Characterization of the Canine Type C Enterotoxin Produced by *Staphylococcus intermedius* Pyoderma Isolates

VINCENT M. EDWARDS,¹ JAMES R. DERINGER,¹ SCOTT D. CALLANTINE,¹ CLAUDIA F. DEOBALD,¹
PHILIP H. BERGER,² VIVEK KAPUR,³ CYNTHIA V. STAUFFACHER,⁴
AND GREGORY A. BOHACH^{1*}

Department of Microbiology, Molecular Biology, and Biochemistry¹ and Department of Plant, Soils, and Entomological Sciences,² University of Idaho, Moscow, Idaho 83844; Department of Veterinary Pathobiology and Advanced Genetic Analysis Center, University of Minnesota, St. Paul, Minnesota 55108³; and Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907⁴

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The type C staphylococcal enterotoxins (SECs) are a group of highly conserved proteins with substantial antigenic cross-reactivity. Although *Staphylococcus intermedius* and coagulase-positive species of staphylococci are reported to produce SEC and other SEs, toxins produced by species other than *Staphylococcus aureus* have not been previously characterized. In this study we report the molecular, biological, and immunological properties of the canine SEC (SEC_{canine}) expressed by pathogenic isolates of *S. intermedius*. The mature form of SEC_{canine} has 239 amino acid residues and a pI of 7.0. Typical of the SEs, purified SEC_{canine} induces an emetic response in monkeys and the proliferation of T cells in a V β -dependent manner. Although SEC_{canine} has >95% sequence identity to previously described SEC variants, its sequence is most related to SEC2 and SEC3. In contrast to the sequence similarity, the V β profile induced by SEC_{canine} is typical of that induced by SEC1. This result is likely explained by the conservation of a cysteine residue at position 26 in SEC_{canine}; residues at this position have been previously shown to determine subtype-dependent differences in T-cell receptor interactions of other SEs. Overall, these results show that superantigen toxins produced by the multiple members of the genus *Staphylococcus* are highly conserved in respect to biological and structural properties. Further, the frequent association of SEC_{canine} with pyoderma in dogs supports the notion that the toxins are important for staphylococcal survival and pathogenesis.

Staphylococcal enterotoxins (SEs) are members of the pyogenic toxin (PT) family (6, 8). The PT family includes several SE antigenic variants (staphylococcal enterotoxin A [SEA], SEB, SEC1, SEC2, SEC3, ovine type C SE [SEC_{ovine}], SEC_{bovine}, SED, SEE, and SEH) (7, 34, 41), toxic shock syndrome toxin 1 (TSST-1) (5), TSST_{ovine} (32), streptococcal pyrogenic exotoxins (streptococcal pyrogenic exotoxin A [SPEA], SPEB, SPEC, and SPEF) (3, 24, 29), and streptococcal superantigen (40). The PTs have been typically associated with two gram-positive cocci, *Staphylococcus aureus* and *Streptococcus pyogenes* (8). The PTs are confirmed virulence factors in several human and animal diseases caused by these two species, which have a broad host range.

We previously demonstrated that *S. aureus* bovine and ovine isolates produce unique molecular variants of SEC (SEC_{bovine} and SEC_{ovine}, respectively) (34). The slightly divergent sequences of these two toxins affect their biological properties and have been proposed to represent bacterial adaptation to their broad range of potential hosts. Similar conclusions were made in regard to molecular variants of TSST (32). Molecular heterogeneity resulting from allele differences and potential posttranslational modification has also been reported for several of the streptococcal toxins (37, 44).

The PTs studied to date exhibit a diverse group of shared and unique biological activities. All PTs bind to major histocompatibility complex class II molecules outside the antigen binding groove. This causes them to stimulate T lymphocytes via a unique mechanism by which they interact with the V β

region of α/β T-cell receptors (TCR). As a result, each PT stimulates a characteristic, or fingerprint, V β profile (12, 15, 16, 26). Molecules with these properties are termed superantigens (SAGs) (35). Presumably, superantigenicity is responsible for many shared PT activities. Some of these activities include shock, cytokine induction, pyrogenicity, lymphocyte proliferation, and immunosuppression (8). Although SEs are SAGs, they possess a unique activity not shared by other PTs. They have long been known to induce emesis and diarrhea following oral ingestion and are the agents of staphylococcal food poisoning (25).

Although both organisms can be isolated from dogs, Hajek (20) differentiated *Staphylococcus intermedius* from *S. aureus* on the basis of several biochemical reactions. *S. intermedius* isolates had previously been classified as *S. aureus* biotypes E and F (21, 22). *S. intermedius* is the predominant organism isolated from canines and is most often associated with canine pyoderma or hot spots (2, 14, 19, 23, 27, 28). *S. intermedius* has also been isolated from the gingivae of certain canine breeds and from three cases of human skin infections caused by dog bite wounds (45).

Based on analysis of culture supernatants, between approximately 5 and 40% of *S. intermedius* isolates produce a protein that is serologically related to currently recognized *S. aureus* SEs (1, 2, 14, 18, 19, 22, 23, 27, 28). Although *S. aureus* SEs have been studied extensively, information on SEs from other species of staphylococci is extremely limited (7). Our laboratories collected several SEC-producing *S. intermedius* pyoderma isolates over a period of two years. The *sec* gene was isolated from three independent isolates and sequenced. In addition, the biological properties of the toxin were determined by using protein purified from one of the three isolates.

* Corresponding author. Phone: (208) 885-6666. Fax: (208) 885-6518.

MATERIALS AND METHODS

Bacterial isolates. Specimens obtained from dogs with confirmed pyoderma were isolated initially on sheep blood agar plates. *S. intermedius* was identified by the methods described by Hajek (20); the definitive tests were lack of growth on P-agar supplemented with acriflavine (7.0 µg/ml) and the production of β-galactosidase when grown in ONPG broth (20, 42). Each *S. intermedius* isolate was screened for expression of SEA, SEB, SEC, SED, and TSST-1 by immunodiffusion techniques as described previously (38). Three *S. intermedius* isolates that produced SEC (strains 95-011195, 95-062195, and 95-072895) were chosen for further analysis of the toxin.

Toxin purification. *S. intermedius* 95-011195 was used to purify SEC_{canine}. Cultures were grown to stationary phase in dialyzable beef heart medium at 37°C with aeration and then precipitated with 4 volumes of ice-cold ethanol. Insoluble material was recovered after 4 days of precipitation at 4°C. A crude toxin concentrate, prepared by resolubilizing the precipitate in pyrogen-free distilled water, was clarified by centrifugation and dialyzed overnight against distilled water. SEC_{canine} in the retentate was purified by preparative flatbed isoelectric focusing (IEF) as described previously (8). The proteins were first resolved in a pH gradient from pH 3.5 to 10. Fractions containing SEC_{canine} were identified by immunodiffusion, pooled, and refocused in a narrow pH gradient from pH 6.0 to 8.0 to achieve maximum purification. Fractions containing homogenous SEC, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (31), were pooled, aliquoted, and stored in a lyophilized state until used.

PCR amplification and cloning of the SEC_{canine} gene. Chromosomal DNA was prepared from *S. intermedius* isolates 95-011195, 95-062195, and 95-072895 by standard techniques (9). Primers that had been used previously for successful *sec* gene amplifications (34) were used to amplify the SEC_{canine} structural gene from DNA from all three isolates. The primers corresponding to regions that are conserved among all known *sec* genes plus 9-base flanking extensions with *HindIII* or *BamHI* restriction sites to facilitate cloning were GGGAAGCTGTGAATTTT GATATTCGACT and CCCGGATCCTATCAAAATCGGATTAACA, respectively.

PCR amplification was performed with an AmpliTron II instrument (Barnstead/ThermoLyne Corp., Dubuque, Iowa) by using the following thermal profile: 5 min at 97°C; 45 cycles of PCR, with 1 cycle consisting of 30 s at 92°C, 1 min at 40°C, and 1 min at 72°C; and 5 min at 72°C. The PCR mixtures were held at 4°C until further use. A 1% agarose gel was used to purify the amplified nucleic acids (approximately 800 bp long) which were excised from the gel, extracted, and stored as precipitates in ethanol.

For cloning, the PCR products were digested with either *HindIII* or *BamHI* and ligated to appropriately digested phagemid pBluescript II KS+ (Stratagene, La Jolla, Calif.) by standard molecular biology techniques (33). Following ligation, the recombinant phagemids were transformed into *Escherichia coli* DH5α followed by growth under ampicillin selection on Luria-Bertani agar plates. Several representative colonies were picked and screened for the presence of *sec* by miniplasmid analysis.

Nucleotide sequencing and analysis. Nucleotide sequencing of *sec*_{canine} from *S. intermedius* was determined initially by manual methods employing the dideoxynucleotide sequencing methodology (43) with the Sequenase version 2.0 commercial kit (U.S. Biochemical Corp., Cleveland, Ohio). By this method, the sequence was determined for *sec*_{canine} amplified from each of the three *S. intermedius* isolates. To achieve these reactions, the PCR products were cloned into pBluescript II KS+ (see above) and converted into single-stranded template according to the manufacturer's suggestions. The nucleotide sequence of *sec* from all three strains (one PCR from each isolate) was identical by this method. These results were confirmed by performing three additional PCRs for each isolate and sequencing the products by two different automated techniques below.

The first automated sequencing method used miniplasmid DNA isolated from three separate *E. coli* DH5α clones containing the *sec* gene insert (one for each PCR). Purified plasmid DNA was denatured and sequenced by using the Sequi-therm Long Read cycle sequencing kit (Epicentre Technologies, Madison, Wis.) and an LI-COR 4000L automated DNA sequencer (LI-COR, Lincoln, Nebr.) per the manufacturer's instructions. Dye-labeled M13 forward and reverse primers (LI-COR) were used for all reactions. Approximately 800 bases of sequence were routinely obtained.

The second automated method directly sequenced the 800-bp PCR-generated *sec* fragment without cloning. Unincorporated nucleotides and primers were separated from amplified DNA by filtration through Micron 100 microconcentrators (Amicon, Inc., Beverly, Mass.). Sequencing reactions with the Taq FS DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, Calif.) were performed with 3 µl of PCR-amplified DNA as the template and 3.2 pmol of either primer used for PCR amplification. Unincorporated dye terminators and primers were separated from the extension products by spin column purification (Centri-Sep; Princeton Separations Inc., Adelphi, N.J.). The products were dried in a vacuum centrifuge, resuspended in 4 µl of loading buffer (5:1 deionized formamide–50 mM EDTA; pH 8.0), heat denatured for 2 min at 90°C, and immediately loaded on an acrylamide gel in an Applied Biosystems model 377 automated DNA sequencer. The DNA fragments were completely sequenced in both orientations.

Eight individual alleles of *sec* had been previously reported (34) and deposited in GenBank (accession nos. L13374 and L13379). These alleles and *sec*_{canine}

(accession no. U91526) were analyzed for pairwise comparisons of the number of synonymous site (d_s) and coding substitutions per nonsynonymous site (d_N) by the method of Nei and Gojobori (36).

Emesis assay. Assessment of SEC_{canine} for emetic activity was accomplished by a modification of the standard monkey feeding assay (4) using young adult pigtailed monkeys (*Macaca nemestrina*) weighing 5 to 10 kg. The animals were manually restrained while the toxin (10 µg per kg of body weight) was administered through a nasogastric tube (infant feeding tube; Becton Dickinson, Rutherford, N.J.). The animals were returned to their cages and observed for emesis.

Lymphocyte proliferation assays. Peripheral blood mononuclear cells (PMBC) were obtained from samples of heparinized whole blood collected from human volunteers by venipuncture and then fractionated by centrifugation through a Ficoll-Paque gradient (blood-Ficoll-Paque [6:4, vol/vol]) (11). PMBC were resuspended in RPMI 1640 for washing and then recovered by centrifugation. The cells were resuspended in complete RPMI 1640 medium (RPMI 1640 containing 2% fetal bovine serum, 2 mM glutamine, 200 U of sodium penicillin G per ml, and 200 µg of streptomycin sulfate per ml). The PMBC were resuspended in 25 ml of complete RPMI, plated in petri plates, and allowed to incubate overnight at 37°C (6.5% CO₂). The nonadherent lymphocyte-enriched cells were collected, washed, resuspended at a concentration of 1.0×10^6 cells/ml, and separated into aliquots (200 µl/well) in 96-well tissue culture plates (Costar, Cambridge, Mass.).

The ability of SEC molecular variants to induce lymphocyte proliferation in these cultures was determined in a standard 4-day assay (39). Serial dilutions of each toxin were added in triplicate to each culture to stimulate PMBC proliferation. After 3 days, 1 µCi of [³H]thymidine (NEN Research Products, Boston, Mass.) was added to each culture which was incubated for an additional 18 to 24 h. Radiolabeled DNA was harvested onto glass-fiber filters (Skatron, Sterling, Va.) with a semiautomatic cell harvester (Skatron). The amount of radiolabel incorporation into cellular DNA was quantified by liquid scintillation counting.

Quantitative PCR to measure Vβ expansion. The PCR technique described by Choi et al. (12) and modified by Kotb et al. (30) and Deringer et al. (16) was used to quantify Vβ expression in cultures stimulated by SEC_{canine}. This assay is based on the ability to distinguish 22 different Vβ-expressing human α/β TCR subsets. Human PBMC were collected from multiple donors and processed as described above. The lymphocyte-enriched cell suspensions were adjusted to a concentration of 2.5×10^6 cells/ml. Aliquots (3 ml each) were stimulated for 4 days (37°C, 6.5% CO₂) with toxins at a final concentration of 0.5 µg/ml. Each experiment included cultures stimulated with an anti-CD3 (human) monoclonal antibody (final concentration, 33 ng/ml; Sigma Chemical Co., St. Louis, Mo.) to determine the basal levels of the 22 detectable human Vβs.

Total RNA was isolated from cell cultures after stimulation by using Trizol reagent (Life Technologies, Gaithersburg, Md.). cDNA was prepared from 2 to 6 µg of total RNA by using Superscript II reverse transcriptase (Life Technologies) and random hexamers. Expression of each TCR Vβ element was detected by PCR Vβ-specific primers in combination with a primer that recognizes the constant segment on all TCR β-chain transcripts (Cβ primer). A quantitative determination of the total α/β TCR T-cell population was performed with two PCR primers that recognize a conserved region of the TCR α chain (Cα primers). All primers used in this study, including those for amplification of 22 known human Vβ subfamilies, have been previously described (12). To quantify the PCR-generated Cα and Vβ TCR transcripts, the 3' Cα and 3' Cβ primers were 5' end labeled with [γ-³²P]dATP. Coamplified Cα and Vβ PCR products from each of the 22 reaction tubes were resolved on 2% agarose gels, visualized by autoradiography, and quantified by densitometry as previously reported (16). The percentage of each Vβ in toxin-stimulated cultures was compared to the percentage of the same Vβ in the corresponding anti-CD3-stimulated culture and expressed as a ratio (the expansion index value). Expansion index values greater than 1.0 indicate proliferation.

N-terminal sequencing. The N-terminal sequence for SEC_{canine} was determined by automated Edman degradation with an Applied Biosystems protein sequencer 475A.

RESULTS

General properties of SEC from *S. intermedius*. The three isolates used in this study were identified as *S. intermedius* based on their inability to grow on P-agar supplemented with acriflavine (7.0 µg/ml) and their production of β-galactosidase. Each isolate produced a protein that was immunologically related to previously described human and animal SEC variants as determined by their reactivity with anti-SEC1 and anti-SEC2 hyperimmune rabbit sera. SEC, purified to homogeneity from cultures of *S. intermedius* 95-011195 by IEF, resolved into a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (results not shown). The apparent size of the protein (approximately 27,000 Da) was consistent with other SEC subtypes and animal-associated molecular variants re-

GAG AGT CAA CCA GAC CCT ATG CCA GAT GAT TTG CAC AAA TCA AGT	45
E S Q P D P M P D D L H K S S	15
GAG TTT ACT GGT ACG ATG GGT AAT ATG AAA TGT TTA TAT GAT GAT	90
E F T G T M G N M K C L Y D D	30
TAT TAT GTA TCA GCA ACT AAA GTT AAG TCT GTA GAT AAA TTT TTG	135
Y Y V S A T K V K S V D K F L	45
GCA CAT GAT TTA ATT TAT AAC ATT AGT GAT AAA AAA CTG AAA AAC	180
A H D L I Y N I S D K K L K N	60
TAT GAC AAA GTG AAA ACA GAG TTA TTA AAT GAA GAT TTA GCA AAG	225
Y D K K V K T E L L A N E D L A K K	75
AAG TAC AAA GAT GAA GTA GTT GAT GTA TAT GGA TCA AAT TAC TAT	270
K Y K D E V V D V Y G S N Y Y	90
GTA AAC TGC TAT TTT TCA TCT AAA GAT AAT GTT GGT AAG GTT ACA	315
V N C Y F S S K D N V G K V T	105
GGT GGT AAA ACT TGT ATG TAT GGA GGA ATA ACA AAA CAT GAA GGC	360
G G K K T G T A T G G A I T K H E G	120
AAC CAC TTT GAT AAT GGG AAC TTA CAA AAT GTA CTT ATA AGA GTT	405
N H F D N G N L Q N V L I R V	135
TAT GAG AAT AAA AGA AAC ACA ATT TCT TTT GAC GTG CAA ACT GAT	450
Y E N K R C N Y I T V D V Q T D	150
AAG AAA AGT GTA ACA GCT CAA GAA CTA GAC ATA AAA GCT AGG AAT	495
K K S V T A Q E L D I K A R N	165
TTT TTA ATT AAT AAA AAT TTT TAT GAG TTT AAG AGT TCA CCA	540
F L I N K M Y I E F N S S P	180
TAT GAA ACA GGA TAT ATA AAA TTT ATT GAA AGT AAC GAC AAT ACT	585
Y E T G Y I K F I E S N D N T	195
TTT TGG TAT GAT ATG ATG CCT GCA CCA GGC GAT AAG TTT GAC CAA	630
F W Y D M M P A P G D K F D Q	210
TCT AAA TAC TTA ATG ATA TAC AGC GAC AAT AAA ACG GTT GAT TCT	675
S K Y L M I Y S D N K T V D S	225
AAA AGT GTG AAG ATA GAA GTC CAC CTT ACA ACA AAG AAT GGA TAA	720
K S V K I E V H L T T K N G	239

FIG. 1. Nucleotide and deduced amino acid sequences of the mature form of SEC_{canine}. The sequences at the amino acid and nucleotide levels were identical for all three isolates used in this study. Numbering is in reference to the first codon of the predicted mature protein.

ported thus far (34). Based on its localization in the pH gradient during IEF, we concluded that the toxin had a pI of approximately 7.0.

Sequencing results. Nucleotide sequencing was performed on the *sec* genes amplified by at least three independent PCRs for each *S. intermedius* strain. The nucleotide and deduced amino acid sequences of the amplified *sec* genes encoding SEC_{canine} were 100% conserved among all three isolates (Fig. 1). In addition, N-terminal sequencing of purified SEC_{canine} showed that the site for cleavage of the signal peptide is identical to that of SEC1 (10).

Figure 2 shows an alignment of SEC_{canine} from *S. intermedius* with other SEs including the currently known SEC subtypes produced by *S. aureus* (SEC1, SEC2, SEC3, SEC_{ovine}, and SEC_{bovine}). The translated amino acid sequences showed 93 to 96% sequence identity with the various *S. aureus* SEC proteins, and the structural genes had 95% to 97% homology at the nucleotide level (Table 1). SEC_{canine} was the least related to SEC_{ovine} and most homologous to SEC3-FRI913. Amino acids 20, 22, and 26 are key residues for determining the antigenic epitope which distinguishes SEC1 from SEC2 and SEC3 (46). SEC_{canine} is identical to SEC2 and SEC3 at residues 20 and 22. Position 26, occupied by cysteine in SEC_{canine}, is unique from all previously sequenced SEC subtypes and molecular variants.

Patterns of nucleotide substitution among *sec* alleles. To determine whether there is evolutionary pressure acting on the *sec* genes to mutate in response to host selection or to increase the fitness of the organism, we calculated the ratio of the number of nucleotide substitutions that result in amino acid

replacement (nonsynonymous substitutions) to those that do not (synonymous substitutions). The number of potential nucleotide substitutions among all possible pairs of sequences is large, and in many instances there are two or more nucleotide differences within a single codon. We therefore calculated the numbers of synonymous changes per synonymous site (d_s) and coding changes per nonsynonymous site (d_N) by the unweighted pathway of Nei and Gojobori (36). The results indicate an excess of synonymous over nonsynonymous substitutions in the *sec* gene. The d_s values for all nine known *sec* alleles were 0.040 ± 0.010 , while the d_N values were 0.016 ± 0.000 , a d_s/d_N ratio of 2.5:1.

Emesis. In the standard monkey feeding assay using *M. nemestrina*, SEC1 and SEC_{canine} dissolved in saline caused an emetic response after approximately 2 h when administered in a dose of 10 μ g/kg. Control animals, given saline only, did not exhibit signs of emesis after 12 h.

Mitogenicity. The ability of SEC_{canine} to stimulate lymphocytes in cultures of PMBC was compared to that of SEC1, SEC_{bovine}, and SEC_{ovine} (Fig. 3). Stimulation was assessed by measuring the incorporation of [³H]thymidine into cellular DNA following stimulation with each of the four SECs. Consistent with our prior observations, SEC1 and SEC_{ovine} were similar to each other in T-cell proliferation induction ability (34). Further, both were significantly more potent than either SEC_{bovine} or SEC_{canine}, which had a similar mitogenicity dose response. Tenfold more SEC_{canine} or SEC_{bovine} was required to induce a level of T-cell proliferation comparable to that of SEC1 or SEC_{ovine}. Although the peak response for all four toxins was achieved at the same dose (1.0×10^{-3} μ g), the maximum proliferation induced by SEC_{canine} and SEC_{bovine} was significantly less than for the other two SEC variants tested (Fig. 3).

V β profile. The ability of SEC_{canine} to act as a SAg by stimulating specific T-cell subpopulations was determined from experiments with cells from three different human donors. SEC_{canine} consistently induced the proliferation of T cells bearing TCR V β elements 3, 12, 13.2, 14, 15, 17, and 20 (Fig. 4). Although weak proliferation of T cells bearing V β s 5.2, 10, and 11 was produced in some experiments, this was not a consistent observation. Inconsistent with their relative overall sequence relatedness, the V β expansion profile of SEC_{canine} was more similar to SEC1 than to SEC3. SEC_{canine} had a strong proliferative effect on V β 3-bearing T cells but was unable to expand V β 13.1 above basal levels. SEC_{canine} also induced a consistently low-level expansion of V β 20 compared to other SECs which expand V β 20 to levels approximately three times greater.

DISCUSSION

The goals of this project were to clone, purify, characterize, and compare the SECs from several representative *S. intermedius* isolates. Although enterotoxin production by *S. intermedius* has been documented previously, this is the first detailed characterization of an SE produced by a species of staphylococci other than *S. aureus*. The mature form of the toxin contains 239 amino acids, has a predicted molecular weight of 27,600, and has a pI of 7.0. Based on its reactivity with SEC-specific antisera, the relatedness of its sequence with those of the other SEC toxins, and its unique but consistent association with canine infections, we propose the designation SEC_{canine} for this toxin.

Overall, SEC_{canine} shares numerous properties with other SEs. First, its ability to induce an emetic response when administered to monkeys via the oral route confirmed that this

FIG. 2. Alignment of the predicted primary sequences of SEC_{canine} with other SEs including SEC subtypes and host-specific molecular variants reported previously by Marr et al. (34). Identical amino acid residues are shown in boxes, and similar residues are indicated by shading. The sequences were aligned using the program PILEUP from the Genetics Computer Group suite of software (17). Similarity is based on the Dayhoff PAM matrix (13). The dashes indicate gaps introduced to optimize alignment.

toxin has enterotoxic activity and is a potential source of food poisoning. In addition, SEC_{canine} was able to stimulate T cells. Its potency in vitro was similar to that of SEC_{bovine}, but both SEC_{canine} and SEC_{bovine} were 10-fold less effective than either SEC1 or SEC_{ovine} in T-cell proliferation dose-response assays.

SEC_{canine} induced the proliferation of T cells in a V β -dependent manner, confirming that it is an SAg. This toxin consistently induced expression of human TCR V β elements 3, 12, 13.2, 14, 15, 17, and 20. It has been proposed that the significant relatedness of these receptors at the amino acid sequence level, as reflected in their assignment to the same V β subgroups, corresponds to their similar expansion by related SAGs such as the SECs (16). Consistent with this prediction, the V β expansion profile of SEC_{canine} was identical to that of SEC1 with the exception of two minor differences. One difference is the weak but inconsistent expansion of V β 5.2, which has not been observed for other SEC variants (16). In addition, the

consistently low expansion of V β 20 is uncharacteristic of SEC toxins, which typically induce efficient expansion of this V β . The V β profile induced by SEC_{canine} is interesting from several standpoints. Although the amino acid sequence of SEC_{canine} is most similar to those of SEC2 and SEC3, its V β induction profile is more typical of that induced by SEB or SEC1. SEC_{canine} had a strong proliferative effect on V β 3-bearing T cells but was unable to expand T cells expressing V β 13.1 above basal levels. We reported previously that residue 26 is a key residue for determining the V β specificity of the SEC toxins (and SEB) (16). For example, SEC2 and SEC3, which have a bulky tyrosine residue at this position, are unable to expand cells expressing V β 3. Based on mutagenesis and molecular modeling experiments, this finding could be explained by potential steric conflict between large amino acid side chains at position 26 (i.e., tyrosine) and two large side chains (lysine-70 and arginine-73) of the hypervariable 4 element on V β 3 (16).

TABLE 1. Sequence identity between SEC_{canine} and other SECs

SEC	% Identity to SEC _{canine}	
	Protein ^a	Gene ^b
SEC _{bovine}	93.72	95.56
SEC _{ovine}	93.31	95.56
SEC1	93.72	95.83
SEC2	95.40	97.22
SEC3-FRI909	95.40	97.50
SEC3-FRI913	96.23	97.78

^a The percent identity for each toxin compared to SEC_{canine} is given for the predicted mature protein.

^b The percent identity for each toxin gene compared to the SEC_{canine} gene is given for the region coding the predicted mature protein.

Presumably, the smaller side chain of the cysteine residue in the analogous position of SEC_{canine} behaves more like valine-26 of SEC1 in this regard. It is noteworthy that streptococcal SAg has cysteine in the analogous location which is presumably its TCR binding cavity (40). In contrast, the two cysteine residues (three for SEC_{ovine}) are typically limited to the area of the disulfide bond and cystine loop, two hallmark SE structural features (25).

SEC_{canine} produced by *S. intermedius* isolated from dogs with pyoderma is the third reported example of host-specific SEC molecular variants produced by staphylococci isolated

from animals (34). To date, nine different *sec* alleles from human and animal staphylococcal isolates have been sequenced. The analysis of the sequences of these nine alleles indicates an excess of synonymous substitutions compared with nonsynonymous ones, suggesting that there has been evolutionary pressure to conserve most of the primary structure of the SEC toxin in isolates of *S. aureus* and *S. intermedius*. Despite this conservation, molecular heterogeneity in biologically important regions, such as the TCR binding cavity, raises some interesting questions regarding the potential role of PT SAg in staphylococcal diseases. For example, the ability of each SEC and TSST variant to induce quantitatively different levels of proliferation among T cells from various animals has been documented previously and in this study (32, 34). At the present time one cannot conclude with certainty whether the minor differences in SAg sequences provide a selective advantage to the organism. Although immunomodulatory and potentially immunosuppressive activity of SAg can be demonstrated, the in vivo relevance of this phenomenon is currently unknown. One could speculate that inducing immunosuppression offers an advantage to the pathogen and that PT molecular variants represent adaptations which allow the SAg to interact more efficiently with receptors in certain hosts. This type of adaptation could be beneficial to organisms such as staphylococci, which have a broad host range and encounter significant receptor diversity among its cadre of potential hosts. Confirmation of this possibility will require experimental in-

FIG. 3. Stimulation of human PBMC by SEC1, SEC_{bovine}, SEC_{ovine}, and SEC_{canine}. The results shown are from one representative experiment, which was performed three times. The data shown are the means $\pm$ the standard errors of the means, using data collected in triplicate.

FIG. 4. V β -dependent stimulation of human T cells by SEC_{canine}. (A) Autoradiograms from a representative experiment using PCR to coamplify segments of the TCR C α (internal control) and the indicated TCR V β s. Anti-CD3 monoclonal antibody (X-CD3) and SEC_{canine} were used as stimulants in this experiment. Both stimulations for this figure were done using cells from the same PBMC preparation. (B) The V β expansion index for SEC_{canine} is determined from autoradiograms as previously described (16). V β expansion profiles show the mean expansion index values $\pm$ standard errors of the means for SEC1, SEC3-FRI913, and SEC_{canine} used in this study. The data shown represent the combined results from human PBMC from three different donors. The mean V β expansion index represents the n -fold difference in the percentage of V β in toxin-stimulated cultures compared to the anti-CD3 stimulation (basal level) using the same PBMC preparation. Mean expansion index values greater than 1.0 indicate expansion.

fections in various animals comparing the virulence of isogenic strains differing only in the toxin variant produced.

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