Unique Superantigen Activity of Staphylococcal Exfoliative Toxins


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Unique Superantigen Activity of Staphylococcal Exfoliative Toxins


Certain strains of Staphylococcus aureus express one or both of two related, but immunologically distinct, exfoliative toxins (ETA and ETB). These toxins induce the symptoms associated with staphylococcal scalded skin syndrome. Both ETs have been shown to stimulate T cell proliferation. Recently, it was reported that ETA is a superantigen that stimulates T cells bearing human Vβ2 or several murine Vβs. However, other investigators have proposed that the superantigenicity reported for ETA resulted from contaminants in commercial preparations. This present study addresses those conflicting reports by assessing the biological and immunologic activities of highly purified RETs. ETA and ETB required APCs to induce selective polyclonal expansion of several human Vβs (huVβs), although, neither toxin expanded huVβ2. ETB induced expansion of murine T cells bearing Vβs 7 and 8, those that have the highest homology to the huVβs expanded by ETA and ETB. Although flow cytometry of ETB-stimulated T cells matched PCR results, stimulation by ETA reduced percentages of T cells positive for several huVβs that had been shown to have increased levels of mRNA transcripts. ETA and ETB induced contrasting reactions in vivo. In rabbits, ETB was moderately pyrogenic and enhanced susceptibility to lethal shock, while ETA lacked both activities. Predictions based on comparisons with other superantigens suggest molecular regions potentially involved in receptor binding in the ETA crystal structure and a modeled ETB three-dimensional structure. These results show that ETs are superantigens with unique properties that could account for the discrepancies reported. The Journal of Immunology, 1999, 162: 4550–4559.

One or both of two serologic variants (A and B) of exfoliative toxins (ETs) are expressed by a low, albeit significant percentage, of Staphylococcus aureus isolates (1). Both ETs are produced predominantly by phage group II staphylococci and induce intraepidermal skin peeling, characterized by separation of the epidermis at the desmosomes, leading to a positive Nikolsky sign (2). This biological activity is associated with the etiology of an illness termed staphylococcal scalded skin syndrome, which occurs predominantly in the very young before the development of protective Ab.

Although several mechanisms (i.e., protease activity and T cell proliferative ability) have been proposed, a definitive mechanism for the pathogenesis of ET-induced intraepidermal skin peeling remains elusive. A significant amount of sequence and structural information has been determined for both ETA and ETB (1, 3). This work has provided several lines of evidence that suggest that the ETs may act as serine proteases. The crystal structure of ETA shows similarity to the chymotrypsin-like family of serine proteases (3, 4). Moreover, substitution of serine residue 195 in the putative catalytic site abrogates the ability of ETA to induce skin peeling in the murine model, although the biologically relevant substrate remains to be determined (3, 5). The structure of ETA also suggests that enzymatic activity may be, unlike typical serine proteases, directly regulated by unique conformational attributes that restrict access to the putative catalytic site until association with a specific cellular receptor occurs.

The ability of ETs to induce potent T cell proliferation has long been recognized (6). Most reports have shown that nanogram quantities of either ETA or ETB are sufficient to induce a substantial proliferation of T cells in human PBMC cultures (1). Presently, it is not clear whether the interactions with immune cells contribute to ET-mediated pathogenesis. However, while it has been shown that the ETA (S195C) mutant toxin is unable to induce skin peeling, induction of T cell proliferation by this mutant toxin is fully achieved (3). The mechanism responsible for T cell stimulation by ETs is controversial. Like many other staphylococcal exotoxins, efficient ET-induced T cell proliferation reportedly 1) requires MHC class II accessory cells (7), and 2) occurs in a Vβ-dependent manner (1). Molecules with these two properties have been termed superantigens (SAGs) (8), and some staphylococcal and streptococcal exotoxins are prototypic microbial SAGs. Most currently known staphylococcal and streptococcal SAGs have been grouped into a large family of toxins known as the pyrogenic toxins (PTs) based on a number of shared biological properties (9, 10). This family of toxins includes the staphylococcal enterotoxins.
(SEs), toxic shock syndrome toxin-1 (TSST-1), the streptococcal pyrogenic exotoxins (SEs), and other more recently described toxins.

Although information regarding Vβ reactivity of ETB is limited, several studies suggest that ETA recognizes a limited repertoire of human and murine Vβ elements (huVβ and muVβ, respectively), consistent with its classification as a SAg. Choi et al. (11) first proposed that ETs are SAgs based on their observation that stimulation of human T cells with ETA induced a slight but significant increase in huVβ2 mRNA. Callahan et al. (12) used several techniques to assess muVβ skewing in murine T cells stimulated with ETA. They reported that ETA caused increased numbers of cells expressing muVβ15. Although the ability to interact with muVβ15 parallels its high degree of relatedness (45% homologous) to huVβ2, other less related muVβs, including 1, 3, 8, 2, 10, and 11, were also stimulated by ETA in that former study (12).

A recent report by Fleischer (13) disputed the evidence that ETs are SAgs, and proposed that previously reported skewing of Vβ profiles induced by ETA resulted from contaminants in impure toxin preparations obtained from a single commercial source. Several lines of evidence obtained in his laboratory suggested that ETs were not SAgs. Specifically, although a commercially obtained ETA preparation stimulated proliferation of human and murine T cells, purified ETA expressed by S. aureus 8325-4 was nonmitogenic (14). Furthermore, it was reported that his group was able to further purify the commercial ETA. In doing so, the huVβ2 stimulation previously attributed to ETA was separated entirely from ETA in this preparation. Although the putative huVβ2-stimulating contaminant was not identified, it was proposed to be a previously unrecognized SAg, consistent with results of others who have found that S. aureus UT0003 does not harbor structural genes for any of the classical staphylococcal SAgs (1).

The present study was undertaken to characterize the T cell proliferative ability and other biological properties relevant to the potential ability of ETA and ETB to function as SAgs. Both ETs were expressed in S. aureus on a multicopy plasmid, purified to homogeneity, and used to stimulate human PMBC or mouse splenocytes or were used to test ET toxicity in vivo. In this study, each ET stimulated polyclonal expansion of T cells in a similar Vβ-dependent manner. However, the Vβ profiles obtained were not the same as those reported previously for ETA. These observations, plus some unusual properties of ETA, could provide an explanation for the contradictory reports in the literature.

Materials and Methods

Bacterial strains and purification of ETA and ETB

Recombinant ETA and ETB, expressed in an isogenic S. aureus background, were used in most experiments. Cloning of the eta and eth structural genes from the genomes of S. aureus strains UT0003 and UT0007, respectively, was described previously (15, 16). S. aureus RN4220, a nontoxicogenic laboratory cloning strain, was transformed with recombinant plasmids (pCE117 (3) and pDH560:pLT7 (17)), harboring the subcloned eta and eth genes, respectively. RN4220 has been used to express recombinant toxin genes for numerous SAgs studies, and its nontoxicogenic background has been confirmed using a variety of biological, immunological, and molecular biology techniques (18–20). Transformation of RN4220 was performed according to the method of Chang and Cohen (21). For some experiments, ETA was expressed in Escherichia coli DH5α (supE44, U169 (B06000,ZAM15) hsdR17 recA1 endA1 g69 thi-1 relA1) (22) transformed with pCE117.

ETA and ETB were purified to apparent homogeneity from the recombinant organisms by preparative isoelectric focusing (IEF), as described previously (10, 23). Although IEF-purified ETA and ETB resolved as a single band in gel electrophoresis, for most studies, the IEF-purified ETs were subjected to HPLC to insure purity. Reverse-phase HPLC was performed using a Hewlett Packard HP1090 (Waldbonn, Germany) apparatus equipped with a 15-cm C18 column (VYDAC, Hesperia, CA). Toxins were loaded onto the column in 0.1% trifluoroacetic acid and eluted in a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid with a flow rate of 1 ml/min. The resulting highly purified toxins were quantified by the Bio-Rad protein assay (Richmond, CA) and assessed by SDS-PAGE (24) (Fig. 1). In addition, because antiserum against ETB was not available, N-terminal amino acid sequencing (10 residues) was done to verify the identity of the protein. All toxins were lyophilized for long-term storage.

Processing and stimulation of human and murine lymphocytes in culture

To assess huVβ expansion by ETA and ETB using a quantitative PCR assay or flow cytometry (FC), PBMCs were isolated from healthy human donors. Venous blood was mixed with heparin (14 U/ml blood) and fractionated by gradient centrifugation to isolate PBMCs according to procedures described previously (25). For huVβ analysis, lymphocyte-enriched cell suspensions were prepared as follows: The PBMCs were washed and resuspended in RPMI 1640 supplemented with 2% FBS, 100 U penicillin G, and 100 μg/ml streptomycin (complete RPMI), and incubated overnight at 37°C and 7% CO2 in plastic petri plates. Nonadherent lymphocyte-enriched PBMCs were collected, washed, and resuspended at a final concentration of 2.5 × 106 cells/ml.

To determine whether expansion of specific huVβ-bearing T cells occurred following stimulation by ETA or ETB, each toxin (final concentration of 0.25 μg/ml) was added to a 3-ml aliquot of the enriched lymphocyte cell suspension. Cell cultures were incubated for either 2 or 4 days (37°C and 7% CO2). Control cultures, stimulated by adding a soluble murine mAb specific for the human CD3 molecule (33 μg/ml final concentration; Sigma, St. Louis, MO; product number C-7048), were used to quantify basal levels of huVβ.

The effect of ETs on muVβs was assessed using spleen cells from C3HeB/FeJ mice. The spleenocytes culture used in these experiments were processed and stimulated in a manner similar to that for human PBMCs, except that murine cultures were supplemented for 3 days with either medium alone or medium containing either ETB or SEB (as a positive control) (100 ng/ml each). After 3 days, the cells were resuspended at a final concentration of 106 cells/ml (provided by Dr. Robert Zimmerman, Chiron, Emeryville, CA) for an additional 3 days. Cells were then processed for FC, as described below.

PCR assay of huVβ transcript levels in ET-stimulated lymphocyte cultures

RNA (2–6 μg) isolated from human lymphocyte cell cultures using Trizol reagent (Life Technologies, Gaithersburg, MD) was used to generate cDNA with Superscript II reverse transcriptase (Life Technologies) and random DNA hexamers. The primers used in PCR assays to analyze huVβ expansion by SAgs have been previously described (11).

The bulk mixture method as described by Kob et al. (26) was used, but with a 10-fold reduced concentration of 32P-labeled primers per reaction (105 cpm/reaction). PCR conditions were described previously (25), with minor modifications as follows: final concentrations per reaction were 1× Taq PCR buffer (supplied with enzyme; Life Technologies), 0.3 μM 3′ Ca primer, 0.3 μM 5′ Ca primer, 0.3 μM 3′ CB primer, 0.3 μM huVβ-specific primer, 1.5 mM MgCl2, dNTP mix (200 mM each A, T, C, and G), 1 × 105 cpm 32P-labeled 3′ Ca primer (5′ end labeled with T4 polynucleotide kinase; Life Technologies), 1.5 × 105 cpm 32P-labeled 3′ CB primer (5′ end labeled with T4 polynucleotide kinase; Life Technologies), 1 × 105 cpm 32P-labeled 3′ Vβ primer (5′ end labeled with T4 polynucleotide kinase; Life Technologies), and 1 × 105 cpm 32P-labeled 3′ CX primer (5′ end labeled with T4 polynucleotide kinase; Life Technologies). Thermocycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 25 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The process was terminated with a single cycle at 72°C for 15 min.

Ca and huVβ PCRs were resolved on 2% agarose gels. Radioactive PCR products were quantified by scanning dried gels using a GS525 Molecular Image System (Bio-Rad, Hercules, CA). Each PCR product was quantified in pixel density units using Molecular Analysis Software (Bio-Rad). Calculations to determine the extent of huVβ expansion were done as described by Deringer et al. (25). Briefly, the values for each specific huVβ product were normalized by dividing the huVβ value by the corresponding Ca control value. The normalized huVβ values were used to determine the percentage of each of the 22 different amplified huVβs in stimulated cultures. Results were expressed as an expansion index value that is defined as the ratio of the percentage of each huVβ in a toxin-stimulated culture compared with the percentage of the same huVβ in an identical culture stimulated with anti-CD3 (basal levels of huVβs).
Assessment of VB expansion by FC analysis

Human PBMCs were harvested from culture dishes and processed immediately for FC staining. Cells were pelleted by centrifugation and suspended in Ca2+- and Mg2+-free PBS containing 10% horse serum and 20% acid citrate dextrose. Aliquots (50 μl) of cell suspension (5 × 10⁶ cells) were added to wells of 96-well plates containing a mixture of mAbs to background staining of the negative control. The percentage of positive cells was calculated by subtracting the conjugated mouse anti-rat RR3-15, a rat IgG; Mr12-4, a mouse IgG; KJ23, a mouse IgG2a; and 14-2, F23.1, a mouse IgG2a; F23.2, a mouse IgG2a; MR10-2, a mouse IgG1; a mouse IgG1; RR4-7, a rat IgG2a; TR-310, a rat IgG2b; KJ16, a rat IgG; 2C11, a hamster IgM; B20.6, a rat IgG2a; KJ25, a hamster IgG1; MR9-4, a mouse IgG1; RR4-7, a rat IgG2a; TR-310, a rat IgG2b; KJ16, a rat IgG; F23.1, a mouse IgG2a; F23.2, a mouse IgG2a; MR10-2, a mouse IgG1; RR3-15, a rat IgG; MR12-4, a mouse IgG; KJ23, a mouse IgG2a; and 14-2, a mouse IgM. Cells were labeled with primary Abs and then either a FITC-conjugated mouse anti-rat κ-chain, a FITC-conjugated goat anti-hamster Ig, or a FITC-conjugated goat anti-mouse Ig. Fluorescence was measured using a FACScan with CellQuest software (Becton Dickinson Immunocytometry Systems). Between 5 × 10⁵ and 1 × 10⁶ cells were analyzed per sample. The percentage of positive cells was calculated by subtracting the background staining of the negative control.

Subcloning and nucleotide sequencing of selected huVB gene segments

Using the techniques described above, human PBMC cultures were stimulated with ETA or ETB. After extraction of RNA from the PBMC, cDNA was synthesized and amplified by PCR, employing several selected sets of huVB-specific primer pairs. The resulting DNA fragments were agarose gel purified and subjected to a second round of PCR amplification using Vent DNA polymerase (2 U/reaction) (New England Biolabs, Beverly, MA) to generate products with blunt ends for facilitation of molecular cloning. Following agarose gel purification, these blunt-ended fragments were ligated to EcoRV-linearized pBluescript KS+ phagemid (Stratagene, La Jolla, CA). After ligation, the recombinant plasmids were transformed into E. coli DH5α (22) and screened for failure to hydrolyze X-gal. Plasmids were isolated from randomly selected transformants, and the huVB-derived insert in each was sequenced using a commercial kit employing the dideoxynucleotide sequencing methodology (27) with the Sequenase Version 2.0 commercial kit (United States Biochemical, Cleveland, OH) and the T7 promoter primer. Routine molecular biology techniques were employed throughout (22).

Quantification of lymphocyte proliferation induced by ETs

Incorporation of [3H]thymidine into cellular DNA in a standard 4d assay (28) was used as an index for quantifying the level of lymphocyte proliferation induced by ETA and ETB. PBMCs were processed as described above, adjusted to a concentration of 1 × 10⁶ cells/ml in complete RPMI 1640 medium. Each toxin was added to PBMC cultures (200 μl of PBMC suspension in 96-well culture plates) and incubated at 37°C in 7% CO₂ for 72 h. Cultures were pulsed (18–24 h) with 1 μCi of [3H]thymidine. Cellular DNA was harvested on glass fiber filters, and the radioactivity incorporation was quantified by liquid scintillation counting.

A modification of this procedure was performed to determine whether APCs were required for ET-induced T cell proliferation. Adherent cells were removed by incubating in plastic petri plates, as described above, and then additionally by passing the lymphocyte-enriched cell suspensions through a Sephadex G10 column. The lymphocytes were processed to obtain purified T cells by two successive rounds of E-rosetting with sheep erythrocytes and density-gradient centrifugation, as described by Fendal et al. (29). After lysing the erythrocytes in ammonium chloride (0.15 M), the T cells were plated in 96-well plates, with or without toxin, as described.
above, for the standard 4-day proliferation assay, except that $1 \times 10^5$ T cells were used for each culture. In some wells, the purified T cells were reconstituted with adherent cells ($3 \times 10^4$/well), which were dislodged and recovered from plastic petri plates (see above).

Assessment of ET pyrogenicity and lethality in vivo

Most staphylococcal and streptococcal SAg exotoxins have been grouped into a family of PTs based on a number of shared biological properties. These shared properties include, but are not limited to, pyrogenicity and induction of lethal shock (10). These two representative activities were assessed using the rabbit model described by Kim and Watson (30) as an indication of the relatedness between the ETs and other staphylococcal exotoxins in the PT family. Each animal (1–2 kg, young adult Dutch-Belted rabbits; Birchwood Farms, Grantsburg, WI) was given an initial i.v. injection containing ETA or ETB (0.1–50 μg/kg) dissolved in PBS. Positive and negative control animal groups were given injections of either TSST-1 or PBS, respectively. After monitoring rectal temperature for 4 h, an i.v. injection of endotoxin extracted by established techniques (31) from Salmonella typhimurium was administered at a dose of 10 μg/kg. Mortality was monitored for 48 h.

Homology modeling of ETB

ETB was modeled using the HOMOLOGY module in INSIGHT II 95.0 (BioSym, La Jolla, CA). Briefly, side chain residues in the published crystal structure of ETA (3) were changed to the aligned residues in the primary sequence of ETB (15). The short loops between helix αN and strand βN, helix αNa and strand βA1, and strands βB2 and βC2 were modeled using templates from proteins in the Protein Data Base. The energy of the structure was minimized to reduce unlikely contacts and poor geometry. Solvent-exposed areas of ETA and ETB were calculated using GRASP (32).

Results

ETA and ETB induce expression of similar Vβ gene segments

mRNA was obtained from human lymphocyte-enriched cultures stimulated for 4 days with ETA or ETB. Following cDNA synthesis, PCR reactions employing huVβ-specific primers were used to assess the relative quantity of each huVβ transcript. By comparing the mRNA levels following stimulation by toxin with baseline levels (induced by anti-CD3), it was possible to ascertain whether the ETs induced selective huVβ skewing. ETA and ETB induced similar huVβ profiles; both toxins caused an expansion of huVβs 3, 12, 13.2, 14, 15, and 17 mRNA (Fig. 2A). Although ETA also induced an expansion of huVβ20, no significant increase was observed with this huVβ after stimulation with ETB.
Table I. Deduced amino acid sequences encoded from selected regions of huVβ transcripts induced by ETs

<table>
<thead>
<tr>
<th>Toxin</th>
<th>huVβ Primers</th>
<th>3' Vβ Sequence</th>
<th>Dβ Sequence</th>
<th>Jβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETA</td>
<td>huVβ3</td>
<td>MYLCASS</td>
<td>GE</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>huVβ3</td>
<td>MYLCASS</td>
<td>PGG</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>huVβ3</td>
<td>MYLCASS</td>
<td>NPG</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>huVβ15</td>
<td>LYFCATS</td>
<td>DPGV</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>huVβ15</td>
<td>LYFCATR</td>
<td>DR</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>huVβ15</td>
<td>LYFCATS</td>
<td>DPASG</td>
<td>2.3</td>
</tr>
<tr>
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<td>LYFCATS</td>
<td>ESGPS</td>
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<td></td>
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<td>LYFCATS</td>
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<td>ETB</td>
<td>huVβ3</td>
<td>MYLCASS</td>
<td>FN</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>huVβ3</td>
<td>MYLCASS</td>
<td>FMTR</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>huVβ15</td>
<td>LYFCATS</td>
<td>DSFGSG</td>
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<td>LYFCATS</td>
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<td>LYFCATS</td>
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<td></td>
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<td>LYFCATS</td>
<td>D</td>
<td>2.3</td>
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<tr>
<td></td>
<td>huVβ17</td>
<td>KNPTAFYLCAS</td>
<td>TDRVG</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* Human PBMC cultures were stimulated with either ETA or ETB and processed as described in the text. The amino acid sequences were determined from the DNA sequence information obtained from reverse-transcribed RNA isolated from stimulated T cells.

† Used for PCR amplification following cDNA synthesis.

‡ Jβ family to which the amplified RNA transcript belonged. Information was determined from DNA and deduced amino acid sequences.

In contrast to previous reports (11), huVβ2 was not expanded over baseline levels by either ETA or ETB, even though, as expected, a control preparation of TSST-1 efficiently up-regulated expression of huVβ2 mRNA (Fig. 2B). The huVβs induced by both ETs are most related to each other and have been grouped into subgroup 4 according to the classification recommended by Choiha et al. (33). Interestingly, this huVβ profile resembled profiles induced by SEB and various SEC subtypes and species-specific molecular variants (Ref. 25 and Fig. 2B). Strain 4220 is well documented to lack SAg activity and has been used to express other recombinant proteins for use in similar studies (18–20). However, as an additional control, a separate preparation of rETA, purified from an E. coli clone, was tested. The E. coli-derived toxin generated the same conclusions compared with experiments using recombinant toxin expressed in S. aureus RN4220 (results not shown). Combined, this evidence eliminated the unlikely possibility that SEB, SEC, or a novel and unrecognized staphylococcal contaminant expressed by strain RN4220 was responsible for the profile.

**ETA and ETB cause polyclonal T cell stimulation**

Unlike conventional Ags, SAgS bind to the TCR in a Vβ-dependent manner regardless of the Ag specificity of the receptor. Thus, SAgS and conventional AgS may be differentiated on the degree of junctional diversity they generate. SAgS induce a polyclonal expansion that leads to extensive junctional diversity within the complementarity-determining region 3 (34). To rule out the possibility that the T cell stimulation by ETs was the result of an Ag-specific stimulation of one or a limited number of T cell clones, RNA extracted from ET-stimulated T cell cultures was reverse transcribed and amplified by PCR (using primers specific for huVβ3, huVβ15, or huVβ17). The resultant products were cloned and sequenced.

The sequencing results summarized in Table I show that ETA and ETB induced a polyclonal T cell proliferation in a manner consistent with SAg activity. As expected, DNA fragments generated by using the huVβ-specific PCR primers contained nucleotide sequences identical to those reported for the corresponding huVβ gene segments in the literature (35, 36). Importantly, the deduced amino acid sequences of 17 randomly cloned cDNAs revealed that the TCR primary sequence had extensive junctional diversity within each individual huVβ element. The sequences within complementarity-determining region 3 (combined D and J regions of the β-chain transcripts) were unique for every clone.

**ETA-induced Vβ-dependent stimulation does not result in phenotypic skewing of the corresponding Vβ**

Generally, SAg activation of T cells results in increased Vβ gene expression due to proliferation of compatible T cell subpopulations so that an increase in certain Vβ RNA species is detectable within stimulated cultures (11, 25, 26). This typically is also associated with a corresponding increased phenotypic expression of the TCR due to expansion of the reactive Vβ T cell subpopulation. Thus, FC analysis may be used to assess skewing of the Vβ repertoire by SAgS on the basis of their ability to selectively induce increased phenotypic expression of certain Vβ elements on the cell surface (8). Since minor differences between PCR and FC analysis of SAg activation have been reported (25), it was of interest to analyze ET-stimulated PBMC by the FC method. Human PBMC cultures were stimulated with either ETA or ETB for 48 or 96 h, harvested, and analyzed for expression of several huVβ epitopes on the cell surface. This was accomplished using mAbs specific for huVβ2, huVβ3, huVβ8, huVβ12, or huVβ14, which represented randomly selected expanded and nonexpanded huVβs, as determined by semiquantitative PCR.

Unlike results based on the PCR, FC analysis showed significant differences between ETA- and ETB-induced huVβ profiles (Fig. 3). ETB stimulation for 4 days resulted in the expansion of huVβs in a fashion closely matching the results obtained by PCR analysis. For example, a significantly increased level of CD3+ T cells expressing huVβ3, huVβ12, and huVβ14 was noted in PBMC cultures stimulated with ETB. Also consistent with results of PCR analysis was the observation that levels of huVβ2 or huVβ8 were not elevated.

In contrast to ETB, stimulation of T cells with ETA did not lead to elevated percentages or numbers of T cells bearing the appropriate Vβ on the cell surface, despite the elevated levels of Vβ mRNA as observed by PCR. For example, FC analysis showed that ETA exposure did not result in elevated percentages of T cells expressing huVβ3, huVβ12, and huVβ14, whereas it consistently elevated mRNA levels for each of these huVβs (Figs. 2A and 3). Interestingly, in ETA-stimulated T cell cultures, there was a reduction in the percentages of these huVβ T cells to levels below baseline. This reduction apparently resulted from the Vβ-dependent interaction of ETA with T cells, since the percentages of T cells bearing huVβ2 and huVβ8, previously shown by PCR assay to be nonreactive Vβs, were not significantly reduced. Neither toxin induced major alteration of the huVβ levels on the surface of CD3+ cells after 48 h of incubation (results not shown).

**Quantification of T cell proliferation by ETA and ETB**

The apparent discrepancy between the PCR and FC analysis observed while assessing the ability of ETA to induce huVβ-dependent T cell stimulation suggested that either 1) ETA stimulation did not result in expansion of T cells bearing reactive huVβ elements, or that 2) the cells may have proliferated, but expression of huVβ on the cell surface was down-regulated. To differentiate between these two possibilities, ETA and ETB were compared for quantitative differences in T cell proliferation. Fig. 4 shows that the minimal amount of ETA and ETB sufficient to induce T cell proliferation was the same for both
toxins (1–10 ng/culture). This concentration represents a level of activity approximately 100-fold less potent than TSST-1 or the SEs. Both toxins had nearly identical abilities to stimulate human lymphocytes at all doses tested, as measured by incorporation of radioactivity into cellular DNA. Similarly, increases in cell numbers induced by stimulation with ETA were identical to results for ETB (results not shown). These data suggest that the decline in Vβ associated with ETA exposure is likely to result from down-regulation of huVβ expression on the surface, and not from a failure of ETA to induce T cell proliferation.

SAGs induce T cell proliferation via a mechanism requiring MHC class II molecules on APCs (8). Although ET-induced T cell proliferation was reported previously to have this same requirement (1, 7), it was of interest to determine whether this observation could be confirmed using highly purified rETs used in this current study. Experiments in which human T cells were stimulated with ETA or ETB in the presence or absence of APCs confirmed that APCs were required for efficient T cell activation by the toxins. In the absence of accessory adherent cells, levels of [3H]thymidine incorporation into T cell cultures upon exposure to the toxins were comparable with background levels (Table II). In contrast, T cell cultures supplemented with APCs displayed comparable and significant levels of stimulation by either ETA or ETB.

ETB expands murine T cells in a Vβ-specific fashion
To further evaluate the ability of ETB to expand T cells in a Vβ-specific fashion, murine T cells were treated with ETB, collected, and assayed by FC for the expression of specific muVβ. The analysis showed that only T cells bearing muVβ7 and muVβ8.1–8.3 were significantly expanded above the levels observed in untreated cells (Table III). These muVβ are, like those huVβ stimulated by ETB, classified in the subgroup 4 as designated by Chothia et al. (33) and share a high degree of sequence homology. Murine T cells, bearing Vβ outside of subgroup 4, were not stimulated by either ETB or SEB.

Table II. APC requirement for ET-induced T cell proliferation

<table>
<thead>
<tr>
<th>Culturesa</th>
<th>Toxinb</th>
<th>Proliferation (cpm ± SEM)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells only</td>
<td>None</td>
<td>640 ± 73</td>
</tr>
<tr>
<td>T cells only</td>
<td>ETA</td>
<td>386 ± 64</td>
</tr>
<tr>
<td>T cells only</td>
<td>ETB</td>
<td>720 ± 76</td>
</tr>
<tr>
<td>T cells plus adherent cells</td>
<td>ETA</td>
<td>30,362 ± 220</td>
</tr>
<tr>
<td>T cells plus adherent cells</td>
<td>ETB</td>
<td>28,389 ± 153</td>
</tr>
</tbody>
</table>

a Conditions were as described above for the standard 4-day proliferation assay using cultures containing E-rosetted T cells alone or with adherent cells from the same human donor.
b Cultures were either unstimulated (“None”) as background controls or stimulated with the ET indicated (1 μg/well).
c Cellular DNA synthesis was quantified by measuring incorporation of [3H]thymidine.
Table III. FC analysis of muVβ expression on murine splenocytes after stimulation with SAgsa

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Percent Positive Cellsb (%) When Exposed to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Medium</td>
</tr>
<tr>
<td>L3T4</td>
<td>CD4</td>
<td>54.0</td>
</tr>
<tr>
<td>2C11</td>
<td>CD3</td>
<td>98.9</td>
</tr>
<tr>
<td>B20.6</td>
<td>muVβ2</td>
<td>7.1</td>
</tr>
<tr>
<td>K25</td>
<td>muVβ3</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>MR9-4</td>
<td>muVβ5</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>RR4-7</td>
<td>muVβ6</td>
<td>0</td>
</tr>
<tr>
<td>TR-310</td>
<td>muVβ7</td>
<td>0</td>
</tr>
<tr>
<td>K16</td>
<td>muVβ8.1–8.3</td>
<td>17.5</td>
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<tr>
<td>F23.1</td>
<td>muVβ8.1–8.3</td>
<td>30.0</td>
</tr>
<tr>
<td>F23.2</td>
<td>muVβ8</td>
<td>13.2</td>
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<td>muVβ9</td>
<td>1.7</td>
</tr>
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<td>MR12-4</td>
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<td>2.3</td>
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<tr>
<td>K23</td>
<td>muVβ13</td>
<td>0</td>
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<tr>
<td>14-2</td>
<td>muVβ14</td>
<td>0</td>
</tr>
</tbody>
</table>

a CHSHeb/FcII splen cells were stimulated with medium ± ETB or SEB (10 μg/ml) for 3 days followed by 3 additional days with rIL-2 (10 U/ml).

b Numbers represent percent of total splenocytes in culture from a single representative experiment.

Toxicity of ETA and ETB in vivo

Most of the currently known staphylococcal exotoxins with SAg activity have been classified in the family of PTs on the basis of several shared biological activities. Since it was of interest to determine whether the staphylococcal ET SAgS also elaborate these PT-associated activities, we determined their ability to induce two PT-associated activities, induction of fever and enhancement of susceptibility to lethal shock, in a standard rabbit model. ETB behaved in a manner closely approximating the activity of TSST-1, a known PT, in this model (Table IV). Both ETB and TSST-1 were pyrogenic and induced lethality in the rabbit model, although, compared with TSST-1, ETB had somewhat reduced potency. Only four of eight rabbits given a relatively high dose (10 μg/kg) of ETB died, whereas lower doses of TSST-1 consistently induced lethality. In contrast, ETA was minimally active in this model. At doses of 10 μg/kg and higher, ETA was significantly less pyrogenic than ETB (p < 0.01 using Student’s t test of unpaired data) and was not lethal at any dose tested.

ETB model

Based on sequence identity with ETA, ETB was predicted to have a fold similar to chymotrypsin-like serine proteases. This fold consists of two perpendicular six-strand β barrels and a carboxy-terminal α helix (αC). ETB most likely has an amino-terminal domain similar to ETA, which includes an amphipathic aminoterminal α helix that is unique to the ETs. This large loop may lay near the S1 binding site of ETB, as seen in chymotrypsin, and is large enough to contact residues of the N-terminal domain after the N-terminal helix. ETB also has features that suggest a proteolytic activity similar to that proposed for ETA, including a catalytic triad (His65, Asp114, and Ser166) and a motif that may bind glutamic acid residues in the S1 binding site (Thr181, His201, and Lys204) (3).

Discussion

The present study was undertaken to address the controversial classification of the staphylococcal ETs as microbial SAgS by some investigators. The current literature in this area is based mainly on studies using ETA, which have yielded conflicting results. The results of this present study extend the prior investigations by comparing the activity of both ETA and ETB. In the landmark report by White et al. (8), SAgS were defined as molecules that induce Vβ-dependent T cell proliferation in association with MHC class II. Using highly purified recombinant toxins, we confirmed prior publications showing that efficient ET-induced T cell proliferation requires MHC class II-expressing accessory cells (1, 7). Furthermore, we demonstrated that these two toxins stimulate T cells in a Vβ-dependent manner. Together these two findings suggest that the ETs fulfill the basic criteria required to be classified as SAgS. Despite this, our results and the unique clinical picture of staphylococcal scalded skin syndrome suggest that ETA and ETB have unique properties compared with other staphylococcal PT SAgS. For example, the ETs are approximately 100-fold less potent in inducing T cell proliferation and less toxic in a rabbit TSS model than PT SAgS.

Although higher concentrations of ETs were required to induce efficient T cell proliferation in vitro, the peak level of de novo DNA synthesis (i.e., approximately 1–2 × 10⁶ cpm of radioactivity incorporated) generated in ETA-stimulated and ETB-stimulated PBMC cultures (Fig. 4) was comparable with that reported for other microbial SAgS, including type C SEs (38), TSST-1 (39), and streptococcal pyrogenic exotoxins (40) when characterized using the method employed in this current study. This high level of stimulation precludes the possibility that a significant amount of the ET-induced T cell proliferation was a result of their activity as conventional Ags. Typically, conventional Ags analyzed in our laboratories using this method generate very low levels of radioactivity incorporation (approximately 1–5 × 10⁴ cpm over background) (41). In addition, FC analysis showing a high blastogenesis rate in ET-stimulated cultures further discounted the possibility that effects caused by the ETs reflected their function as conventional Ags in this study. For example, after 48 h, 29% of T cells achieved blast size in cultures stimulated (results not shown). This value is entirely consistent with results observed with other SAgS, which reported can activate between 5 and 30% of human T cells (42). In contrast, conventional Ags require a much more specific interaction to induce T cell proliferation and typically are able to activate less than 0.1% of human peripheral T cells (42).

The patterns of Vβ mRNA expression induced by ETA and ETB indicated that several huVβS were substantially enriched following stimulation with these two toxins. It is noteworthy that much of the controversy in the literature has focused largely on the inability of some investigators to confirm initial reports that demonstrated a minor expansion of huVβ2 by ETA. Interestingly, in this present study, highly purified ETA and ETB failed to significantly expand this huVβ. Occasionally, a slight expansion of huVβ mRNA levels, similar to that reported by Choi et al. (11), was observed, however, this was attributed to routine experimental variation seen with all huVβ mRNAs, and was not found to be

Table IV. Toxicity of ETA and ETB in vivo

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Dose (μg/kg)</th>
<th>ETA</th>
<th>ETB</th>
<th>TSST-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Δ Temperature (°C) (lethality)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.45 (0/2)</td>
<td>1.2 (5/5)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>1.3 (0/3)</td>
<td>1.1 (4/8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.3 (0/3)</td>
<td>0.15 (0/2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.17 (0/5)</td>
<td>0.1 (0/6)</td>
</tr>
</tbody>
</table>

a Data are given as μg toxin per kg body weight (administered i.v.).
b Mean rise in body temperature (°C) after 4 h.
c Number of dead animals/number of animals tested.
d Diluent PBS.
statistically significant after running numerous replicate experiments with multiple donors. Instead, a profile of Vβ skewing similar to that induced by two well-characterized SAgs, staphylococcal enterotoxins B and C (SEB and SEC), was generated. Although this Vβ profile has not been reported previously for either ETA or EBT, the possibility of SEB or SEC contamination was eliminated by several methods. Highly purified rETs, isolated from an isogenic, nontoxicogenic S. aureus (RN4220) background, were used for these experiments. Additional experiments, using ETA expressed in E. coli, generated similar huVb profiles. Finally, although the profiles are similar to SEB and SEC, as well as to each other, there were unique aspects of each profile that would not be expected if a contaminant was responsible. For example, while huVβ20 is routinely expanded by SEB, SECs 1–3, and ETA, no significant expansion of this Vβ was observed in cultures stimulated with EBT.

Fleischer and coworkers (13, 14) reported that impure commercial ETA preparations stimulated huVβ2, but that it was possible to separate huVβ2 expansion away from ETA. Based on their data, they suggested that ETA is not a SAg. Instead, Fleischer (13) proposed that reported huVβ2 expansion was due to a contaminating SAg in the commercial preparations. We suggest a modification of that proposal, as follows. We suspect that contamination in some preparations may account for the reported huVb2 expansion by ETA. However, we also propose that the subsequent assessment of superantigenicity for highly purified ETA on the basis of huVβ2 skewing would not detect its true activity. It has been reported that UT0003, the native strain often used for purification of ETA, does not express previously characterized SEs or TSST-1 (1). However, tsST would not detect its true activity. It has been reported that UT0003, the native strain often used for purification of ETA, does not express previously characterized SEs or TSST-1 (1). However, this does not preclude the possibility that UT0003 expresses a not express previously characterized SEs or TSST-1 (1). However, this does not preclude the possibility that UT0003 expresses a novel, undescribed SAg capable of inducing proliferation in a huVβ2-dependent manner. Several new SAgs have been recently discovered in S. aureus, Staphylococcus intermedius, and Streptococcus pyogenes, and it is likely that the SAg family will continue to grow as additional research is conducted in this area. We are currently investigating ET-producing strains, including UT0003 for novel SAgs.

Because of the deviation of the results in this study from previous reports, we confirmed the ability of EBT to cause Vβ skewing in the murine system. Interestingly, and entirely consistent with the results obtained with human cells, only T cells expressing muVβ7 or muVβ8.1–8.3 were significantly elevated in relation to background levels. Like the human system, the profile generated was similar to that of SEB. Also, in regard to sequence homology, muVβ7 and muVβ8 are most related to the huVβs stimulated by ETA, EBT, SEB, and SEC. These Vβs have all been classified in the same subgroup (subgroup 4), according to the criteria of Choithia et al. (33). huVβ 20, which is stimulated by ETA (and the SECs) but not by EBT, is less related (subgroup 5) and presumably represents a divergence in the specificity for these two groups of toxins.

The ETs were found during the course of this investigation to have unique attributes differing somewhat from staphylococcal exotoxins in the PT family, which contains the prototypic bacterial SAgs. All PTs have similar molecular masses (22–30 kDa) and share several structural characteristics. They all are able to induce similar biological effects, including induction of T cell proliferation at nanomolar ranges, and enhance susceptibility to lethal endotoxin shock. In contrast, based on information from crystal structure data and homology modeling, the ETs do not share significant structural similarities to the PTs. Instead, they more closely resemble proteins in the serine protease family. In comparison with the PT SAgs, the amount of ET required to induce T cell proliferation is significantly greater. Furthermore, ETA was only minimally pyrogenic and did not enhance susceptibility to lethal shock when tested in the rabbit model. EBT retained these activities, but were less potent than TSST-1. Thus, although the ETs have the ability to induce Vβ-specific T cell proliferation, it is possible they represent a novel, uncharacterized class of SAg with a unique mechanism of T cell stimulation. Also importantly, some of the controversy as to whether or not these two staphylococcal toxins are SAgs is most likely the result of these unique differences.

Since the Vβ profiles of ETA and EBT are similar to each other and to those induced by SEB and SEC, it was of interest to investigate potential regions of the molecules that could account for these findings. Although the ETs have significant (40%) amino acid sequence identity with each other, they are not structurally related to the SEs (3). The binding characteristics of one TCR/SAg complex have been analyzed in detail through the crystal structure of the muVβ8.2/SEC3 complex (43). The surface area at the interface between these two molecules is relatively large (~1300 Å²) and lacks charged residues. Of the SEC3 contact residues, the surface area of the SECs. In contrast to these criteria on the ETs that are evident in the structures shown in Fig. 5. However, in both regions, there are few conserved residues and no residues that are identical, suggesting that the ETs have unique TCR-binding modes.

One likely outcome of potential differences in the modes of ETA and EBT binding to the TCR is that they could be expected to induce different pathways of cellular activation. Our results are consistent with this possibility. For example, a significant and unexpected finding was the demonstration that ETA-induced elevated levels of Vβ mRNA were not associated with increased percentages of T cells bearing the corresponding Vβs. In contrast, EBT stimulation led to increased levels of both Vβ mRNA and Vβ-bearing T cells. Although stimulation of T cells through an interaction involving their Vβs with a compatible SAg may result in deletion of the stimulated subpopulation (8), this effect is unlikely to explain the effects seen with ETA within the time frame of our in vitro experiments. Since we could not detect quantitative differences in T cell proliferative activities of ETA and EBT, it is possible that ETA-activated cells down-regulate phenotypic expression of the TCR. Some other SAgs have been reported to cause a rapid down-regulation of surface TCR and CD3 with sequestration and intracellular accumulation of the receptor in endocytic vesicles (44). Similarly, Salio et al. (45) reported that agonist-induced activation of T cells involves internalization and degradation of the TCR, which may result in a several-fold reduction of the number of TCR complexes on the cell surface. This mechanism was demonstrated in peptide-induced and also in SAg-induced activation (46). Although TCR internalization may be one potential fate of T cells stimulated with many, if not all SAgs, ETA binding to the TCR may be more likely than other SAgs to commit the cell to internalize their receptors. Clearly, this mechanism was not confirmed in the present study, and other possibilities exist. For example, one may also speculate that, following binding to the toxin, the TCR might serve as a substrate for the putative ET protease activity. Experiments to differentiate these possibilities are ongoing.

The difference between ETA and EBT in determining the fate of lymphocytes is likely to result from differences in signal transduction following their interaction with the TCR on the cell surface. If so, it is logical to assume that the two toxins would be likely to
induce different patterns of cytokine release, a key factor influencing their fate in vivo. We showed that ETA and ETB behave differently in vivo using the rabbit pyrogenicity and lethal shock model. Specifically, ETB was pyrogenic and lethal, which, although not as potently, mimicked the properties of TSST-1, a classical member of the PT family. In contrast, ETA was not lethal and minimally pyrogenic. Based on these data, one should consider whether the divergence in activity of these toxins in vivo represents an adaptation by staphylococci, as with many other microorganisms, to coexist with their host. In consideration of whether SAgs offer an advantage to microorganisms that express them, one proposal is that they promote persistence through immunomodulation of the host (47). Most staphylococcal SAg toxins, such as TSST-1, ETB, and other PTs, are both lethal and immunosuppressive. Accordingly, these toxins may represent traits that have been relatively recently acquired by S. aureus. This reasoning would also suggest that the lack of lethality by ETA represents an adaptation that benefits the organism by promoting host-parasite coexistence, while maintaining its ability to modulate the immune status of the host.

FIGURE 5. Two views of the secondary structure of ETA (A) and the solvent-exposed areas of ETA (B) and ETB (C). The bottom figures are rotated 120° along the vertical axis. A, α-Helices are red, β-strands are blue, and the putative protease active site residues (His72, Asp120, and Ser195) are yellow. B and C, Acidic residues are red, basic residues are blue, histidines are cyan, hydrophobic residues are green, and Ser195 of the active site is yellow. Boxes indicate possible binding sites.

Acknowledgments
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References


