Two Adjacent Residues in Staphylococcal Enterotoxins A and E Determine T Cell Receptor Vβ Specificity

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Summary

The T cell receptor (TCR) Vβ-determining region of two bacterial superantigens, staphylococcal enterotoxin A (SEA) and SEE, has been mapped to the COOH-terminal region of SEA and SEE using a panel of recombinant SEA/SEE hybrids. Total TCR Vβ mRNA enrichment in human peripheral blood T cell cultures was determined by a novel single-tube amplification technique using a redundant Vβ-specific primer. SEA routinely enriched mRNA coding for hVβ1.1, 5.3, 6.3, 6.4, 6.9, 7.3, 7.4, and 9.1, while SEE, which is 83% homologous to SEA, enriched hVβ5.1, 6.3, 6.4, 6.9, and 8.1 mRNA. Exchanging residues 206 and 207 was sufficient to convert in toto the TCR Vβ response of human peripheral T lymphocytes. In addition, an SEA-reactive murine T cell line, SO3 (mVβ17), unresponsive to wild-type SEA responded to SEE-S206P207, while an SEE-specific human T cell line, Jurkat (hVβ8.1), unresponsive to SEA was stimulated strongly by SEA-P206D207. Exchanging all other regions of SEA and SEE except residues 206 and 207 did little to change the Vβ response. Thus, the Vβ binding region appears to be a stable, discrete domain localized within the COOH-terminal region that is largely unaffected by the considerable amino acid variability between SEA and SEE. This region may interact directly with TCR Vβ.

The interaction of staphylococcal enterotoxins and murine retroviral products (Mls) with class II MHC molecules and TCR Vβ domains is a remarkable departure from classical MHC-restricted T cell recognition of peptides (1–3). T cell activation by superantigens appears to be an integral part of the host immune response to some bacteria and viruses. Staphylococcal and streptococcal toxins possess the ability to bind to MHC class II antigens (4–7) and stimulate a large but distinct set of T cells based solely on the expression of the TCR Vβ domain with apparently little or no contribution from other parts of the TCR normally involved in peptide/MHC recognition (1, 8–10). The staphylococcal and streptococcal toxins fall into two homologous groups, staphylococcal enterotoxin (SE)tA, SEE, and SED in one; and SEB, SEC1, 2, and 3, and SPE-A, B, and C in another. Homology between the two groups is ~30%. The exact mechanism of interaction between toxin, MHC, and the TCR Vβ domain remains unresolved. Toxin binding to MHC class II is readily detectable but interactions between toxin and TCR in the absence of MHC class II have so far been undetectable by all methods. A single soluble TCR Vβ chain alone in the absence of a TCR α chain is sufficient to bind to SEA but only when SEA is first bound to MHC class II (10). This is strong evidence that TCR Vα and junctional domains formed between Jα and Dβ and Jβ have no part in binding to toxin/MHC. This excludes the requirement of “classical” interaction between MHC polymorphic residues, peptide residues, and the TCR antigen binding “face.” All residues involved in toxin/MHC recognition must be exclusive to TCR Vβ and are probably located within a predicted loop structure around residues 70–74 (11). The mapping of functional residues in SEA and SEB has been attempted. Disruption of the disulphide loop in SEA leads to substantial loss in activity of SEA although binding to MHC class II is unaffected (12). A second study has shown that the MHC binding region of SEA is located in the COOH-terminal region 103–233, which is just beyond the disulphide bridge (13). Other studies have reported the opposite for SEB (14, 15), where both the MHC binding domain and TCR Vβ-determining domain mapped to the NH2-terminal half of the molecule.

In this paper, the region of SEA and SEE that influences Vβ stimulation has been identified by comparing the Vβ response profile of human PBL T cells with a panel of SEA/SEE hybrids. Strikingly, despite the considerable amino acid diversity between SEA and SEE, variation in Vβ response is caused only by residues 206 and 207 in the COOH-terminal domain.

Materials and Methods

Hybrid Construction. The genes coding for SEA and SEE were originally cloned from Staphylococcus aureus strains FDA196E and...
FR1 326 and sequenced to ensure identity. An in-frame BamHI restriction site was created by PCR. 5' of the first NH2-terminal amino acid codon of both SEA and SEE genes to remove the leader sequence and inserted into the pGEX expression vector fused with the glutathione-S-transferase (GST) gene (16). Because the same oligonucleotide primer was used to subclone both SEA and SEE, the latter had an extra three amino acids in the NH2 terminus identical to SEA. An EcoRI site was introduced using PCR, 3' of the stop codon of both toxin genes. Hybrids ABE, AAE, EAA, and EEA were generated by restriction cleavage of both toxin genes at either a shared AccI site or XhoI site 36 and 66% downstream from the start codon. Mixing and relocation of respective 5' and 3' fragments generated four separate hybrid genes that were reinserted into the pGEX plasmid and expressed in Escherichia coli DH5α. Cell lysates were lysed in a French press and dialyzed overnight against 5 liters 10 mM Tris, pH 8.0, 0.15 M NaCl to remove endogenous glutathione. The inactive 50-kD fusion protein was purified from whole cell lysates in a single step on a 10-ml column of glutathione agarose and eluted with 30 ml of 20 mM Tris, pH 8.0, 0.15 M NaCl, 10 mM GSH (16). Mature toxin was released by digestion for 30 min at room temperature with 0.1% (wt/wt) TPCK-treated trypsin (Sigma Chemical Co., St. Louis, MO), which cleaves at a single site in the hinge region between the GST and toxin. Released GST was removed by passing the sample over the washed GSH column. Toxin was then purified on CM sepharose with a gradient of 10 mM NaPO4, pH 6.0, to 50 mM PO4, pH 6.8. Trypsin was used because it quantitatively cleaved all the hybrids without fragmentation of either the GST or toxin proteins. All hybrids gave a single band on SDS-PAGE of the expected molecular weight (not shown).

Hybrids (C16, C17, C18, C19, D16, D17, D18, and D19) were constructed by using overlapping PCR technology. Gene fragments were amplified from SEA and SEE using a pair of complementary oligonucleotides designed to hybridize to both plus and minus strands at the junction point selected along with oligonucleotide to both ends of the toxin genes outside of the two utility restriction sites (BamHI and EcoRI). Fragments from the first round of PCR were gel purified, denatured, mixed together, and reamplified using the utility oligos at either end of the full-length gene. The resulting hybrid genes of the correct size were gel purified, cut with BamHI and EcoRI, and reinserted into the pGEX vector for the production of fusion protein. Each construct was sequenced to check for misincorporations by Taq polymerase.

**Vβ Enrichment Analysis by Anchored Multiprimer Amplification.**

PBL (107) from a single donor were stimulated for 5 d with 0.1-10 ng/ml toxins or 5 μg/ml PHA. 20 ng/ml human rIL-2 was added on day 4. Cells were harvested, washed once with PBS in a microfuge tube, and RNA was made by acid phenol extraction (17). RNA was precipitated from ethanol at room temperature, washed thoroughly with 80% ethanol, and resuspended in 90 μl diethylpyrocarbonate (DEPC)-treated water. Crude RNA was digested for 20 min at room temperature with 2 U of RNAse-free DNase (Promega Biotec, Madison, WI) in 10 mM Tris, pH 7.4, 10 mM MgCl2, acid phenol/chloroform extracted, and ethanol precipitated and resuspended in 100 μl of DEPC water (≈0.1 μg/ml). 1 μg (10 μl) of total RNA was reverse transcribed with 0.05 μM of a Cβ transmembrane-specific primer, JF5 5'-GATCTTTCTAGAG-GATGGTGGCAGACAGGAC-3', in 20 μl 0.5 mM dNTP, 9.0 U M-MLV reverse transcriptase was added to each sample and incubated for 30 min at 42°C. 80 μl of water was added and samples were heated at 95°C for 5 min. 1 μl (200 U) of M-MLV reverse transcriptase was added to each sample and incubated for 40 min at 42°C. 80 μl of water was added and samples were heated at 95°C for 5 min. 1 μl (1%) of the double-stranded TCR β chain cDNA was amplified with 0.2 μM of JF3 5'-GCTAAGTTCG(TT)TGTTA(A3'), which hybridizes to the complementary strand of the 5' end of JF1/JF2 and either Cβ-1 (5'-TTCTTGATGCTCAACAC-3'), which anneals to a region 50 nucleotides down from the JC junction, or Cβ-2 (5'-TCAGGCCA-TGTCTGGGATCA-3'), which anneals to a region 200 nucleotides down from the JC junction. Cβ-2 not only provides a longer C-region but also is a more efficient amplifier. The Vβ anchor primer JF3 was 100-fold excess over the total redundant primer concentration and 10,000-fold excess over each individual sequence. Amplification was carried out in 50 μl 10 mM Tris, pH 8.3, 50 mM KCl, 0.01 mg/ml gelatin, 3.0 mM MgCl2, 0.05 mM dNTP, and 2.5 U Taq polymerase (Cetus Corp., Emeryville, CA). Conditions applied were 2 min at 55°C, 1 min at 72°C, 0.5 min at 94°C for between 25 and 30 cycles using thin-walled tubes (Cetus Corp.). The single 300-bp (Cβ1) or 500-bp (Cβ2) VβDββBCβ product was separated from cold nucleotides by adsorption onto glass milk (Bio-101, La Jolla, CA) and 10% of the amplified product was labeled with 10 μCi [3P]dCTP (5,000 Ci/mmol) in 20 μl with 1 U Klenow fragment overnight, using random hexanucleotides. Labeled Vβ probes were heated to 95°C for 5 min, then hybridized 4-5 h (10 ml 5 5× SSC, 0.5% SDS, 0.5 μg/ml fish DNA, 0.1 μg/ml PBS DNA at 65°C to Hybond N+ (Amersham Corp., Arlington Heights, IL) strips spotted (dot blottter; Bio-Rad Laboratories, Richmond, CA) with exactly 1.0 μg of a plasmid containing the Vβ 3' end (β and Cβ removed) of all the major hVβ families along with one spot representing 1.0 μg of a plasmid containing just Cβ1 and Cβ2. Strips were washed twice at room temperature in 2× SSC, 0.5% SDS and once at high stringency (0.1× SSC, 0.1% SDS, 65°C for 20 min). After autoradiography, spots were punched out and counted by scintillation radiography. All Vβs were assigned family numbers by the system of Toyonaga and Mak (18) based on >75% nucleotide homology. In this system, Vβ13.1 and 13.2 become Vβ12.2 and 12.4, respectively, while Vβ14 becomes Vβ3.3. Vβ21.1 is highly homologous but not identical to a recently published Vβ21 (19). All new sequences (Vβ7.3, 7.4, 12.5, 21.1) will be published elsewhere (J. D. Fraser, manuscript in preparation).

**Stimulation of T Cell Lines.**

5 × 105 T cells (Jurkat-hVβ8.1 or SO3-mVβ17) were cultured for 24 h in the presence or absence of 105 GM4672A B cells expressing MHC class II with 1 or 10 ng/ml toxins. Cultures medium contained PMA at 10 ng/ml for Jurkat stimulations to enhance release of IL-2. Supernatants were tested for levels of IL-2 using 3-d Con A-activated mouse spleen cells as an indicator line. One unit was defined as the amount of supernatant to support 50% of the maximal [3H]thymidine incorporation overnight.

**Results**

**Analysis of TCR Vβ Levels by Anchored Multiprimer Amplification.** Full analysis of Vβ-specific stimulation in humans is restricted by the lack of anti-Vβ mAbs and human T cell lines expressing all of the 21 hVβ families and >50
different TCR Vβ gene segments (18). One solution is to analyze TCR Vβ mRNA levels instead of surface expression of protein (9). This is based on the assumption that the intracellular level of Vβ mRNA transcripts is the same for all activated T cells so that levels of a particular Vβ mRNA relate directly to the number of activated cells expressing that Vβ on the surface. A comparable approach has been to utilize a large panel of oligonucleotides to amplify each hVβ family separately (9). This has several disadvantages by being both expensive and labor intensive, allowing only a few samples to be analyzed at one time.

We sought to simplify the analysis of individual Vβ mRNA transcripts in a mixed population to enable the analysis of the overall Vβ response to SEA, SEE, and SEA/SEE hybrids. Two inosine-containing redundant primers with fully conserved 5' ends were designed to anneal to a region containing an invariant tryptophan codon midway along all hVβ genes. Initial experiments with the redundant primers and the Cβ-I primer produced a 300-bp VβDβJβCβ fragment from all 13 hVβ-cloned cDNAs templates tested. Yields for each were very similar, indicating that amplification efficiency for any one Vβ sequence was not compromised with respect to other Vβ sequences (not shown). The redundant primers were used in the first cycle after initial cDNA synthesis at high concentration, then either removed by glass-milk extraction or simply diluted to a concentration >100 less than the Vβ anchor primer. The resulting product of the amplification was radiolabeled with [32P]dCTP and hybridized to a collection of immobilized Vβ probes representing the 3' end of each Vβ gene. Each strip was standardized with an immobilized Cβ probe. The rate of annealing of a soluble double-stranded DNA probe [Cγ] to a large excess of filter-bound DNA [Cf] has been well described (20) and is governed by the pseudo-first-order reaction: \(-d[Cγ]/dt = k_1[Cγ][Cf]\). Because [Cf] is constant and much greater than [Cγ], reannealing of Cγ is negligible. Thus, the rate of annealing to Cf is directly proportional to [Cγ] \(x t\) with a slope of \(1/k_1\). Rate is unaffected by the specific activity of the probe so that amplified products that incorporate less [32P]dCTP/μg of probe can still be compared if an internal standard is included. For a complex mixture of Cγ such as in mixed Vβs, crosshybridizing species annealing to Cf during the hybridization step do not compete with the correct species because Cf is in vast excess. These can later be removed with high-stringency washes.

Fig. 1 shows that the technique accurately reflects the original Vβ template ratio. It also demonstrates the lack of crosshybridization between homologous Vβs, for example within the Vβ6 family. Plasmids containing Vbβs 1.1, 2.1, 5.1, 6.4, 6.9, and 7.4 were mixed at fixed ratios or either 1:1 or 2:1 and amplified in duplicate as described in Materials and Methods. Spots from each strip were cut out and counted. The counts obtained for the Cβ spot were 2.5 times less than the total Vβ region counts because the C region in the amplified product was only 50-bp long while the Vβ region was 200 bp. All Cβ internal control spots were therefore multiplied by 2.5 to provide a 100% figure to which each Vβ could be normalized (Fig. 1). The normalized values for Vβs are given in Fig. 1 above each bar.
The ratio of \( V_\beta \) spot intensities in all cases was the same as the initial mixing ratios, indicating that amplification was not biased towards any one \( V_\beta \). In addition, the method was accurate to 10%. No crosshybridization with other members on the strip was detected for \( V_\beta 1, V_\beta 7.4, V_\beta 2, \) and \( V_\beta 6.9 \). Some slight degree of crosshybridization was detected between \( V_\beta 5.1 \) and \( V_\beta 5.3 \), and \( V_\beta 6.4 \) and \( V_\beta 6.9 \), although the level of this crosshybridization was very low (<10%) and would only be significant when a single \( V_\beta \) species contributed a very high proportion of the total probe. All other \( V_\beta \) clones have been tested in isolation and found to have no detectable crosshybridization with other immobilized \( V_\beta \)s (not shown). Two highly homologous cDNA clones, \( V_\beta 7.3 \) and \( V_\beta 7.4 \), and \( V_\beta 12.5 \) and \( V_\beta 12.3 \), were 100% crossreactive with each other. This indicated that immobilized probes almost certainly detected very similar family members. Nevertheless, those \( V_\beta \)s represented on the strips did not crossreact with each other.

Reproducibility of the method was tested by determining the \( V_\beta \) profile of a single individual's peripheral blood on a monthly basis stimulated with either PHA or SEA and SEE. Each in vitro culture was analyzed immediately after extraction of RNA, so each profile represents a separate analysis made over a 3-mo period (Fig. 2). The profiles were clearly consistent for each stimulating antigen. Unstimulated cells displayed a high proportion of \( V_\beta 3, V_\beta 8, \) and \( V_\beta 12 \) mRNA, which accounted for >30% of the total. This was probably a result of both a high proportion of activated cells expressing these \( V_\beta \)s as well as the fact that the immobilized probes almost certainly hybridized to an undetermined number of homologous family members. The \( V_\beta 3 \) family has at least 10 members, \( V_\beta 8 \) has at least 5, and \( V_\beta 12 \) has as many as 11, determined by medium stringency crosshybridization (21).

Interestingly, this high percentage of \( V_\beta 3 \) is not consistently seen in all individuals tested and may indicate significant differences in \( V_\beta \) usage within the human population as a possible result of superantigen influences (our unpublished results). In contrast to unstimulated cells, PHA gave a more even profile. This was not surprising since in a healthy individual there is always a small proportion (~1–5%) of activated peripheral T cells (i.e., IL-2R positive) at any given time. Because unstimulated cells express low levels of TCR mRNA in comparison with activated cells, the \( V_\beta \) profile from unstimulated cells probably reflected the \( V_\beta \) usage of the small activated subset while the PHA profile reflected all T cells.

SEA and SEE produced two distinct profiles that were consistent over 3 mo. \( V_\beta 3.2 \) and 12, which had been the major species in the unactivated samples, were no longer represented and the predominant \( V_\beta \) species were \( V_\beta 1.1, 5.3, 6.3, 6.4, 7.4, \) and 9.1 in SEA-, and \( V_\beta 5.1, 6.3, 6.4, 6.9, \) and 8.1 in SEE-stimulated T cells. The most significant differences between the SEA and SEE profiles were \( V_\beta 3.1 \) and 8.1 enrichment for SEE, and \( V_\beta 1.1, 5.3, 7.4, \) and 9.1 enrichment by SEA. \( V_\beta 6.3, 6.4, \) and 6.9 were strongly activated by both toxins. Thus, even though SEA and SEE are 83% homologous in amino acid sequence, there were subtle but significant differences in \( V_\beta \) stimulation profiles between the two toxins reproducibly detected by this technique.

The method also detected major differences in \( V_\beta \) enrichment in response to other staphylococcal toxin (Fig. 3). Each toxin gave a very distinct profile that could only have resulted from selective activation and expansion of \( V_\beta \)-bearing T cells. In particular, SEB and SEC1 strongly enriched for \( V_\beta 3 \) (40 and 30%), while toxic shock toxin (TST) strongly enriched for \( V_\beta 2 \) (39%). The intensity of each spot was determined by counting, and the results are provided in Table 1. These
Figure 3. Vβ enrichment to wild-type staphylococcal enterotoxins. Autoradiograph of Vβ strips from PBL (10^7) from a single donor stimulated with 0.1 ng/ml of either rSEA, rSEB, SEC1, rSEE, TST, or 5 μg/ml PHA. The primer used was Cβ-I.

Results compare very favorably with other published data on staphylococcal toxin stimulations (9). In summary, this method we have developed can routinely distinguish both major and minor differences in Vβ/Cβ ratios between highly complex RNA samples. No evidence of amplification-induced bias was detected. This would have resulted in one particular profile occurring irrespective of the stimulating antigen.

Table 1. Staphylococcal Toxin Stimulation Alters the Vβ/Cβ mRNA Ratios in PBL T Cells

| Vβ | Unstimulated | PHA | SEA | SEB | SEC1 | SEE | TST |
|----|--------------|-----|-----|-----|------|-----|-----|
|    | cpm %Vβ | cpm %Vβ | cpm %Vβ | cpm %Vβ | cpm %Vβ | cpm %Vβ | cpm %Vβ |
| 1.1 | 343 <1 | 440 2.3 | 468 2.9 | 860 1.8 | 292 <1 | 368 <1 | 643 2.2 |
| 2.1 | 1,021 7.1 | 297 1.0 | 198 <1 | 958 2.1 | 500 1.0 | 323 <1 | 9,380 39.0 |
| 3.2 | 1,886 15.6 | 1,388 10.7 | 172 <1 | 11,987 40.0 | 10,150 30.0 | 488 1.9 | 2,711 11.0 |
| 4.1 | 950 6.4 | 440 2.3 | 210 <1 | 640 1.0 | 491 1.0 | 420 1.2 | 2,096 8.3 |
| 5.1 | 987 6.8 | 657 4.2 | 777 5.8 | 857 1.8 | 682 1.5 | 1,293 10.6 | 1,170 4.4 |
| 5.3 | 731 4.3 | 626 3.9 | 1,747 15.0 | 482 <1 | 767 1.8 | 615 3.0 | 1,028 3.8 |
| 6.3 | 192 <1 | 400 1.9 | 1,031 8.3 | 511 <1 | 302 <1 | 877 6.0 | 325 <1 |
| 6.4 | 271 <1 | 275 0.8 | 803 6.1 | 706 1.3 | 1,033 2.5 | 985 7.3 | 461 1.5 |
| 6.9 | 400 1.0 | 436 2.3 | 2,414 18.9 | 841 1.7 | 1,148 3.0 | 3,068 30.0 | 915 3.3 |
| 7.4 | 850 5.5 | 518 3.0 | 1,630 14.0 | 645 1.1 | 307 <1 | 461 1.6 | 352 1.0 |
| 8.1 | 1,450 12.0 | 352 2.3 | 232 <1 | 956 2.1 | 632 1.4 | 2,717 26.0 | 850 3.0 |
| 9.1 | 325 <1 | 200 <1 | 395 1.4 | 406 <1 | 198 <1 | 231 <1 | 115 <1 |
| 12.3 | 1,250 9.3 | 1,152 8.7 | 201 <1 | 2,072 6.0 | 2,160 6.0 | 345 <1 | 620 2.1 |
| 15.1 | 460 3.1 | 472 2.6 | 246 <1 | 2,228 6.5 | 2,007 5.6 | 518 2.2 | 525 1.7 |
| 21.1 | 293 <1 | 93 <1 | 246 <1 | 336 <1 | 160 1.2 | 263 <1 | 113 <1 |
| 0 | 290 | 183 | 251 | 621 | 565 | 310 | 310 |
| Cβ | 4,375 | 4,655 | 4,352 | 11,967 | 13,637 | 4,008 | 9,670 |
| (x2.5) | 10,205 | 11,180 | 10,465 | 29,077 | 33,692 | 9,362 | 23,892 |

Spots from filters (Fig. 3) were cut and counted. For background (0), 1 μg of plasmid (pBS) was included on each filter. All Vβ spots were expressed as a percentage of the internal Cβ. Counts for Cβ spot were first multiplied by 2.5 to account for the shorter length of the Cβ region. Any Vβs showing enrichment are underlined.
levels in unstimulated cells (Table 2). Similarly, the D series response to wild-type SEA with enrichment of V~3.1, 5.3, hybrids, except for V~8.1 enrichment, were identical to the population were observed. All the C series hybrids produced over several months duration because each hybrid was tested as it was constructed and purified. This resulted in a variation in intensities for different strips due to different specific radioactivities as well as total concentration of each probe. Neither of these parameters affected the proportional rates of hybridization within each sample however. For instance, compare hybrid EEA to D18, for example (Table 3), where the total counts vary by 11-fold but the individual Vβ/Cβ ratios were not altered. To indicate the similarity between the C and D series stimulations, the mean value of each enriched Vβ and the standard deviation among hybrids was determined to indicate the similarity within each series (Tables 2 and 3). SEA- or SEE-like profiles were clearly distinguishable, particularly with respect to Vβ1.1, Vβ5.1/5.3 ratios, Vβ7.4, and Vβ9.1. Vβ8.1 was the only Vβ that did not segregate entirely with the C series but this may be due to the fact that a portion of Vβ8-bearing T cells are responsive to SEA especially at higher concentrations of toxin (see below). Consistent with the Vβ-determining region mapping to the very COOH-terminal region and not to the variable region 187–200, hybrids C17 and D17 were SEA- and SEE-like, respectively.

The Vβ Response to SEA and SEE Is Strongly Influenced by Residues 206 and 207. Two more hybrids (D19 and C19) were constructed to isolate residues 206 and 207 (see Fig. 4). The results of initial experiments using the Cβ-3 oligonucleotide as a downstream amplifying primer are given in Tables 2 and 3, compared with results for other hybrids. In a second experiment, RNA samples from cultures stimulated with 10 ng/ml (100-fold higher) of SEA, SEE, C19, D19, and PHA cultures were amplified in duplicate with the more efficient Cβ-2 amplimer instead of Cβ-1. This provides a longer (200 bp) C region equivalent to Vβ in length. Clearly, the SEA-induced profile matched with D19 while the SEE profile was indistinguishable from C19. The normalized Vβ values were calculated by counting each spot and the averaged values of duplicate strips are shown in Table 4. Significantly, both SEA and C19 selectively enriched Vβ5.1 over Vβ5.3, and Vβ8 strongly. SEA and D19 selectively enriched Vβ1.1, Vβ3.1 over Vβ5.1, Vβ7.4, Vβ9.1, and Vβ21.1. Only the partial enrichment of Vβ5.1 by D19 suggested that it was more like SEE than SEA. In contrast to the previous experiments, Vβ8 was a significant proportion in the SEA profile. However, this only reflected an enrichment of Vβ8 when compared with PHA (4.8%) but not with unactivated PBL, where the Vβ8 proportion was significantly higher at 10%. As this was the starting proportion before SEA activation, there was in effect no net change in the level of Vβ8 mRNA. This suggested that only a portion of Vβ8-bearing T cells were stimulated by SEA and D19, while SEA and C19 enriched a much larger number. Stimulation of Vβ8 cells by SEA also appeared dependent on the concentration of toxin in culture. Results in Table 4 represent stimulation with 10 ng/ml, while the data in Tables 2 and 3 represent 1 ng/ml. These data suggest that residues 206 and 207 strongly influence the Vβ response to both SEA and SEE, and that changing these two residues alone can convert SEA into SEE and vice versa in terms of the general TCR Vβ response of peripheral T cells. In contrast, interchanging other large regions of both toxins did little to alter the recognition by TCR Vβ. Thus, the region that interacts with TCR Vβ must

| HYBRID | STRUCTURE | JUNCTION | PBL Vβ PROFILE |
|--------|-----------|----------|----------------|
| SEA    |           | 233      | Vβ1.1, 5.3, 5.6.4, 6.9, 7.4, 9.1 |
| SEE    |           | 233      | Vβ5.1, 5.3, 5.6.4, 6.9, 9.1 |
| AEE    |           | 85       | SEE            |
| AAE    |           | 156      | SEA            |
| C16    |           | 187      | SEA            |
| C17    |           | 187-196  | SEA            |
| C18    |           | 200      | SEE            |
| C19    |           | 206-207  | SEE            |
| EAA    |           | 85       | SEA            |
| EEA    |           | 156      | SEA            |
| D16    |           | 187      | SEA            |
| D17    |           | 187-200  | SEE            |
| D18    |           | 196      | SEE            |
| D19    |           | 200-207  | SEA            |

Figure 4. Structure of SEA/SEE hybrids. Diagrammatic representation of all hybrids constructed and the position of their junctions between SEA and SEE. C17 and D17 have been grouped with the C series and D series, respectively, even though their Vβ response profiles were SEA- and SEE-like.

major changes to three-dimensional structure. For ease of identification, the panel was divided into two groups based on the derivation of the COOH-terminal residues. The C series (AEE, AAE, C16–19) retained diminishing portions of the COOH terminus of SEA, while the D series (EAA, EEA, D16–19) retained COOH-terminal residues derived from SEA (Fig. 4). Hybrids C17 and D17 were different in that the region of SEA and SEE exchanged was not the final COOH-terminal region but a variable segment between residues 187–200.

Each hybrid was used to stimulate the same individuals PBL in vitro. The Vβ response to each hybrid was characteristic either SEA- or SEE-like (Tables 2 and 3). No new Vβ enrichments other than those seen in SEA and SEE stimulation were observed. All the C series hybrids produced profiles indistinguishable from the responses to wild-type SEE with enrichment of Vβ3.1 and 8.1 and no evidence of Vβ1.1, 5.3, 7.4, or 9.1 activation when compared with the starting levels in unstimulated cells (Table 2). Similarly, the D series hybrids, except for Vβ8.1 enrichment, were identical to the response to wild-type SEA with enrichment of Vβ1.1, 5.3, 7.4, and 9.1 (Table 3). The strips represented analyses made over several months duration because each hybrid was tested as it was constructed and purified. This resulted in a variation in intensities for different strips due to different specific radioactivities as well as total concentration of each probe. Neither of these parameters affected the proportional rates of hybridization within each sample however. For instance, compare hybrid EEA to D18, for example (Table 3), where the total counts vary by 11-fold but the individual Vβ/Cβ ratios were not altered. To indicate the similarity between the C and D series stimulations, the mean value of each enriched Vβ and the standard deviation among hybrids was determined to indicate the similarity within each series (Tables 2 and 3). SEA- or SEE-like profiles were clearly distinguishable, particularly with respect to Vβ1.1, Vβ5.1/5.3 ratios, Vβ7.4, and Vβ9.1. Vβ8.1 was the only Vβ that did not segregate entirely with the C series but this may be due to the fact that a portion of Vβ8-bearing T cells are responsive to SEA especially at higher concentrations of toxin (see below). Consistent with the Vβ-determining region mapping to the very COOH-terminal region and not to the variable region 187–200, hybrids C17 and D17 were SEA- and SEE-like, respectively.

The Vβ Response to SEA and SEE Is Strongly Influenced by Residues 206 and 207. Two more hybrids (D19 and C19) were constructed to isolate residues 206 and 207 (see Fig. 4). The results of initial experiments using the Cβ-3 oligonucleotide as a downstream amplifying primer are given in Tables 2 and 3, compared with results for other hybrids. In a second experiment, RNA samples from cultures stimulated with 10 ng/ml (100-fold higher) of SEA, SEE, C19, D19, and PHA cultures were amplified in duplicate with the more efficient Cβ-2 amplimer instead of Cβ-1. This provides a longer (200 bp) C region equivalent to Vβ in length. Clearly, the SEA-induced profile matched with D19 while the SEE profile was indistinguishable from C19. The normalized Vβ values were calculated by counting each spot and the averaged values of duplicate strips are shown in Table 4. Significantly, both SEA and C19 selectively enriched Vβ5.1 over Vβ5.3, and Vβ8 strongly. SEA and D19 selectively enriched Vβ1.1, Vβ3.1 over Vβ5.1, Vβ7.4, Vβ9.1, and Vβ21.1. Only the partial enrichment of Vβ5.1 by D19 suggested that it was more like SEE than SEA. In contrast to the previous experiments, Vβ8 was a significant proportion in the SEA profile. However, this only reflected an enrichment of Vβ8 when compared with PHA (4.8%) but not with unactivated PBL, where the Vβ8 proportion was significantly higher at 10%. As this was the starting proportion before SEA activation, there was in effect no net change in the level of Vβ8 mRNA. This suggested that only a portion of Vβ8-bearing T cells were stimulated by SEA and D19, while SEA and C19 enriched a much larger number. Stimulation of Vβ8 cells by SEA also appeared dependent on the concentration of toxin in culture. Results in Table 4 represent stimulation with 10 ng/ml, while the data in Tables 2 and 3 represent 1 ng/ml. These data suggest that residues 206 and 207 strongly influence the Vβ response to both SEA and SEE, and that changing these two residues alone can convert SEA into SEE and vice versa in terms of the general TCR Vβ response of peripheral T cells. In contrast, interchanging other large regions of both toxins did little to alter the recognition by TCR Vβ. Thus, the region that interacts with TCR Vβ must
Table 2.  TCR Vβ Enrichment to the C Series of Hybrids Is SEE-Like

| Vβ | Unstim. | SEE | AEE | AEA | C16 | C17 | C18 | C19 | Mean |
|----|--------|-----|-----|-----|-----|-----|-----|-----|------|
| cpm | %Vβ | cpm | %Vβ | cpm | %Vβ | cpm | %Vβ | cpm | %Vβ | cpm | %Vβ | cpm | %Vβ | cpm | %Vβ | cpm | %Vβ | x ± σ |
| 1.1 | 417 | <1 | 783 | <1 | 109 | <1 | 84 | <1 | 642 | <1 | 224 | 1.4 | 486 | <1 | 782 | <1 |
| 2.1 | 1,207 | 5.0 | 933 | <1 | 147 | <1 | 115 | <1 | 650 | <1 | 150 | <1 | 496 | <1 | 608 | <1 |
| 3.2 | 2,623 | 13.3 | 1,157 | 1.8 | 221 | 2.0 | 369 | 3.0 | 1,381 | 4.4 | 296 | 2.5 | 500 | <1 | 1,382 | 1.8 |
| 4.1 | 1,678 | 7.8 | 883 | <1 | 214 | 1.9 | 251 | 1.7 | 652 | <1 | 219 | 1.3 | 516 | <1 | 1,148 | 1.2 |
| 5.1 | 1,508 | 6.7 | 4,246 | 15.8 | 546 | 8.1 | 1,205 | 11.9 | 3,741 | 18.2 | 160 | <1 | 1,956 | 17.0 | 7,030 | 14.8 |
| 5.3 | 1,286 | 5.5 | 1,885 | 5.1 | 253 | 2.6 | 167 | 1.9 | 1,442 | 4.7 | 296 | 2.5 | 4,982 | 9.4 | 4.8 | ± 2.9 |
| 6.3 | 482 | <1 | 1,551 | 3.6 | 304 | 3.6 | 255 | 1.8 | 1,812 | 6.9 | 212 | 1.2 | 851 | 4.5 | 2,422 | 4.2 |
| 6.4 | 668 | 1.8 | 4,232 | 7.6 | 499 | 7.2 | 702 | 6.5 | 2,795 | 12.6 | 641 | 7.5 | 1,215 | 8.6 | 7,386 | 14.8 |
| 7.4 | 1,303 | 5.6 | 1,885 | 5.1 | 221 | 2.0 | 206 | 1.3 | 1,352 | 4.2 | 1,075 | 4.1 | 856 | 4.5 | 3,800 | 7.3 |
| 8.1 | 2,050 | 10.0 | 6,001 | 24.0 | 1,051 | 17.6 | 1,814 | 18.4 | 3,010 | 25.6 | 1,682 | 23.0 | 2,771 | 26.3 | 7,386 | 15.6 |
| 9.1 | 470 | <1 | 716 | <1 | 118 | <1 | 117 | <1 | 588 | <1 | 833 | 10.0 | 443 | <1 | 1,632 | 2.4 |
| 12.3 | 1,466 | 6.5 | 1,432 | 3.0 | 127 | <1 | 163 | <1 | 658 | <1 | 117 | <1 | 517 | <1 | 1,738 | 2.6 |
| 15.1 | 385 | 1.3 | 1,158 | 1.8 | 103 | <1 | 85 | <1 | 638 | <1 | 115 | <1 | 336 | <1 | 726 | <1 |
| 21.1 | 363 | <1 | 756 | <1 | 136 | <1 | 71 | <1 | 662 | <1 | 157 | <1 | 448 | <1 | 446 | <1 |
| 0 | 355 | 7.6 | 761 | 113 | 86 | 632 | 132 | 457 | 606 | &n...
Table 4. Similarity of Vβ/Cβ Ratios between SEA/D19 and SEE/C19

| Vβ | PHA | SEA  | D19  | SEE  | C19  |
|----|-----|------|------|------|------|
| 1.1| 2.1 ± 0.2 | 5.0 ± 0.3 | 5.0 ± 1.1 | <1   | <1   |
| 2.1| 2.2 ± 0.2 | <1   | <1   | <1   | <1   |
| 3.2| 19.3 ± 0.1 | 1.4 ± 0.2 | 1.3 ± 0.1 | 1.1 ± 0.05 | 1.6 ± 0.05 |
| 4.1| 2.9 ± 0.03 | <1   | <1   | <1   | <1   |
| 5.1| 4.2 ± 0.2 | 5.1 ± 0.2 | 11.7 ± 0.6 | 19.5 ± 1.3 | 15.6 ± 0.6 |
| 5.3| 3.0 ± 0.1 | 13.6 ± 0.4 | 13.3 ± 1.1 | 6.4 ± 0.1 | 9.0 ± 1.6 |
| 6.3| <1   | 7.5 ± 1.3 | 3.6 ± 0.3 | 4.6 ± 0.4 | 6.6 ± 0.5 |
| 6.4| <1   | 8.2 ± 1.4 | 5.0 ± 0.4 | 5.2 ± 0.5 | 7.2 ± 0.3 |
| 6.9| 2.8 ± 0.04 | 18.6 ± 3.4 | 12.3 ± 0.5 | 13.2 ± 1.0 | 16.8 ± 0.4 |
| 7.4| 2.7 ± 0.06 | 16.4 ± 1.0 | 11.5 ± 1.7 | 6.0 ± 2.0 | 8.1 ± 1.0 |
| 8.1| 4.8 ± 0.6 | 11.5 ± 1.8 | 10.5 ± 1.3 | 33.1 ± 0.5 | 17.2 ± 0.6 |
| 9.1| <1   | 3.3 ± 0.2 | 3.1 ± 0.6 | <1   | <1   |
| 12.3| 9.6 ± 0.3 | 1.5 ± 0.05 | 1.1 ± 0.1 | 1.7 ± 0.2 | 1.7 ± 0.4 |
| 15.1| 1.7 ± 0.02 | <1   | <1   | <1   | <1   |
| 21 | <1   | 1.3 ± 0.7 | 0.9 ± 0.5 | <1   | <1   |
| **Total (%)** | 55  | 93  | 80  | 91  | 84  |

PBL T cells were stimulated with 10 ng/ml of each toxin or 5 μg/ml of PHA. Duplicate RNA samples were reverse transcribed then amplified with the Cβ-2 downstream primer for 25 cycles. The 500-bp product was labeled and hybridized to duplicate strips. The Cβ figure in these experiments is multiplied by a factor of 1.2 to account for the slightly lower G/C content of the C-region with respect to Vβ. The error for each Vβ/Cβ ratio was calculated from duplicate strips.

be a highly localized region that incorporates residues 206 and 207 as an integral part of its structure. Results below indicate that for Vβ8.1 recognition other variable toxins regions can also influence this determinant but residues 206 and 207 are by far the most important.

*Response to Hybrids by Two T Cell Lines.* The panel of hybrids was tested on the SEA-reactive murine SO3 T cell line (mVβ17) and the human Jurkat cell line (hVβ138.1) (Fig. 5). SO3 was consistently stimulated by all D series hybrids, including D19, which only had Ser→Asn 2→ from SEA. None of the C series hybrids stimulated SO3, except C17, because this hybrid still had an SEA-derived COOH terminus including residues 206 and 207. Only hybrid C19 gave a very weak response that could not be increased with higher concentrations of toxin (not shown). These data confirmed that the only difference between selective reactivity by mVβ17 towards SEA was the presence or absence of Ser→Asn 2→. The results for Jurkat (Vβ8.1) also reflected the importance of residues 206 and 207. For instance, C19 differed from SEA only by two residues, Pro→Asn 2→, but stimulated Jurkat strongly while SEA was completely inactive. However, Vβ8.1 recognition was slightly more complex than mVβ17 and depended on other toxin regions as well as residues 206 and 207. For instance, D18 and D19 both stimulated strongly even though 206 and 207 were from SEA. Because D18 was active on Jurkat while EEA was not, it was concluded that the region bounded by the junctions of these two hybrids (156–200) in SEA acted in a noncooperative fashion. This noncooperative effect could be abolished by changing residues 206 and 207 in the TCR Vβ binding site. Hybrid D16, which bisects this region and is SEE up to 187, could have provided significant information but was found to have a single point mutation of a histidine involved in the MHC class II binding site (22). This reduced its potency by >1,000-fold. Because of this, no stimulation of either SO3 or Jurkat was seen with D16 even at highly toxin concentrations. Nevertheless, D16 stimulated PBL T cells very well when used at 1 ng/ml. This concentration is sufficient for peripheral T cells but not T cell lines, reflecting the much greater sensitivity of PBL-derived T cells to toxin stimulation compared with isolated T cell lines.

**Discussion**

The single-tube TCR Vβ amplification technique is reproducible, faithful, and relatively robust in its ability to determine relative levels of individual Vβ mRNAs. Duplicate analyses of 10 or more samples at once can be made and results obtained within 2 d with most of that time in hybridization.

In this paper we have used the method to determine the general TCR Vβ response of peripheral T cells to bacterial toxins. Thus, we have not been limited to individual T cell lines, which may not always reflect the general Vβ response due to influences by the Vα and junctional domains. 16 in-
dependent profiles have been presented in this paper for SEA, SEE, and the SEA/SEE hybrids, and all were either SEA- or SEE-like, which in turn were very different from other wild-type toxin profiles. This was strong evidence that the predictions concerning the Vβ determinant in SEA and SEE are valid. These predictions were further validated by stimulation of two T cell lines. We strongly emphasize that this method at present does not indicate absolute levels of Vβ-bearing T cells in culture, only the relative ratio of Vβ mRNA to the total β chain message. The reasons for this are two-fold. First, incorporation of [3H]dTTP is not identical for all Vβs due to varying G/C content. However, this did not influence individual Vβ/Cβ ratios between samples. Second, immobilized probes such as Vβ3, 7, 8, and 12.3 (previously 13.1) clearly detected more than one family member. For instance, we find that a Vβ7.3 cDNA clone hybridizes equally well to the Vβ7.4 probe (95% homology), and likewise the Vβ12.5 cDNA (new) to the Vβ12.3 probe (95% homologous). This is an accepted limitation of this method and would explain discrepancies seen between PCR data and staining with the few available anti-Vβ mAbs. For instance, Vβ8 consistently hybridized to 25–30% of SEE-activated TCR β chain cDNA while the anti-Vβ8.1 mAb C305 (23) stained only 13% of the CD3-activated cells from the same sample. For this reason we have purposefully excluded anti-Vβ mAb staining as a comparison in these experiments, even though generally the two correlate, to avoid implying they are mutually compatible. Exact specificities of both mAbs and probes must first be determined before they should be compared. For this study, it has been necessary only to indicate reproducible changes in Vβ profiles as a reflection of superantigen stimulation.

We chose to construct hybrids of SEA and SEE in order to create molecules that would closely resemble the parent toxins and minimize the chance of introducing catastrophic changes, a situation more likely to occur with random mutagenesis, thus avoiding significant alterations in both MHC binding and Vβ response. Each hybrid stimulated with identical potencies to the wild type toxins (except D16, which had a mutation in the MHC class II binding site) and gave only SEA- or SEE-like patterns of Vβ enrichment. This confirmed that each hybrid closely resembled the parental toxins and that the individual Vβ determinants were structurally stable. Notably, binding affinities towards MHC class II, which differ fivefold between SEA and SEE, are also conserved between the series but appear to map to another region (K. Hudson, S. Lowe, and J. Fraser, manuscript in preparation).

The most striking result from this study was that TCR Vβ segregated with residues 206 and 207 in the very COOH-terminal region. These conclusions were drawn from mutually consistent results obtained by both Vβ mRNA amplification and stimulation of two Vβ-bearing T cell lines. Perhaps not surprisingly, these data establish that individual Vβs see the same region of SEA and SEE but in different ways. For instance, the mVβ17 + SO3 cells responded to a slightly different hybrid pattern than hVβ8.1 + Jurkat. Stimulation of the latter Vβ was clearly dependent on another region of SEA, which mapped to a region between 156 and 200. The conclusions from these data are that residues 206 and 207 are the most influential variable residues in a broader Vβ determinant. Other amino acids that also contribute to the Vβ determinant may be invariant between SEA and SEE, so their influence was not observed in these domain-shuffling experiments. In another study, Kappler et al. (14) have shown residues in the NH2 terminus of SEB are important for Vβ binding also.

The implication that the COOH terminus of SEB is important for activity is consistent with a report in which SEA lacking the final seven residues of the COOH terminus was completely inactive (24) but conflicts slightly with a more recent report that TCR Vβ binding residues in SEB are localized in the NH2 terminus (14, 15). We would argue that the Vβ binding site is likely to be made up of residues from both regions, but in SEA and SEE, residues 206 and 207 are the only variable amino acids in this site. Further studies support the proliferation experiments present in this paper. Residues
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