TCR Binding Differs for a Bacterial Superantigen (SEE) and a Viral Superantigen (Mtv-9)

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Summary

Both superantigens (SAG) and many anti-TCR monoclonal antibodies (mAb) have specificity for the VB region of the TCR encoded by TCRBV genes. For instance the bacterial SAG staphylococcal enterotoxin E (SEE), the retroviral SAG MTV-9 and the mAb OT145 each react with human T cells expressing BV6S7. This BV gene encodes two common alleles. We found that SEE and the mAb preferentially activate T cells expressing BV6S7‘1 as opposed to BV6S7‘2, but Mtv-9 activates T cells expressing either allele. Thus binding to the TCR differs between the two SAGs. A mutation in the TCR HVR-4 region of BV6S7‘1 (G72E), where the two BV6S7 alleles differ, indicated that HVR-4 is a component of the binding site for SEE and for the mAb OT145. BV6S7‘2 has a charged E72 which may result in electrostatic repulsion of SEE, as SEE contains a similarly acidic aspartic acid residue at a TCR interaction site (204D).

Superantigens are powerful T cell mitogens produced by bacteria, mycoplasma and certain viruses. In contrast to nominal peptide antigens they do not require antigen processing and presentation to T cells is not restricted to individual MHC alleles. However, efficient presentation does require binding of the superantigens (SAG) to an MHC class II molecule at the surface of an antigen presenting cell. Typically superantigens activate T cells that express T cell antigen receptors (TCR) using specific BV genes. The bacterial SAG SEE (1), the retroviral SAG Mtv-9 (2), and the mAb OT145 (3), have each been shown to react with human T cells expressing BV6S7, previously designated VB6.7 (4). There are two naturally occurring alleles of this gene, which differ by two nonconservative amino acids substitutions at positions 38 and 72 (4, 5). BV6S7‘1 (VB6.7a) encodes S38 (serine) and G72 (glycine), whereas BV6S7‘2 (VB6.7b) encodes R38 (arginine) and E72 (glutamic acid). Position 72 is located in hypervariable region-4 (HVR-4) of the TCR β chain. HVR-4 is a region of the TCR β chain previously implicated in recognition of bacterial and viral SAGs, including SEB, SEC1, SEC2, Mtv-7, and Mtv-9 (6–9). Therefore, we tested T cell responses to SEE and Mtv-9 SAG to assess whether these SAG distinguish between the two alleles of BV6S7.

In addition to HVR-4 residues, TCR residues outside of HVR-4 have also been shown to influence SAG reactivity (10–14). Moreover, the precise topology of the interaction between SAG encoded residues and TCR residues is still unresolved as co-crystals involving SAG and TCR have not yet been described. There is data on the TCR residues involved in interactions with TCR. For instance, the COOH-terminal residues of the Mtv SAGs are polymorphic and therefore thought to be responsible for TCR BV specificity (15, 16). Several studies have pinpointed two residues on SEE that provide for the TCR BV specificity of this SAG (P203 and D204) (17–19). Using these previous results and the data described herein we propose a model of interaction between TCR BV6S7 position 72 with specific residues of SEE and describe relative positions within the trimolecular complex for TCR, superantigen and MHC.

Materials and Methods

Cells and mAbs. Heparinized peripheral blood was obtained from normal donors with known homozygous or heterozygous BV6S7 genotype (4). Mononuclear cells were isolated by Ficoll Hypaque gradient separation and in some cases T cells were further separated by rosetting with neuraminidase-treated sheep red blood cells. Cell separations with GAM-coated magnetic beads
were washed and then incubated with phycoerythrin-conjugated mAb to class I in (4), is a 19-mer with complete sequence identity with primers were able to amplify DNA from the specified subsets sized with random hexanucleotide primers and m-MLV reverse transcriptase. The transfectants were cultured in RPMI-1640 10% fetal bovine serum, 1% t-glutamine, 1% penicillin-streptomycin in the presence of G418. The mAb OT145 is specific for BV6S7 as previously described (4, 21). Other TCR BV-specific mAbs are described elsewhere (22). The Jurkat transfectants, YP59.44 and m72, have previously been described (3). Staphylococcal enterotoxin E (SEE) was obtained from Toxin Technology Inc., and used at 0.1 ng/ml.

Culture. For co-cultures of T cells with Mtv-transfectants, the latter were first treated with mitomycin C, 50 mcg/ml, to stop further proliferation. For other co-cultures irradiated (3,000 rads) autologous non-T cells were pulsed with the superantigens SEE or SEA (1 ng/ml or as indicated), then washed and added back to the autologous T cells at a 1:1 ratio. T cells were preincubated with OT145 ascites (dil. 1:500) for 1 h and then washed and cultured with APCs (irradiated non-T cell fraction from PBMC). PHA was used at 1 μg/ml. Cultures were in RPMI 1640, 10% fetal calf serum, 1% penicillin and streptomycin with added glutamine. hIL-2 (50 U/ml; Boehringer Mannheim, Indianapolis, IN) was added after 3–4 d.

Immunofluorescence. One-color and two-color flow cytometric analysis was performed as follows. 2 x 10⁶ cells were first incubated with saturating concentrations of anti-BV mAbs for 40 min on ice. The cells were washed and cultured with APCs (irradiated non-T cell fraction from PBMC). PHA was used at 1 μg/ml. Cultures were in RPMI 1640, 10% fetal calf serum, 1% penicillin and streptomycin with added glutamine. hIL-2 (50 U/ml; Boehringer Mannheim, Indianapolis, IN) was added after 3–4 d.

Intracellular Ca ++ Studies. The Jurkat transfectants, YP59.44 and m72, were collected, washed and suspended at 10⁷ cells/ml. The cells were labeled with Indo-1 (Molecular Probes, Eugene, OR) at 5 mM for 1 h at 37°C in the dark. The washed cells were resuspended in PBS, 1 mM Ca ++, 1 mM Mg ++. Fluorescence was monitored in a spectrofluorometer. Stimuli were added under the conditions of 10⁶ cells in a 0.4 ml volume. For the addition of SEE in the presence of antigen-presenting cells (APCs) the MHC class II positive 8666-P B lymphoblastoid cell line (10⁶ cells) was added at either a 1:1 or a 3:1 cell ratio before addition of SEE.

TCR BV Use by PCR. RNA was isolated using RNeasy B (CINNA/BIOTECX Labs., Houston, TX) and cDNA synthesized with random hexanucleotide primers and m-MLV reverse transcriptase. Primers for BV genes paired with a BC primer were used to amplify cDNA by PCR as described elsewhere (23). cDNA from PHA-activated normal T cells indicated that all BV primers were able to amplify DNA from the specific subsets (BV1, 2, 3, 4, 5S1, 5S2/3, 6, 7, 8S1, 8S4, 9, 10, 11, 12, 13S1, 13S2, 14, 15, 16, 17, 18, 19, 20). The BV6 sense primer used, primer I in (4), is a 19-mer with complete sequence identity with all BV6 genes and nearly all of their allelic variants. The A/T overhang vector PCR2000 was used to subclone PCR products as described elsewhere (24). Single transformed bacterial colonies were selected for sequencing of the plasmid inserts. Sequencing reactions were performed with sequenase, version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH).

PCR to Detect BV6S7 Allele Use. BV6S7*1 contains a BamHI site which is not present in BV6S7*2 (4). Therefore, BV6S7 allele usage was assessed by PCR with primers that straddle the polymorphic BamHI site and by digestion of the PCR product with BamHI as described by Visisinga et al. (25). RNA was prepared and cDNA synthesized. BV6S7 primers were 5’ GTACA-GAGAAAGGAAAGG3’ (sense) and 5’ CGGGCCCAGTCTCTC CCTGCTG3’ (anti-sense). These primers yield a 237-bp PCR product. The sense primer was end-labeled with γ-32P ATP using phosphonucleotide kinase (Boehringer Mannheim). The PCR mix contained template cDNA from 50,000 cells, 100 ng of each primer, 2.5 mM MgCl2, 0.4 mM dNTPs, 0.8 U Taq polymerase (Promega, Madison, WI) in 50 mM KCl, 10 mM Tris HCl (pH 9), 1% Triton-X. PCR was performed for 30 cycles: 94°C X 1 min, 60°C X 1 min, 72°C X 1 min, with a hot start and a final extension at 72°C X 10 min. The amplified cDNA was then digested using BamHI (New England Biolabs, Beverly, MA) under optimal conditions, run on a 10% polyacrylamide gel which was exposed to x-ray film. The intact PCR product measures 237 bp (representing BV757*2) and the upstream labeled fragment from the BamHI digest measures 189 bp (representing BV6S7*1). The relative intensity of these two bands on exposed x-ray film was assessed with a scanner and the program IMAGE®.

The nomenclature used herein for TCR BV genes is according to the GenBank L3692 entry for the entire TCR BV locus. An alternative nomenclature has recently been proposed in which BV6S7 was designated BV6S5 (26) and Rowen et al. propose the name 7-2 (27).

Results

SEE Activates BV6S7*1, but Not BV6S7*1 G72E, Expressing Jurkat T Cells. A Jurkat transfectant expressing TCR BV6S7*1 (YP55.49) was compared with a Jurkat transfectant expressing a mutated TCR BV6S7*1 chain with a substitution in the HVR4 region, G72E. This substitution was chosen because it represents the naturally occurring BV6S7*2 allele which encodes E72. Both transfectants expressed the identical Jurkat derived rearranged TCR A gene (3). SEE pulsed APCs were added to Jurkat cells, loaded with Indo-1, and intracellular Ca ++ concentration was measured over time (Fig. 1). A Ca ++ response was observed with the BV6S7*1 transfectant (YP55.49), but not with the transfectant expressing BV6S7*1 G72E (m72), although both transfectants expressed equivalent levels of TCRβ6 (3) and signaled by increasing intracellular Ca ++ in response to a CD3 mAb. This result suggests that HVR-4 is part of the binding site for SEE presented by class II-positive APCs. Consistent with previous results which showed undetectable binding of the mAb OT145 to m72 cells (3), there was no Ca ++ response to the OT145 mAb, even in the presence of cross-linking rabbit anti-mouse (RAM) antibodies (Fig. 1).

MTV-9 Activates T Cells Expressing Both BV6S7 Alleles. The viral SAG of Mtv-9 was tested as a stable transfectant in conjunction with human HLA DR1 in mouse fibroblasts (2). Co-culture of this transfectant with normal hu-
man T cells results in expansion of the BV6S7 subset which can easily be monitored by staining the T cells with the monoclonal antibody OT145. This is a highly specific BV response, as a survey with a panel of workshop antibodies covering over 60% of the TCR BV repertoire revealed no other expanded BV subsets in response to Mtv-9 SAG stimulation (28).

Previous studies had shown that individuals homozygous for BV6S7*2 had very low levels of OT145 positive cells (4, 21). Together with the poor binding of OT145 to m72 cells (3), this suggested that OT145 preferentially reacts with T cells expressing BV6S7*1.

To determine whether Mtv-9 prefers to react with one or the other allele of BV6S7, cells from two homozygous BV6S7*2 donors and one homozygous BV6S7*1 donor were stimulated with the Mtv-9 transfectant. IL-2 was added on day 3 of culture. On day 10 the T cells were stained by indirect immunofluorescence. As shown in Fig. 2, T cells from all 3 individuals responded with expansions of OT145+ cells. This demonstrates that the Mtv-9 SAG is capable of stimulating T cells expressing either allele of BV6S7. The fluorescence intensity of the OT145 staining was 5–10-fold greater in the cells derived from donor DNP (homozygous for BV6S7*1) as compared to the two donors homozygous for BV6S7*2. This was reproducible in five experiments and demonstrates the lesser reactivity of OT145 with the product of allele BV6S7*2 (see above). In addition, the magnitude of the response (% OT145 positive T cells) is approximately twofold larger with the BV6S7*1 homozygous cells.

To identify the TCR BV genes used among the OT145+ T cells responding to Mtv-9 SAG, the activated T cells from the experiment shown in Fig. 2 were separated into OT145+ and OT145− cells using magnetic beads coated with goat anti-mouse Ig antibody. BV gene usage was assessed by RT-PCR (Fig. 3). A striking predominance of BV6 message was found in the Mtv-9 SAG activated T cells without other BV genes detectable. The findings were identical for both OT145+ and OT145− cells. However, a semi-quantitative RT-PCR indicated that there was at
least 50-fold more BV6 RNA in OT145+ cells compared with OT145− cells (data not shown).

BV6 is the largest human BV family and contains seven functional genes (22, 27). It was therefore necessary to analyze the PCR product by sequencing to determine which BV6 genes were utilized. PCR products amplified with a pan-BV6 and BC (constant region) primer were subcloned and sequenced. We found that 11/17 sequences from the BV6S7*1 homozygous donor (OT145+ cells) were BV6S7*1 with unrelated V-D-J junctions and BJ usage (Fig. 4 A). In contrast, sequences from the homozygous BV6S7*2 donors yielded BV6S7*2 in 13/17 (Fig. 4 C) and 9/9 cases (Fig. 4 B). BV6S7 was less frequently represented in the amplified BV6 sequences derived from OT145− cells. It is possible that T cells using BV6 genes other than BV6S7 can also respond to Mtv-9. These other BV6 genes might include BV6S3, BV6S4, and BV6S5 seen primarily in the OT145− subset (Fig. 4, A−C). As the total amount of BV6 mRNA was about 50× greater in the OT145+ subset, transcripts with these alternative BV6 genes must represent a minority of the T cells responding to the Mtv-9 SAG. The BV sequence data are summarized in Fig. 5. There are some BV6 genes that are conspicuously absent from the Mtv-9-stimulated cells: BV6S1, BV6S11, BV6S14. As a con-

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**Figure 2.** Indirect immunofluorescence staining of T cells cultured for 10 d with Mtv-9 SAG expressing transfectant cells. The T cell donor genotypes were as follows: DNP homozygous for BV6S7*1/1, DP and AA homozygous for BV6S7*2/2. Percentages of positive cells are indicated in each panel. The two different levels of staining intensity with OT145 are indicated by arrows.

**Figure 3.** PCR for indicated TCR BV families in cells stimulated with Mtv-9 SAG. The source of the cells and the culture conditions are indicated on the left and lambda PstI size markers (bp) on the left. PCR product was run on a 1% agarose gel and stained with ethidium bromide. The cells analyzed here were OT145− cells sorted after the 10-d culture (see Fig. 2 and methods); identical results were obtained with OT145− cells from the same cell sort.

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### Table 1. BJ Use in BV6S7 Versus Other BV6 TCR

| BJ segments | 1.1 | 1.2 | 1.3 | 1.4 | 1.5 | 1.6 | 2.1 | 2.2 | 2.3 | 2.4 | 2.5 | 2.6 | 2.7 | n |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|
| DNP BV6S7   | 1   | 1   | 1   | 2   | 1   | 1   | 1   | 1   | 1   | 1   | 3   | 13  |     |    |
| other BV6   | 1   | 3   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 5   | 15  |     |    |
| DP BV6S7    | 2   | 1   |     |     |     |     |     |     |     |     |     |     |     |    |
| other BV6   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 2   | 1   |     |     | 7 |
| AA BV6S7    | 3   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 18 |
| other BV6   | 1   | 3   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 2   | 2   | 8   |     |    |
| all BV6S7   | 6   | 3   | 1   | 2   | 3   | 4   | 3   | 5   | 2   | 3   | 3   | 9   | 44  |     |
| other BV6   | 2   | 3   | 3   | 2   | 5   | 1   | 2   | 3   | 2   | 7   | 30  |     |     |
| as percent  | BVE67 | 14  | 7   | 2   | 5   | 7   | 0   | 9   | 7   | 12  | 5   | 7   | 7   | 21  |
| other BV6   | 7   | 10  | 0   | 10  | 7   | 17  | 3   | 7   | 10  | 0   | 7   | 0   | 23  | 100 |
control for variations in PCR efficiency, some of these sequences could readily be amplified from genomic DNA. Moreover, an additional antibody specific for BV6S1 (22) failed to stain Mtv-9-activated T cells (28).

In unrelated studies effects of BJ encoded (14) or V-D-J junctional encoded (13) amino acids on SAG reactivity have been uncovered. However, BJ usage was equally distributed among BV6S7 and other BV6 TCRs in all three donors tested with only a few possible exceptions: BJ1S6 was used by 0/44 BV6S7 sequences, but 5/30 other BV6 sequences, and BJ2S6 was used by 3/44 BV6S7 sequences, but 0/30 other BV6 sequences (Table 1). There is no amino acid motif from the V-D-J or the J region that is shared exclusively by BV6S7 sequences and not other BV6 sequences (Fig. 4) and it seems unlikely that the minor difference in BJ usage reflects preferences of the SAG.

SEE and OT145 mAb Each Preferentially Activate the BV6S7*1 Allele. To distinguish between reactivity preferences of the SAGs and of the mAb OT145, an experimental system was needed that did not rely on OT145 staining or sorting and in which cells expressing both BV6S7 alleles were present. Therefore, T cells from BV6S7 heterozygous donors were stimulated with either SEE, Mtv-9 SAG or OT145 and cultured with IL-2 for 10 d. Expansion of T cells using BV6S7*1 versus BV6S7*2 was then measured by RT-PCR with two BV6S7-specific primers, of which the upstream primer was end-labeled with 32P (the amplified fragment is 237 bp). The PCR product of the BV6S7*1 allele contains a BamHI site. After BamHI digestion the labeled upstream fragment (189 bp) migrates faster (Fig. 6). To control for partial BamHI digestion, PCR amplified DNA from a BV6S7*1 homozygous donor was used (DNP, Fig. 6 A). Mtv-9 and PHA stimulation of T cells from a heterozygous donor (SF) resulted in expression of both BV6S7 alleles (Fig. 6 A). By contrast, OT145 stimulation led to a marked shift to BV6S7*1 expression.

To assess the effect of SEE in this system, BV8 positive T cells need first be removed, as this BV subset is the preferred target of SEE (29). Effects of SEE on BV6S7 T cells

Figure 5. Summary of the frequency of TCR sequences of the indicated BV families. The seven functional BV6 genes contained in the BV locus (Genbank L36092) and one of three BV6 pseudogenes (BV6S10) are listed. The left hand panels summarize data from Fig. 4 indicating the frequency of BV6 genes used by OT145+ T cells (solid bars) or OT145− T cells (open bars) responding to Mtv-9 SAG in the three donors. The right hand panels are controls in which the same PCR was performed with genomic DNA from two donors showing a random pattern of BV gene frequency.

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Figure 6. BV6S7*1 and BV6S7*2 usage among polyclonal T cells stimulated with Mtv-9 SAG, SEE, SEA, PHA and OT145. Labeled PCR products digested with BamHI are shown on the right and the quantitative scanning image on the left with the percentage of signal corresponding to BV6S7*1. A, B, and C represent different experiments with T cells from two different heterozygous donors. In A cells were stimulated for 10 d with the indicated conditions and then harvested for RT-PCR. In B and C the cells were first stimulated with Mtv-9 SAG and cultured for 10 d and then restimulated with the indicated ligands and cultured for 7 d. B shows staining with OT145 and (C) RT-PCR, for the two BV6S7 alleles. The 5% signal for BV6S7*2 in the *1/*1 homozygous donor represents background due to incomplete BamHI digestion. "AM ex vivo" represents RNA isolated from fresh PBL and indicates the baseline distribution of the two alleles in this heterozygous donor. In D the experiment was repeated with the SEE D19 and SEA C19 mutants. Dtkl denotes stimulation with a DAP-DRBI*0101/DRA transfectant as a control for the Mtv-9 SAG transfectant which also expresses with DRBI*0101. AM has a DRBI*0102, DRBl*1301 genotype by PCR (courtesy of Dr. D. Kostyu, Duke University). The numbers indicate relative percentages for expression of the two BV6S7 alleles.
can otherwise not be discerned, presumably because inter-
action with BV6S7 T cells is of lower affinity. Therefore
BV6S7-expressing T cells from a heterozygous donor were
first expanded by stimulation with Mtv-9 and cultured in
IL-2 containing medium for 10 d: on day 7 OT145+ cells
represented 27% of all CD3+ cells. On day 8 BV8-
expressing cells (~3%) were removed with magnetic beads.
On day 10 the cells were washed and a second culture was
initiated with the additives listed. On day 17 the cells were
stained (Fig. 6 B) and RNA was harvested for RT-PCR
(Fig. 6 C). At this time each culture contained an expanded
subset of OT145+ cells (Fig. 6 B) but BV8 expressing cells
were undetectable (not shown). BV6S7 allele usage was not
skewed in cells stimulated with Mtv-9 or with the control
SAG SEA (Fig. 6 C). SEE used at two different concentra-
tions favored expression of BV6S7*1. OT145 stimulation
resulted in nearly exclusive expression of BV6S7*1. To
control for the possible effects of DR1 expressed on the
Mtv-9 transfectant, we used cocultures with DAP-DR1 fi-
broblasts (with no SAG) and cocultures of SEE presented
by DAP-DR1 fibroblasts. Similar results to those in Fig. 6 C
resulted in nearly exclusive expression of BV6S7*1. To
control for the possible effects of DR1 expressed on the
Mtv-9 transfectant, we used cocultures with DAP-DR1 fi-
broblasts (with no SAG) and cocultures of SEE presented
by DAP-DR1 fibroblasts. Similar results to those in Fig. 6 C
were obtained. Together with the results of the Jurkat
transfectants in Fig. 1, we conclude from these data that
both SEE and OT145 react preferentially with allele *1.

Position 72 of TCR BV6S7 Lies in Close Proximity to Resi-
dues 203-204 of SEE. Previous work has identified two
adjacent positions in SEE and SEA that impart specificity
for BV encoded regions (17-19). Thus, BV specificity was
exchanged between SEE and SEA by replacing only 2-3
residues in an exposed loop between β strand 10 and α he-
elix 5 (18). Therefore we used SEE D197G P203S D204N
(D19), which has acquired the specificity or SEA, and SEA
S206P N207D (C19) with the acquired specificity of SEE,
to see whether BV6S7 cells were stimulated and whether
there was a preference for either allele of BV6S7. Fig. 6 D
demonstrates that SEA and D19 gave less stimulation of
BV6S7 cells as indicated by low intensity PCR bands and
lower percentage of OT145+ cells by FACS® (data not
shown). Both alleles of BV6S7 were equally represented.
By contrast SEE and C19 were more stimulatory and levels
of BV6S7*1 RNA were predominat over BV6S7*2 in 3/3
experiments. C19 usually showed even greater preference
for allele *1 than SEE itself.

These results indicate that the ability to discriminate be-
tween G72 and E72 of the TCR δ chain HVR-4 lies in
residues P203 and D204 of SEE (Fig. 7). Therefore, this
region of SEE is likely in close proximity of HVR-4 posi-
tion 72. This constrains the possible conformations of a tri-
molecular complex (MHC-TCR-SEE) and is most com-
patible with a position of SEE similar to that of SEB and
TSST1 relative to MHC class II, e.g., adjacent to the δ he-
lix of the α1 domain of MHC class II (30, 31).

Discussion

This paper addresses three unresolved questions. (a) Do
all SAGs, including bacterial (bSAG) and viral (vSAG), in-
teract in the same way with TCR residues or not? (b) What
is the relative position of the TCR within the tri-molecular
complex and is it variable from one complex to another? (c)
Do naturally occurring allelic variants of TCR BV genes
differ functionally?

Variable SAG-interactive Sites on TCR β Chain. Generally,
SAGs are thought to bind to the lateral surface of the TCR
β chain and in particular to residues of the HVR4 region.
Positions 73/74 (adjacent to position 72 investigated in this
study) were implicated by mutagenesis in binding of
mBV8S2 to Mtv-7 vSAG (7) and in binding of mBV17 to
Mtv-9 vSAG (9). HVR4 was shown to impart specificity
for the bSAGs SEC2 and SEC3 by molecularly transplant-
ing 11 HVR4 residues of the reactive BV13S2 to the non-
reactive BV13S1 which then acquired SEC2 and SEC3 re-
activity (8). Thus, both vSAG and bSAG seem to focus on
the TCR HVR4 region.

Early studies with the vSAG of Mtv-7 showed that gly-
cosylation of N residues 18, 20, and 74 of mBV8S2 (16, 18,
and 71 in original reference) inhibited reactivity with Mtv-7
(7). However, several bSAGs showed equivalent dissocia-
tion constants for binding of glycosylated versus unglycosy-
lated mBV8S2 in a recent BIAcore binding study (32). Al-
though Mtv vSAG were not examined in this study, the
implication is that the vSAG of Mtv-7 may differ from
bSAGs in terms of the TCR contact residues of mBV8S2
used.

Herein we show that a bSAG (SEE) and a vSAG (Mtv-9) differ
in fine specificity for human TCR BV6S7. This TCR
gene is naturally allelic. The vSAG of Mtv-9 stimulated
both alleles equally well, but the bSAG, SEE, preferentially
reacts with the BV6S7*1 allele. A monoclonal antibody,
OT145, also has preferential reactivity with this allele. Al-
though there are two amino acid differences between the
two alleles, the single residue important for preferential re-
activity of both ligands is position 72 (Fig. 1), at the apex of
HVR-4, right between δ strands D and E at a sharp turn in
the carbon backbone of the TCR δ chain (3, 33). BV6S7*1
encodes G72 and BV6S7*2 encodes a negatively charged E72.
The difference in fine specificity between SEE and the vSAG of Mtv-9 means that each SAG interacts differently with the same TCR. In fact, others have described TCR BV mutations that affect different SAGs in opposite ways. For instance m_BV10 T26A resulted in diminished reactivity with SEC2 but not with ExT and L27A decreased reactivity with ExT but not SEC2 (34). In the D10 TCR mBV8.2 N24H allows recognition of Mtv-7 SAG and SEB, but wild-type N24 allows recognition of only SEB. In the same TCR G51V G53D allows recognition of SEB, but not SEC1.3, which are all recognized by the wild-type TCR (35). Finally, TCR B chains expressing various BV genes, each known to provide for reactivity with the same SAG, do not share common residues when comparing modeled structures based on the crystal structure of the TCR B chain (32). One explanation for these data is that there are several distinct binding sites for the same SAG depending on the TCR involved.

It is possible that there are subgroups of SAGs that interact with TCR B chain in a similar way. Such distinct groups might include the group of Mtv vSAGs, the bSAGs with two MHC class II binding sites of which one is Zn++ dependent (e.g., SEA), or the bSAGs with a single MHC class II binding site (e.g., SEB, TSST-1). For instance SEA cross-links MHC class II molecules (36, 37). With SAG molecules on both sides of an MHC class II molecule the position of the TCR may be constrained.

There are several possible explanations for the relative lack of reactivity of BV6S7*2 with SEE and OT145. A simple hypothesis is that the shape of the HVR4 region is modified in the absence of a large charged R group at position 72. This was examined (Fig. 8) by comparing the modeled structure of the two human TCR BV6S7 alleles using the crystal coordinates of a mouse TCR B chain (38). The model shows no positional differences in the carbon backbone of HVR4 and the CDR loops between the two alleles. The large charged side chain of E72 lies in a trough bordered by CDRI, CDRII, and HVR4 residues (Fig. 8). Thus the reactive BV6S7*1 allele, encoding G72 (with just a hydrogen as a side chain), is characterized by a surface depression which could accommodate an SEE residue. BV8, the favored target of SEE encodes A72, also with a very small side chain (CH3). In contrast, the depression is filled in BV6S7*2 with the charged side chain of E72 (CH2-CH2-COO-). The oxygen atoms of E72 are potential electron donors, but the closest individual atoms, from CDRI (H24) or CDRII (Q51), are at a distance of 3.36 Å or greater. Thus hydrogen bonds and salt bridges are unlikely and E72 is probably fully charged at a physiological pH.

An alternative hypothesis is that the critically located charge at E72 might result in electrostatic repulsion be-

### Table 2. Alignment of HVR4 from the 7 BV6 Genes, BV16 and mBV8S2

| BV6S1     | ERTGGSVST | 68  |
|-----------|-----------|-----|
| BV6S3     | ERTGGSVST |    |
| BV6S4     | ERTGGSVST |    |
| BV6S14    | ERTGGSVST |    |
| BV6S11    | ERTGGSVST |    |
| BV6S5     | ERTGGSVST |    |
| BV6S7*1   | ERTGGSVST |    |
| BV6S7*2   | ERTGGSVST |    |
| BV16S1    | ERTGGSVST |    |
| BV8S1     | KMPNASFST |    |
| mBV8S2    | SRPSQENFS  |    |

Acidic residues are underlined and basic residues are in bold.
tween TCR β chain and the SAG. BV8, like BV6S7*I, does not encode a charged residue at the apex of the HVR4 region (Table 2). The negative charge of E72 could prevent interaction with SEE due to a negatively charged SEE residue. Two amino acids of SEE that determine BV specificity have been mapped (17-19). The SAGs SEE and SEA are closely related (82% identity) but have different BV specificities. Thus, SEA mutated at two residues (SEA $206P N207D$) to emulate the sequence of SEE (Fig. 7), resulted in gain of the BV8 and BV5S1 specificities of SEE. The reverse result was obtained with SEE mutated to look like SEA, SEE D197G P203S, D204N (SEE residue numbering), e.g., gain of BV5S3, BV7S4, and BV9S1 specificities of SEA. Therefore, it is possible that D204 of SEE may lie in close proximity of position 72 of the TCR β chain. In support of this, Mariuzza and colleagues have found that the equivalent regions of SEC3 and mBV8S2 form close contacts in a co-crystal (personal communication).

**Relative Position of TCR within the Tri-molecular Complex.** SEE has not yet been crystallized. However, a crystal structure of the related SEA (39) and a co-crystal of SEB (30) and TSST1 (31) with HLA DR1 have been described. The SAG structures are in general quite similar, in particular the region corresponding to SEE96-204 which represents a solvent exposed loop linking the β10 strand to the α5 helix (39-41), see Fig. 7. In the SEB-DR1 co-crystal structure (30) this loop protrudes such that it overlies the HLA DR1 α helix of the α1 domain (DR1 residues α51-α57) in a position where it could bind to TCR β chain residues. Using this model and assuming close proximity between residue E72 of the TCR β chain and residue D204 of SEE, the TCR β chain CDR1 and CDR2 would be placed over the MHC α1 domain and the CDR1 and CDR2 of the TCR α chain on the α helix of the MHC β1 domain consistent with some models of TCR/MHC positioning (42, 43). However, a recent model of TCR positioning, based on recognition of variant conalbumin peptides presented by IEk (44), places the TCR β chain and HVR-4 over the COOH-terminal portion of the peptide which is inconsistent with binding to known SAG TCR interaction sites and the SAG/DR1 co-crystal data. If these results are generally valid for nominal Ag/MHC recognition, it follows that the rotational orientation of the TCR differs between Ag/MHC versus SAg/MHC recognition. In addition, it is likely that the axial orientation of the TCR differs because SAGs, specially TSST1 (31), occupy space above the class II α helix of the α1 domain and thus are probably wedged in between TCR and MHC on one side. This is consistent with our data, as position 72 of HVR4 would lie on top of SEE D204 in stead of making contact with MHC class II residues (Fig. 9).

**Other TCR Regions That Contribute to SAG Recognition.** The actual TCR residues of BV6S7*I that mediate binding to SEE probably include additional residues beyond residue 72, because the HVR4 motif ERTGGxxST68-76 is shared with BV16 (Table 2) which is neither SEE, Mtv-9 or OT145 reactive (3, and data not shown). Moreover, reactivity of OT145 and SEE with BV6S7 is not completely inhibited in the presence of E72. Thus the interaction between TCR HVR-4 and these ligands is presumably more complex and involves TCR residues other than position 72. TCR residues implicated in SAG reactivity by mutagenesis experiments generally do not affect Ag/MHC reactivity, and vice versa (45). However, from studies of SAG/TCR combinations of presumed “lower affinity,” where an effect of the presenting MHC class II is readily seen, it has become clear that residues beyond HVR4 and the lateral surface of the TCR β chain are involved in determining SAG reactivity, such as residues in TCR β CDR1-III (10, 11, 13, 14, 34) and residues of the TCR α chain (46, 47). It is therefore important to avoid drawing conclusions based solely on experiments with transfected T cell clones in which a single TCR is expressed. SEE has low reactivity with BV6S7 TCRs (compared with BV8) and it was therefore useful to confirm the transfectant data (Fig. 1) with an assay using polyclonal T cells from heterozygous donors. The RT-PCR assay described for detection of BV6S7 alleles (Fig. 6) has not been previously used to monitor SAG responses. Results are independent of all other TCR variable gene segments (BD, BJ, AV, AJ) used by T cells responding to the SAG, because polyclonal T cells are used. Any possible effect of MHC alleles (25) can be ruled out by using T cells from HLA disparate donors. Thus this system
allows one to detect discrete differences in TCR usage (BV6S7 alleles) in a SAG response of polyclonal T cells.

**Evolutionary Significance of TCR BV Alleles.** The data demonstrate that two naturally occurring alleles of a TCR BV gene differ in their functional reactivity with natural ligands such as the bacterial superantigen SEE derived from *Staphylococcus aureus*, a common human pathogen. Most normal adults have antibodies to staphylococcal SAGs indicating prior exposure. Therefore it is likely that the TCR BV6S7 alleles evolved due to ligand interactions leading to selective advantages of one allele versus the other, as opposed to neutral mutations and genetic drift.

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