching a highest level of 44% of the T-cell population in one case. Vβ 5.2 and 6.7 expansions were not as strong, but were still significant ($P = 0.055$ and 0.045, respectively). Consistent

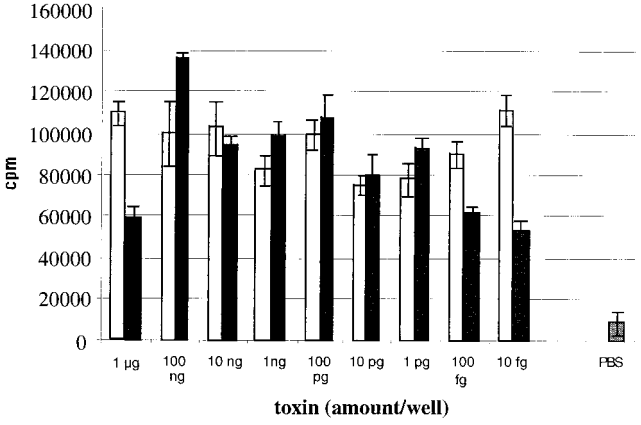

FIG. 6. Superantigenicity assay of SEK (□) versus TSST-1 (■) as assessed by measuring proliferation of rabbit splenocytes (2×10^5/well/200 μl). Splenocytes in complete RPMI were incubated for 4 days in quadruplicate samples in 96-well microtiter plates in the presence of rSEK or TSST-1 used as a control 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 cpm of radiolabel incorporation into DNA. Results are the average cpm of quadruplicate wells. Values represent the mean ± standard error of the mean.

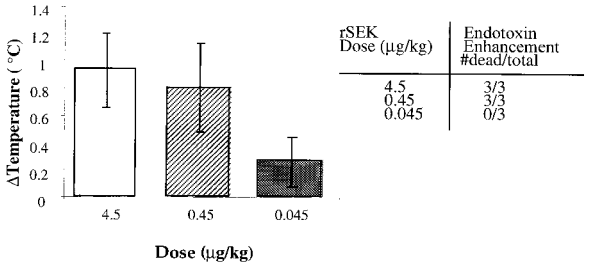

FIG. 7. Pyrogenicity of rSEK in rabbits and the ability of the toxin to enhance host susceptibility to lethal endotoxin shock. rSEK was injected intravenously at doses of 4.5, 0.45, and 0.045 μg/kg/ml in PBS. Fever development was assessed at 0 and 4 h with rectal thermometers. The values presented represent the mean ± standard error of the mean. At the 4-h time point, all rabbits were injected with 10 μg of LPS from *S. enterica* serovar Typhimurium. Lethality was assessed during the first 48 h postinjection; lethality at 4.5 and 0.45 μg/kg was significantly different from that at 0.045 μg/kg at $P = 0.05$, as determined by Fisher's exact test.

with a superantigenic effect, we observed an expansion of Vβs in $CD4^+$ and $CD8^+$ T-cell subsets. In one of the three patients tested, TCR Vβ 22 was also expanded, but the significance of this result was not clear. Some T cells bearing certain TCR Vβ subsets were reduced in relative numbers. Examples of this effect include Vβ 2, 3, 8, 9, and 13.1. This reduction in relative population size was not the result of apoptosis of T cells expressing those Vβs. Rather, the reduction resulted from those T-cell populations being present in smaller numbers through lack of stimulation relative to the T cells that were preferentially expanded by the toxin.

DISCUSSION

The data presented in this work indicate that a novel enterotoxin, designated SEK, is encoded by a gene (*sek*) contained on a SaPI, designated SaPI3, that also contains the gene (*seb*) for SEB. *sek* is also present on SaPI1 (29). SEK shares similar biochemical and biological properties to those described previously for enterotoxins and PTSAgs in general (5, 11). Because the presence of multiple virulence factor genes is a defining feature of pathogenicity islands (29), the presence of both *seb* and *sek* on SaPI3 validates the use of the term "pathogenicity island" in this case.

The presence of multiple expressed toxins also makes it less clear whether any single toxin is responsible for all clinical features of TSS induced by such clinical isolates of *S. aureus*. Rather, the data suggest that these strains make multiple toxins with similar activity and that all could contribute to human disease in the absence of protective antibodies; this does not preclude some toxins being more important than others due to differences in amounts made. There is a precedent for expression of multiple toxins by TSS *S. aureus* in the literature. As many as 15% of TSST-1-positive strains also produce SEC, and 75% of TSST-1-positive strains also make SEA (36). It now appears that certain strains may contain even more toxin genes.

Analysis of the sequence of *sek* and SEK, respectively, shows that SEK fits into a new subfamily of enterotoxins, along with the recently described SEI (34) and SEL (unpublished data). SEK is more closely related to the SEA, -D, and -E subfamily than to SEB and -C (Fig. 3), although *sek* can be genetically linked to *seb*, as on SaPI3. Analysis of the sequence 5′ of the putative *sek* translational start site reveals a Shine-Dalgarno sequence typical of *S. aureus* RBSs previously described (38). *sek* also contains −10 and −35 putative promoter sequences similar to those previously observed (38).

rSEK was superantigenic, capable of stimulating proliferation of both $CD4^+$ and $CD8^+$ T- cells, and pyrogenic and enhanced the lethal effects of endotoxin in a rabbit model. It was also lethal in a model of TSS in which miniosmotic pumps that deliver a constant amount of toxin over a 7-day period were implanted subcutaneously in rabbits. Although we have not evaluated rSEK for emetic activity, its homology to the other SEs suggests that SEK does belong to the SE subfamily of PTSAgs. It should be noted that the protein used in these

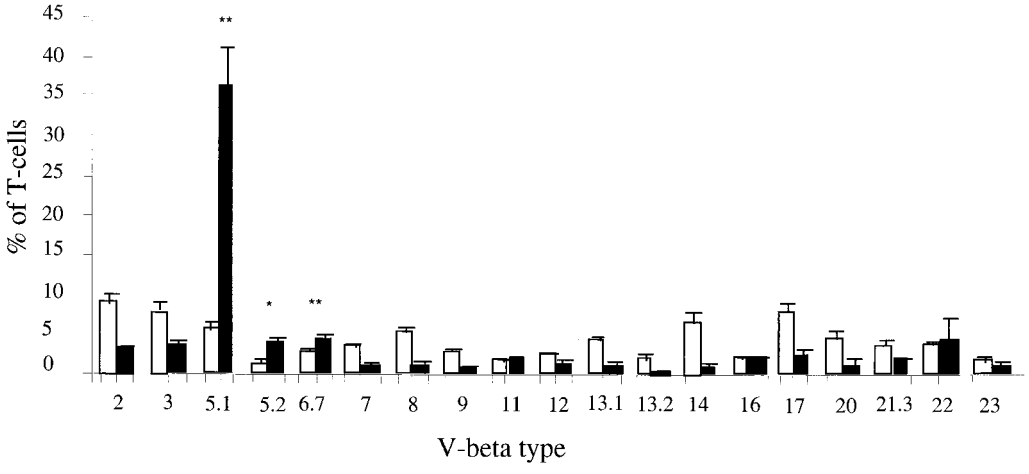

FIG. 8. TCR Vβ profile of rSEK. Three patients' PBMCs were stimulated with either anti-CD3 antibody (white bars), which stimulates all T cells, or rSEK (black bars), which selectively stimulates T cells dependent on the variable part of the β chain (Vβ) of the TCR. Cells were stained with monoclonal antibodies against the listed TCR Vβ chains (V-beta type), and the results were evaluated by flow cytometry. The percentages of T cells expressing the listed TCR Vβ are shown. *P* values were determined by the paired Student's *t* test (**, $P < 0.05$; *, $P = 0.055$). Error bars represent the standard error of the mean for each data set.

studies was cloned without its putative N-terminal signal sequence, and in order to express the protein in the pET system, a methionine and glycine were added to replace the N-terminal glutamine of the recombinant protein. Thus, the N-terminal sequence of rSEK was MGGDIGIDNLR, while the putative N-terminal sequence of SEK was QGDIGIDNLR. This alteration did not appear to have a significant effect on the biological activity of the toxin, however, since rSEK exhibited comparable immunobiological activity to those of TSST-1 and other PTSAgs (11, 51, 52). A putative degradation product of SEK was present in the supernatant of the clinical isolates we tested, as seen in the Western blot presented (Fig. 5). This is also seen for other SEs, where it has been suggested that staphylococcal proteases cleave the toxins, yielding a fairly stable, lower-molecular-weight, antibody-reactive product (5, 6).

The study of SEK is likely to increase our understanding of the structure-function relationships within the PTSAgs. The structures of TSST-1 (1, 42), SEA to -D (46, 55), and several streptococcal pyrogenic exotoxins (40, 45, 49) have been solved, as well as those of a variety of mutant staphylococcal toxins. In addition, SEB and -C have been crystallized in complex with the TCR (13, 28), as well as TSST-1 and SEB in complex with MHC class II (16, 17, 19). These studies have allowed detailed models of PTSAg activation of T cells to be developed. However, there are many facets of PTSAg activities which have not been explained in terms of structural differences. A prime example of this is emetic activity. The enterotoxins are uniquely characterized by their abilities to cause emetic responses when administered orally to monkeys (11), whereas other PTSAgs are not emetic (11). However, we still do not completely understand what parts of the SE molecules are required for this emetic function. It has been proposed that the cystine loop, located in the OB fold of the toxins, is important for emesis (15, 50), but this has only been incompletely studied. Because SEK has only one cysteine, the molecule does not contain a cystine loop at the usual position. One other enterotoxin (SEI) has been identified that lacks the cystine loop, and that protein was shown to be only weakly emetic compared to SEA, -B, and -C (34). Based on primary structure, it is likely that SEK will act more like SEI than the other SEs and thus be less emetic than other toxins. Structural studies of SEs, such as SEK and -I, and comparison to structures of highly emetic SEs may help elucidate what SE structural components are necessary for this activity.

Structural studies of the SEK, -L, and -I subfamily of PTSAgs may also increase our understanding of how superantigens interact with immune cells. For example, SEK generally functions similar to other PTSAgs in stimulation of T cells dependent on the composition of the TCR Vβ, but it has a TCR Vβ profile distinct from those previously observed. Only one other characterized toxin stimulates TCR Vβ 5.1 (SEE), and no other toxins have been observed to stimulate Vβ 5.2 or 6.7. The structure of the PTSAg must dictate the TCR Vβ profile of the stimulated T cells (10, 16, 18). It has been seen with other toxins that a large region at the top front (in the standard view of SEB and -C) or top back of TSST-1 is important for TCR binding (11, 13, 22, 28). Where SEK, SEI, and SEL interact with either TCR or MHC II remains to be determined.

It is noteworthy that SEK has the strongest homology with other SEs in the C-terminal β grasp domain, whereas some PTSAgs (SEA, for example) bind to MHC class II, opposite the usual MHC II site in the OB fold domain (46, 55). In SEA, zinc is coordinated by His187, His225, and Asp227, which are important residues in this MHC II binding site (46, 55). These residues are present in the same position relative to one another in SEK (His169, His208, and Asp210), suggesting SEK may interact with zinc and may have an MHC II binding site in this position. The zinc binding site in SEA also requires Ser1. Homologous residues are not present in either SEK or rSEK, implying that some other residue would be necessary to make up the final piece of the tetrahedral coordination site in this toxin. We do not know if zinc is present in rSEK, but clearly the N-terminal residue normally present on SEK (Gln1) is not required for superantigenicity, as Ser1 is for SEA (46); rSEK, which lacks Gln1, retains superantigenic activity.

We have shown that human PBMCs containing TCR Vβ 5.1, 5.2, and 6.7 were significantly stimulated by rSEK in vitro. TCR Vβ 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 (43). T cells from the Vβ 5 family have also been implicated in juvenile rheumatoid arthritis and periodontitis (32, 37). In the latter case, TCR Vβ 6.7 has also been observed to be overrepresented (37). PTSAgs from both *S. aureus* and group A streptococci have also been implicated in forms of psoriasis and atopic dermatitis (23–27, 53). Previous studies have isolated *S. aureus* from psoriatic lesions, and some of the organisms were categorized as non-enterotoxin producing based on antibody testing against toxins identified at the time (26). It is possible that new SEs, such as SEK, may play a role in previously unexplained cases of these illnesses.

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