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 Vβ 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)[1] 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 Vβ6.7 (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 (Vβ6.7a) encodes S38 (serine) and G72 (glycine) , whereas BV6S7*2 (Vβ6.7b) encodes R38 (arginine) and E72 (glutamic acid). Position 72 is located in hypervariable region-4 (HVR4) 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 SAG 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

[1] *Abbreviations used in this paper:* RAM, rabbit anti-mouse; SAG, superantigens; SEE, Staphylococcal enterotoxin E.

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(Immunotech SA, Marseilles, France) were performed as previously described (20). These separations were monitored by staining before and after the separation. The efficiency of this procedure is ~90%. DAP mtv-9 is a stable transfectant expressing HLA-DR1 and the Mtv-9 *sag* gene cloned in to the expression vector pHAb-Aprl-neo on a mouse DAP fibroblast background. The transfectants were cultured in RPMI-1640 10% fetal bovine serum, 1% L-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.

Cultures. 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 PBMNC). PHA was used at 1 μg/ml. Cultures were in RPMI 1640, 10% fetal calf serum, 1% penicillin and 1% 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. 2×10^5 cells were first incubated with saturating concentrations of anti-BV mAbs for 40 min on ice in RPMI 1640, 1% fetal bovine serum (FBS). Anti-CD3, anti-CD4, anti-CD8, and the negative control mAb FFa26 were used as controls. After incubation, cells were washed with RPMI 1640 1% FBS and then stained with goat anti–mouse Ig conjugated to FITC (GAM-FITC; Tago Inc., 1:50 dilution) on ice for 30 min. After washing, 5,000 cells were analyzed on a Coulter EPICS cytofluorograph (Hialeah, FL). For two-color staining, mAb FFa26 (IgG1) was added for 20 min on ice to block unoccupied binding sites of GAM-FITC antibodies. Cells were washed and then incubated with phycoerythrin-conjugated anti-CD8 or anti-CD4 mAb (Immunotech) for 30 min on ice.

Intracellular Ca^{++} Studies. The Jurkat transfectants, YP59.44 and m72, were collected, washed and suspended at 10^7 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 continuous stirring of 10^6 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 8866-P B lymphoblastoid cell line (10^7 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 RNAzol 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 specified 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 Vissinga et al. (25). RNA was prepared and cDNA synthesized. BV6S7 primers were 5′GTCACAGAGAAGGGAAAGG3′ (sense) and 5′CGGCCGAGTCCTCCTGCTG3′ (anti-sense). These primers yield a 237-bp PCR product. The sense primer was end-labeled with γ-^{32}P ATP using phosphonucleotide kinase (Boehringer Mannheim). The PCR mix contained template cDNA derived from ~50,000 cells, 100 ng of each primer, 2.5 mM MgCl, 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 × 1 min, 60°C × 1 min, 72°C × 1 min, with a hot start and a final extension at 72°C × 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 BV7S7*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^R.

The nomenclature used herein for TCR BV genes is according to the Genbank L36092 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 APC 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 TCR$\alpha\beta$ (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-

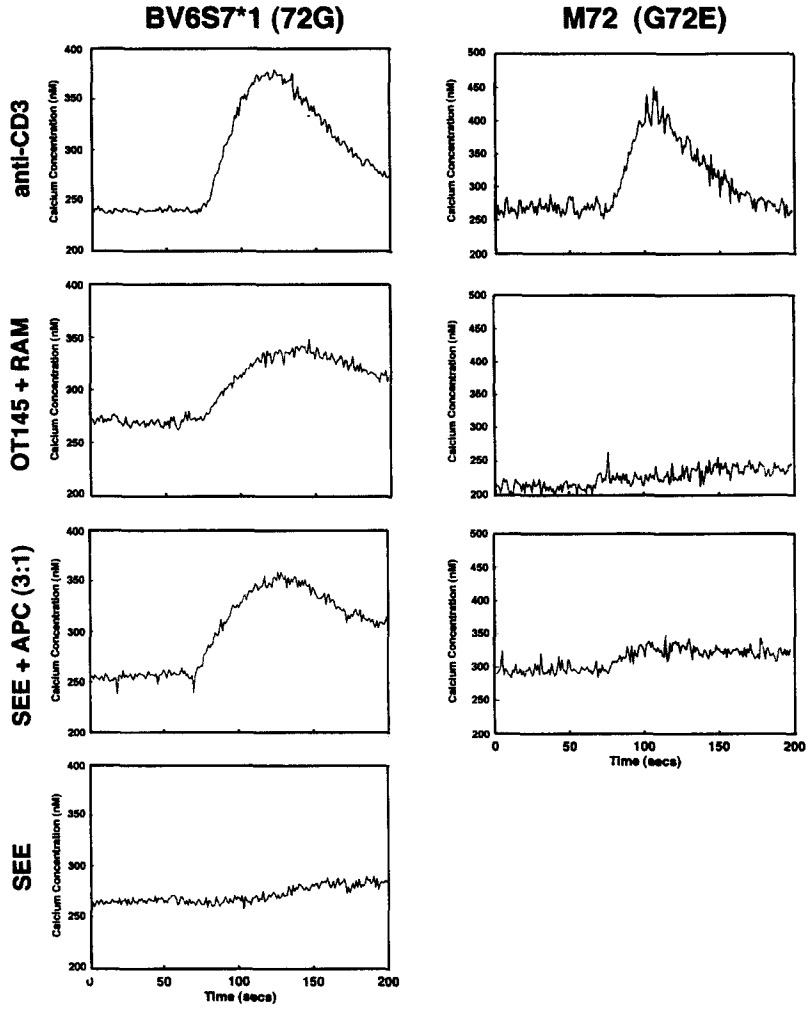

Figure 1. Intracellular calcium signaling in response to TCR ligands. YP55.49 and M72 are stable transfectants of JRT3 (Jurkat subline) expressing wild-type BV6S7*1 and mutated BV6S7*1 G72E (3). The TCR α and β chains are otherwise identical. The indicated stimuli were added at 60 s. The APC were 8866P B lymphoblastoid cells added at a cell ratio of 3:1 (APC: Jurkat T cells).

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 in to 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

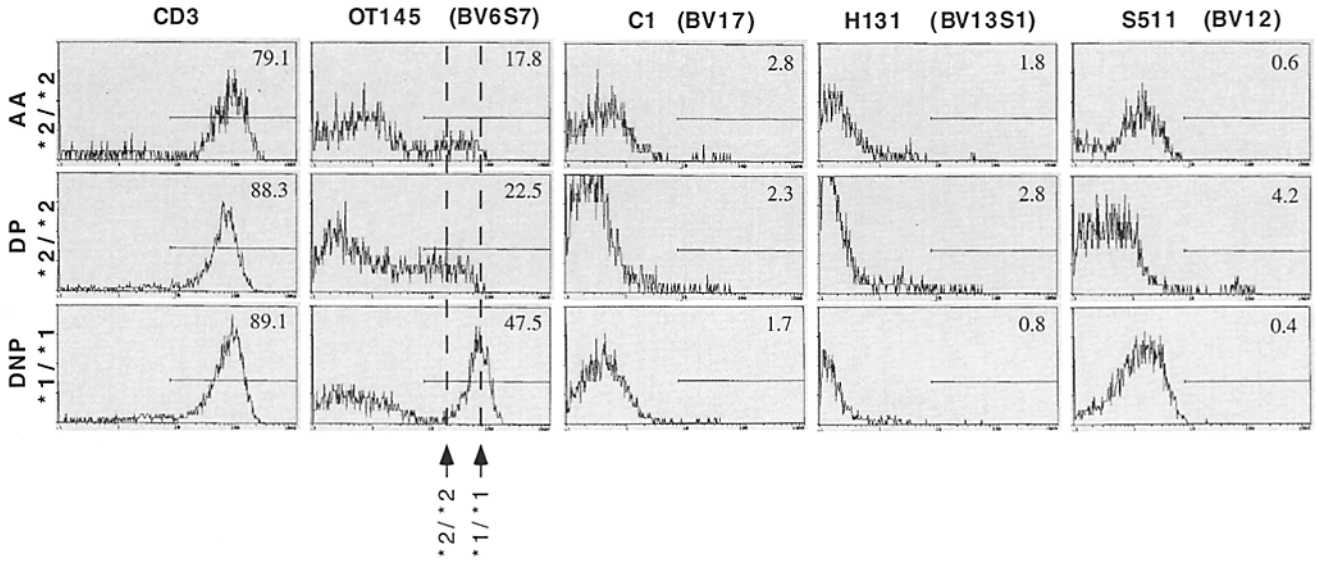

Figure 2. Indirect immunofluorescence staining of T cells cultured for 10 d with Mtv-9 SAG expressing transfectant cells. The T cell donor genotypes were as follows: DNP homozygous for BV6S7*1/1, DP and AA homozygous for BV6S7*2/2. Percentages of positive cells are indicated in each panel. The two different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

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-

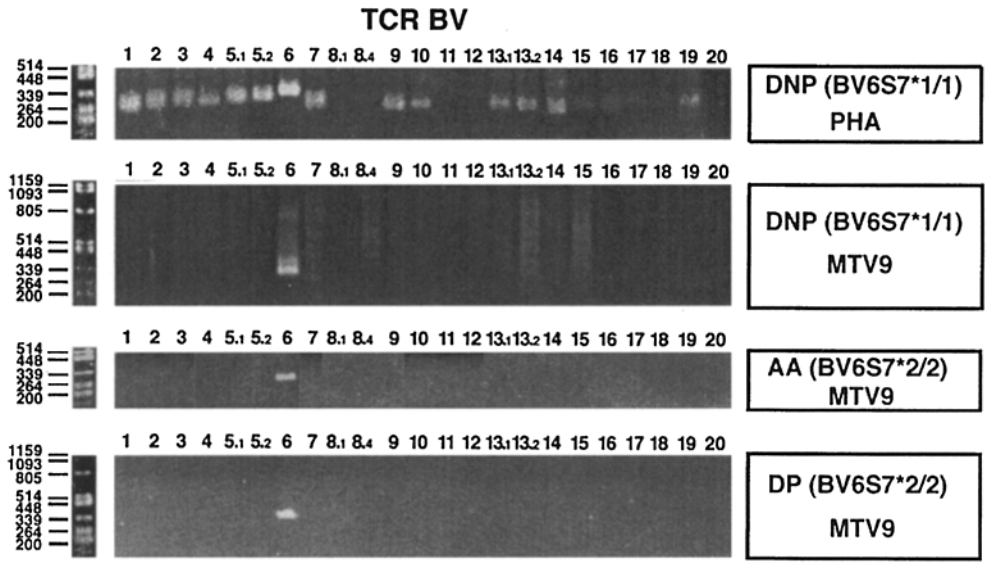

Figure 3. PCR for indicated TCR BV families in cells stimulated with Mtv-9 SAG. The source of the cells and the culture conditions are indicated on the right and lambda PstI size markers (bp) on the left. PCR product was run on a 1% agarose gel and stained with ethidium bromide. The cells analyzed here 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.

A

CLONE	BV gene	CDRIII region	BJ	frame	n
DNP+6/o	**BV6S7*1**	CAS GGSW NTEAFFGQGT	BJ1S1	IF	1
DNP+14/o	**BV6S7*1**	CASS GG YGYTFGSGT	BJ1S2	IF	1
DNP+27/o	**BV6S7*1**	CASS PQEL SGNTIYFGEGS	BJ1S3	IF	1
DNP+17/o	**BV6S7*1**	CASSL GQGKG EKLFFGSGT	BJ1S4	IF	1
DNP+29/o	**BV6S7*1**	CASSL GRG SNQPQHFGDGT	BJ1S5	IF	1
DNP+7/o	**BV6S7*1**	CASSL GGTGIP QPQHFGDGT	BJ1S5	IF	1
DNP+25/o	**BV6S7*1**	CASS PRTGR NTGELFFGEGS	BJ2S2	IF	1
DNP+13/o	**BV6S7*1**	CASS RSTRVSG DTQYFGPGT	BJ2S3	IF	1
DNP+15/o	**BV6S7*1**	CASS PTAAITS IQYFGAGT	BJ2S4	IF	1
DNP+11/o	**BV6S7*1**	CASS QWKS QETQYFGPGT	BJ2S5	IF	1
DNP+30/o	**BV6S7*1**	CASSL GQ SYEQYFGPGT	BJ2S7	IF	1
DNP+34/o	BV6S3	CASS QAGPR QPQHFGDGT	BJ1S5	IF	1
DNP+4/o	BV6S3	CAS IRGGA__ ETQYFGPGT	BJ2S5	OF	1
DNP+28/o	BV6S4	CASSL AGTE QETQYFGPGT	BJ2S5	IF	1
DNP+36/o	BV6S5	CASS PDS YGYTFGSGT	BJ1S2	IF	1
DNP+12/o	BV6S5	CASS LQNDQ GYTFGSGT	BJ1S2	IF	1
DNP+32/o	BV6S5	CASS IASGG SYEQYFGPGT	BJ2S7	IF	1
dnp-2	**BV6S7*1**	CASS LKREGT YEQYFGPGT	BJ2S7	IF	1
dnp-9	**BV6S7*1**	CASS PTGTGY YEQYFGPGT	BJ2S7	IF	1
dnp-18	BV6S3	CASSL ARGATS NEKLFFGSGT	BJ1S4	IF	1
dnp-11	BV6S3	CASSL ALGS DTQYFGPGT	BJ2S3	IF	1
dnp-12	BV6S4	CASS QAGGRA EAFFGQGT	BJ1S1	IF	1
dnp-5	BV6S4	CASS FAGTTS YTFGSGT	BJ1S2	IF	1
dnp-17	BV6S4	CASS LTGGS SYNSPLHFGNGT	BJ1S6	IF	1
dnp-19	BV6S4	CASS AHRVEAF NEQFFGPGT	BJ2S1	IF	1
dnp-10	BV6S4	CASS DGRW QYFGPGT	BJ2S7	IF	1
dnp-4	BV6S4	CASS LSNQ YEQYFGPGT	BJ2S7	IF	1
dnp-7	BV6S4	CASS PAYGGSG EQYFGPGT	BJ2S7	IF	1
dnp-6	BV6S5	CASS LQNDQ GYTFGSGT	BJ1S2	IF	2
dnp-3	BV6S5	CASS FIGSSA YEQYFGPGT	BJ2S7	IF	1

B

CLONE	BV gene	CDRIII region	BJ gene	frame	n
DP+17	**BV6S7*2**	CASS YVR NTEAFFGQGT	BJ1S1	IF	1
DP+26	**BV6S7*2**	CASSL VRAF NTEAFFGQGS	BJ1S1	IF	1
DP+19	**BV6S7*2**	CASS PGGY GYTFGSGT	BJ1S2	IF	1
DP+22	**BV6S7*2**	CASSL RQGT EQFFGPGT	BJ2S1	IF	1
DP+18	**BV6S7*2**	CASSL RAGAL NEQFFGPGT	BJ2S1	IF	1
DP+6/o	**BV6S7*2**	CASS RSSS YNEQFFGPGT	BJ2S1	IF	1
DP+1/o	**BV6S7*2**	CAS RGSA GANVLTFGAGS	BJ2S6	IF	1
DP+2/o	**BV6S7*2**	CASS LGL YEQYFGPGT	BJ2S7	IF	1
DP+19/o	**BV6S7*2**	CASS LMGPF YEQYFGPGT	BJ2S7	IF	1
dp-3	**BV6S7*2**	CASS WEGERG_ TFGSGT	BJ1S2	OF	1
dp-7	**BV6S7*2**	CAS _TFLGAH NEQFFGPGT	BJ2S1	OF	1
dp-24	**BV6S7*2**	CAS NLVA DTQYFGPGT	BJ2S3	IF	1
dp-26	**BV6S7*2**	CAS RGSA GANVLTFGAGS	BJ2S6	IF	1
dp-28	**BV6S7*2**	CASS FRWD ETQYFGPGT	BJ2S5	IF	1
dp-32	**BV6S7*2**	CAS TRTSGGG GELFFGEGS	BJ2S2	IF	1
dp-30	BV6S4	CASS SIQT NSPLHFGNGT	BJ1S6	IF	1
dp-31	BV6S4	CASS FRY TGELFFGEGS	BJ2S2	IF	1
dp-4	BV6S4	CA RYLALG YNQPQHFGDGT	BJ1S5	IF	1
dp-6	BV6S4	CASS PLDV ETQYFGPGT	BJ2S5	IF	1
dp-1	BV6S3	CASS TGQGG TGELFFGEGS	BJ2S2	IF	1
dp-23	BV6S3	CASSL TG TNEKLFFGSGT	BJ1S4	IF	2
dp-27	BV6S3	_ASS TSQVT NYGYTFGSGT	BJ1S2	OF	1
dp-22	BV6S1	CASS SIHMD TEAFFGQGT	BJ1S1	IF	1

C

CLONE	BV gene	CDRIII region	BJ gene	frame	n
AA+21o	**BV6S7*2**	CASSL PR GANVLTFGAGS	BJ2S6	IF	2
AA+24o	**BV6S7*2**	CASS STPSGF LFFGSGT	BJ1S4	IF	1
AA+9o	**BV6S7*2**	CASS LPKAVANK QYFGPGT	BJ2S3	IF	1
AA+8o	**BV6S7*2**	CASS PAGGVRA KNIQYFGPGT	BJ2S4	IF	1
AA+4o	**BV6S7*2**	CASSL EGTSGRY YNEQFFGPGT	BJ2S1	IF	1
AA+12o	**BV6S7*2**	CASSL APTSGRAVA QYFGPGT	BJ2S5	IF	1
AA+26o	**BV6S7*2**	CASS LRGGSY TEAFFGQGT	BJ1S1	IF	1
AA+36o	**BV6S7*2**	CAS RPPG LNPGELFFGEGS	BJ2S2	IF	2
AA+34o	**BV6S7*2**	CASS QGGRGL YGYTFGSGT	BJ1S2	IF	1
AA+16	**BV6S7*2**	CASS YVR NTEAFFGQGT	BJ1S1	IF	1
AA+26	**BV6S7*2**	CASSL MGPF YEQYFGPGT	BJ2S7	IF	1
AA+27	**BV6S7*2**	CASS PNRAGS QPQHFGDGT	BJ1S5	IF	1
AA+19	**BV6S7*2**	CASSL GLLAGGVCA QYFGPGT	BJ2S7	IF	1
AA+31	BV6S4	CASS PQSGLT SYEQYFGPGT	BJ2S7	IF	1
AA+33o	BV6S4	CASS RDRA DTQYFGPGT	BJ2S3	IF	1
AA+14	BV6S3	CASSL TGT NEKLFFGSGT	BJ1S4	IF	1
AA+5	BV6S3	CASS DRLT YEQYFGPGT	BJ2S7	IF	1
aa-1	**BV6S7*2**	CASS PRWREQ QYFGPGT	BJ2S7	IF	1
aa-17	**BV6S7*2**	CASSL ESSG DTQYFGPGT	BJ2S3	IF	1
aa-15	**BV6S7*2**	CASS FSGAVRG EQYFGPGT	BJ2S7	IF	1
aa-33	**BV6S7*2**	CASSL AGYP NTEAFFGQGT	BJ1S1	IF	1
aa-20	**BV6S7*2**	CAS LAW DTQYFGPGT	BJ2S3	IF	1
aa-38	BV6S4	CASS ARVL NSPLHFGNGT	BJ1S6	IF	1
aa-34	BV6S4	CASSL RQA SPLHFGNGT	BJ1S6	IF	1
aa-2	BV6S3	CASSL GR DTQYFGPGT	BJ2S3	IF	1
aa-16	BV6S3	CASS LAA SPLHFGNGT	BJ1S6	IF	1

Figure 4. (*A–C*) Sequences of TCR β chains obtained from the PCR reactions with the pan BV6 primer (Fig. 3). Sequences were obtained from the cultured cells of DNP (*A*), DP (*B*), and AA (*C*), for both $OT145^+$ cells (clones indicated as DNP^+, DP^+, or AA^+) and $OT145^-$ cells (clones named dnp^-, dp^-, aa^-) sorted after the 10-d culture. *n* indicates the number of times a given sequence was found. IF/OF indicates whether the sequence is in frame or not. If not, the underlined area indicates the site at which the sequence comes out of frame.

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	1	2			1	1	1	1		3	13
	other BV6	1	3		1	1	1	1		1		1		5	15
DP	BV6S7	2	1					3	1	1		1	2	2	13
	other BV6	1			1	1	1		2			1			7
AA	BV6S7	3	1		1	1	1	1	1	3	1	1	1	4	18
	other BV6				1		3			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:															
	BV6S7	14	7	2	5	7	0	9	7	12	5	7	7	21	100
	other BV6	7	10	0	10	7	17	3	7	10	0	7	0	23	100

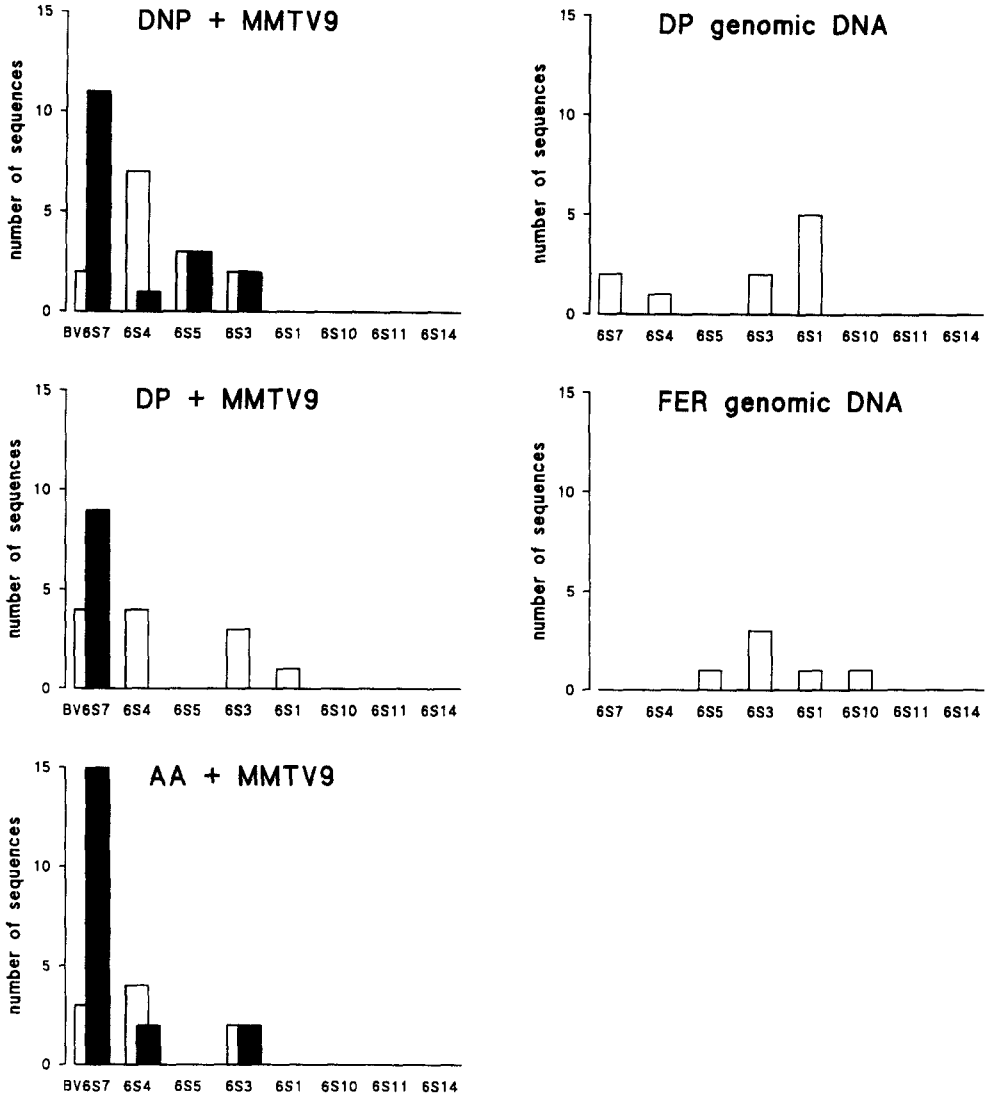

Figure 5. Summary of the frequency of TCR sequences of the indicated BV families. The seven functional BV6 genes contained in the BV locus (Genbank L36092) and one of three BV6 pseudogenes (VB6S10) are listed. The left hand panels summarize data from Fig. 4 indicating the frequency of BV6 genes used 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.

trol 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 ^{32}P (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

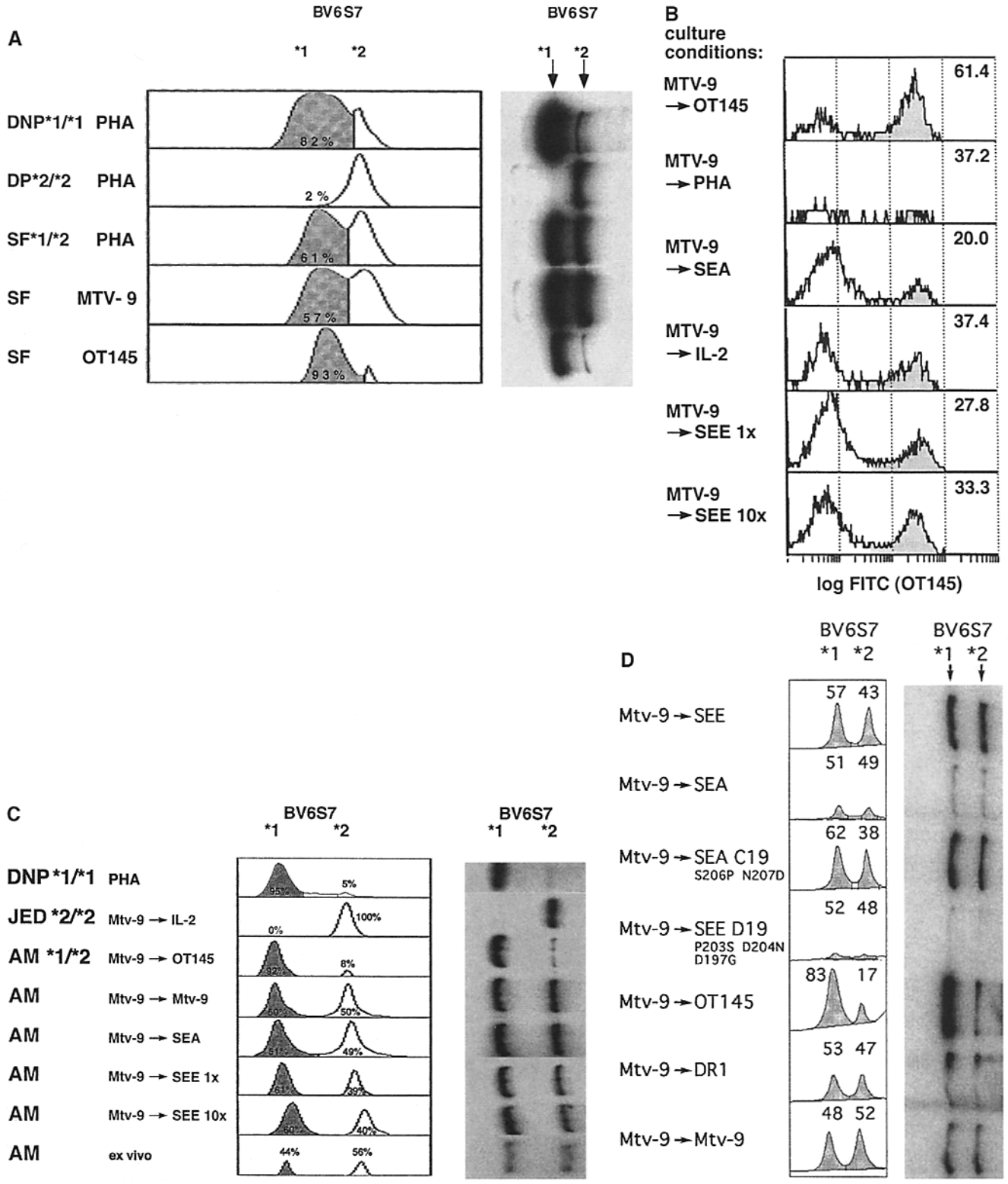

Figure 6. BV6S7*1 and BV6S7*2 usage among polyclonal T cells stimulated with Mtv-9 SAG, SEE, SEA, PHA and OT145. Labeled PCR products digested with BamHI are shown on the right and the quantitative scanning image on the left with the percentage of signal corresponding to BV6S7*1. *A*, *B*, and *C* represent 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. DR1 denotes stimulation with a DAP-DRB1*0101/DRA transfectant as a control for the Mtv-9 *SAG* transfectant which also expresses with DRB1*0101. AM has a DRB1*0102, DRB1*1301 genotype by PCR (courtesy of Dr. D. Kostyu, Duke University). The numbers indicate relative percentages for expression of the two BV6S7 alleles.

Figure 7. Sequence of SEA, SEE and SEB in the region between the β10 strand and the α5 helix. The numbering is that of each superantigen. The circled SEE residues are thought to play a role in determining the BV specificity of SEE.

can otherwise not be discerned, presumably because interaction 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 concentrations 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 fibroblasts (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 Residues 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 helix 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 predominant 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 between G72 and E72 of the TCR β 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 position 72. This constrains the possible conformations of a tri-molecular complex (MHC-TCR-SEE) and is most compatible with a position of SEE similar to that of SEB and TSST1 relative to MHC class II, e.g., adjacent to the α helix 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), interact 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 transplanting 11 HVR4 residues of the reactive BV13S2 to the non-reactive BV13S1 which then acquired SEC2 and SEC3 reactivity (8). Thus, both vSAG and bSAG seem to focus on the TCR HVR4 region.

Early studies with the vSAG of Mtv-7 showed that glycosylation 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 dissociation constants for binding of glycosylated versus unglycosylated mBV8S2 in a recent BIAcore binding study (32). Although 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 TCRBV6S7. 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. Although there are two amino acid differences between the two alleles, the single residue important for preferential reactivity of both ligands is position 72 (Fig. 1), at the apex of HVR-4, right between β strands D and E at a sharp turn in the carbon backbone of the TCR β chain (3, 33). BV6S7*1 encodes G72 and BV6S7*2 encodes a negatively charged E72.

Figure 8. Model of BV6S7*1 and BV6S7*2 based on coordinates of mBV8S2 beta chain (38). 26 residues of the mouse beta chain variable region were substituted with the human sequence excluding the CDRIII region which was not altered. The coordinates were recalculated with the program Modeler (48). For both human BV6S7 proteins the carbon backbone closely overlaps that of the original mouse beta chain with the exception of S62 which represents a supplementary amino acid as the human BV6S7 sequences are one residue longer than the mBV8S2. The image was created with InsightII and shows BV6S7*1 on the left and BV6S7*2 on the right; CDRI = green, CDRII = magenta, CDRIII = yellow, HVR4 = orange, E/G72 = white (with side chain). The side chain of N52 in CDRII and the exposed surface for CDR1, CDR2, and HVR4 are shown.

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 mBV10 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 SEC_{1-3}, which are all recognized by the wild-type TCR (35). Finally, TCR β 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 β 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 β 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 β 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 (CH_3). In contrast, the depression is filled in BV6S7*2 with the charged side chain of E72 (CH_2-CH_2-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*

	68 76
BV6S1	VRPEGSVST
BV6S3	ERPEGSVST
BV6S4	ERPEGSIST
BV6S14	ERPEGSIST
BV6S11	ERPERSVST
BV6S5	ERPKGSLST
BV6S7*1	ERTGGSVST
BV6S7*2	ERTGESVST
BV16S1	ERTGGTYST
BV8S1	KMPNASFST
mBV8S2	SRPSQENFS

Acidic residues are underlined and basic residues are in bold.

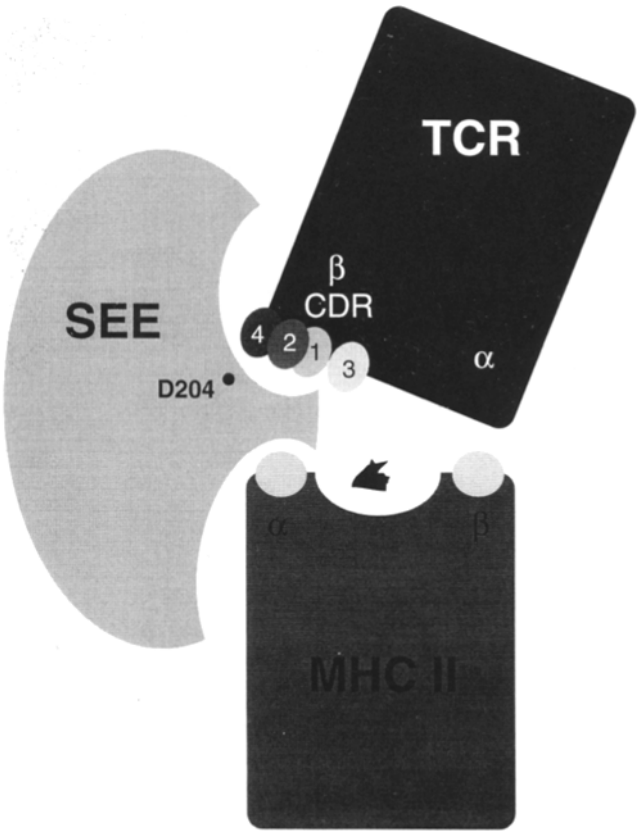

Figure 9. Model of SAG/MHC/TCR complex demonstrating the SAG wedge between TCR and MHC II. The CDR loops of the TCR α chain are not shown.

tween TCR β chain and the SAG. BV8, like BV6S7*1, 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 S206P 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 $SEE_{196-204}$ 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 CDR11 would be placed over the MHC α1 domain and the CDR1 and CDR11 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 IE^k (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*1 that mediate binding to SEE probably include additional residues beyond residue 72, because the HVR4 motif $ERTGGxxST_{68-76}$ 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 *Staphyloccocal 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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