In Vivo Expansion of HLA-B35 Alloreactive T Cells Sharing Homologous T Cell Receptors: Evidence for Maintenance of an Oligoclonally Dominated Allospecificity by Persistent Stimulation with an Autologous MHC/Peptide Complex

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Summary

The nature of alloantigens seen by T lymphocytes, in particular the role of peptides in allorecognition, has been studied intensively whereas knowledge about the in vivo emergence, diversity, and the structural basis of specificity of alloreactive T cells is very limited. Here we describe human T cell clones that recognize HLA-B35 alloantigens in a peptide-dependent manner. TCR sequence analysis revealed that several of these allospecific clones utilize homologous TCR: they all express TCRAV2S3J36C1 and TCRBV4S1J2S7C2 chains with highly related CDR3 sequences. Thus peptide-specific alloreactivity is reflected in homologous CDR3 sequences in a manner similar to that described for T cells that recognize nominal peptide/self-MHC complexes. The in vivo frequency of this TCR specificity was studied in unstimulated PBL of the responding cell donor who was not sensitized against HLA-B35. The vast majority (~75%) of the VA2S3J36 junctional regions obtained from two samples of PBL, isolated at a 9-yr interval, encode CDR3 identical or homologous to those of the functionally characterized HLA-B35 allospecific T cells. These data are most easily explained by a model of alloreactivity in which persistent or recurrent exposure to a foreign peptide/self-MHC complex led to the in vivo expansion and long-term maintenance of specific T cells that show fortuitous crossrecognition of an HLA-B35/peptide complex and dominate the alloresponse against HLA-B35.

T cell recognition is confined to peptide stretches of antigenic proteins that are selected by binding to polymorphic peptide-binding grooves of MHC class I and class II molecules (1–3). Positive and negative selection steps in the thymus prepare T cells for this task (4). Self peptides presented by autologous MHC molecules play an essential role in both selection steps (5). Against this background T cell–mediated alloreactivity remains puzzling since it does not concur with the rules of positive and negative selection: alloreactive T cells respond to MHC molecules for which they have not been positively selected. Likewise they are not negatively selected against self peptides presented by allogeneic molecules (subsequently referred to as "allo-self complexes"), rather they are specifically reactive for such allo-self complexes (6, 7). Characterization of T cell clones specific for foreign peptide presented by self MHC and MHC alloantigens demonstrated that alloreactivity is not a function of a particular population of T cells but is an intrinsic property of many T cells (8, 9) and that this dual specificity is accomplished by one TCR (10). It is assumed that alloreactivity represents fortuitous cross-reactive binding of TCR to particular allo-self complexes but the molecular basis of this cross-reactivity is not yet fully understood (11, 12).

Insight into the structural basis of alloresponses can be gained by analyzing TCR of alloreactive T cells. A three-dimensional model of the TCR proposes that CDR1 and 2, encoded by TCRAV and TCRBV gene segments, interact with the α-helices of the MHC molecules and the hypervariable CDR3, encoded by V-(D)-J junctional regions, contact protruded peptide side chains (13). Analyses of alloreactive T cells in mice and humans revealed a complex TCR usage and support the hypothesis that an alloresponse is mediated by heterogeneous T cells recognizing a wide variety of allo-self complexes (14–17).

In contrast, we show here that a group of HLA-B35 specific, peptide-dependent, alloreactive T cells utilize a set of distinct, yet nearly identical TCR. One of these TCR sequences was found to be predominantly expressed in the unstimulated PBL of the donor whose cells were stimulated in vitro to generate the alloreactive T cells. Furthermore, a second of these TCR sequences was detected with high frequency in a PBL sample obtained from this donor nine years later.

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Thus, this example of allorecognition represents a case in which an allo-self complex is recognized presumably due to its similarity to a nominal peptide/self–MHC complex that selectively activated and maintained these T cells in vivo over a very long time.

Materials and Methods

Cells. PBL of healthy Caucasian donors were HLA typed using local and International Histocompatibility Workshop reagents. HLA-B35 variants were characterized by DNA sequencing (18). EBV-transformed lymphoblastoid cell lines (LCL)[1] were established using culture supernatants of the marmoset cell line producing the B95.8 strain of EBV (American Type Culture Collection, Rockville, MD). LCL or PHA-stimulated PBL were used as target cells for the cell-mediated lympholysis assay and their complete HLA types are as follows:

GM: A2,B8,B40,Cw7,DR2,DR3;
DS: A2,A3,B*3501,B37,Cw4,Cw6,DR1,DR2;
PR: A1,A3,B*3501,Cw4,DR4,DR13
HF: A1,A3,B*3503,B38,Cw4,Cw6,DR3,DR13;
TL: A2,A24,B13,B*3508,Cw4,Cw6,DR3,DR7;
GN: A3,A31,B*3508,B55,Cw3,Cw4,DR4.

The HLA-B*3501 gene was isolated from a genomic library of donor DS. The HLA-A, -B, -C null LCL 721.221 (19) was obtained from the ATCC and transfected by electroporation with HLA-B*3501 cloned into the pHEBo vector (20). P815-HTR mastocytoma cells were also transfected with HLA-B*3501 as described elsewhere (21). T2 is a fusion product of the T-LCL CEM and the LCL721.174 and is MHC hemizygous (22). The residual class I region encodes HLA-A2, -Cw1, -B51; the class II region has a large deletion including the TAP and LMP genes (23). Untransfected T2 cells were kindly provided by Dr. R. Tampe (Max-Planck Institute of Biochemistry, Martinsried, Germany); T2 cells transfected with HLA-B*3501 were kindly provided by Dr. M. Takiguchi (Institute of Medical Science, Tokyo, Japan). The cell lines BM28.7 and BM36.1 were generously provided by Dr. A. Ziegler (Institute of Experimental Oncology and Transplantation, Berlin, Germany); they were derived by mutagenesis from the Burkitt's lymphoma line BJAB; BM28.7 is MHC hemizygous and carries only the A1, Cw4, B*3501 haplotype of BJAB. BM36.1 carries a deletion in its TAP2 gene and lacks a functional peptide transporter (24).

CTL. An HLA-B35–specific CTL line was made by stimulating B35-negative PBL of donor GM, isolated in 1979, with irradiated B*3501-positive lymphocytes of donor DS for 6 d in MLC. After positive selection of $CD8^+$ lymphocytes using anti–CD8-antibody–coated magnetic beads (Dynal, Hamburg, Germany), cloning was performed by seeding 0.3 cells/well in the presence of rIL-2 (40 U/ml) (Proleukin; Eurocetus, Frankfurt, Germany) and irradiated stimulator cells. The remaining $CD8^+$ lymphocytes were cultivated for several months with irradiated stimulator cells (DS) and rIL-2 (40 U/ml). From this $CD8^+$ line $V\alpha2.3$-bearing T cells were positively selected implementing mAb F1 (T Cell Sciences, Cambridge, MA) in combination with goat anti–mouse IgG antibodies coupled to magnetic beads (M450; Dynal).

Cell-mediated Lympholysis Assay. Cytotoxic activity was determined using a standard chromium release assay (25). A percent cytotoxicity was calculated using the formula: % lysis = ([experimental cpm − spontaneous cpm]/[total cpm − spontaneous cpm]) ×100.

Immunofluorescence Analysis and Antibodies. Indirect immunofluorescence was made using undiluted hybridoma culture supernatants or ascites (1:1,000) in the first step followed by FITC-conjugated goat anti–mouse Ig antibody (F261; Dakopatts, Copenhagen, Denmark). The following antibodies were used: anti-Bw6 (Biotest, Dreieich, Germany) for assessment of HLA-B35; F1 (T Cell Sciences) for detection of $V\alpha2.3^+$ T cells; and MPC 11 and UPC 10 (Sigma Chemical Co., St. Louis, MO) as isotype controls. For two-color immunofluorescence cells were incubated with unlabeled primary antibody, washed twice, and stained with PE-labeled rabbit anti–mouse Ig $F(ab')_2$ (Medac, Hamburg, Germany). Free sites were saturated with excess mouse IgG1 and IgG2a (Coulter, Hialeah, FL). Finally the cells were incubated with FITC-conjugated anti-CD8 mAb (Immunotech, Marseille, France). Analyses were carried out on a FACScan® instrument (Becton Dickinson, Mountain View, CA) with gates set to include only living cells.

Analysis of TCR Usage and Determination of TCR Sequences. Total cellular RNA was isolated using RNAzol B (CINNA/BIOTECX, Houston, TX). TCRA and TCRB cDNA of the T cell clones was synthesized using the oligonucleotides Cαe and Cβe (26) and of the PBL, the T cell line and the $V\alpha2.3$ selected T cells using the oligonucleotides CαST (5′ CACTGAAGATCCATCATCTG 3′) and CβST (5′ TAGAGGATGGTGGCAGACAG 3′) with RAV-2 reverse transcriptase (Amersham Buchler GmbH, Braunschweig, Germany). For the T cell clones PCR was performed from cDNA with five sets of TCRAV ($V\alpha$1-6/7-12/13-16,23,24/17-22/25-29,32) and four sets of TCRBV family specific oligonucleotides ($V\beta$1-4, 5.1,5.2,6/7-12/13.1,13.2,14-18/19-24) in combination with biotinylated oligonucleotides derived from TCRAC (T-Cα) (27) and TCRBC (3′Cβ) (28) gene segments, respectively. Oligonucleotides $V\alpha1$ to $V\alpha12$ with $V\alpha17$ to 21 and $V\beta1$ to $V\beta20$ were synthesized according to published sequences (27, 28) and supplemented with the following oligonucleotides:

Vα13: 5′ GAGCCAATTCCACGCTGCG 3′
Vα14: 5′ CAGTCTCAACCAGAGATGTC 3′
Vα15: 5′ GATGTGGAGCAGAGTCTTTTC 3′
Vα16: 5′ TCAGCGGAAGATCAGGTCAAC 3′
Vα22: 5′ TACACAGCCACAGGATACCCTTCC 3′
Vα23: 5′ TGACACAGATTCCTGCAGCTC 3′
Vα24: 5′ GGAGGGAAAGAACTGCACTCTT 3′
Vα25: 5′ ATCAGAGTCCTCAATCTATGTTTA 3′
Vα26: 5′ AGAGGGAAAGAATCTCACCATAA 3′
Vα27: 5′ ACCCTCTGTTCCTGAGCATG 3′
Vα28: 5′ CAAAGCCCTCTATCTCTGGTT 3′
Vα29: 5′ AGGGGAAGATGCTGTCACCA 3′
Vα32: 5′ TGCAAATTCCTCAGTACCAGCA 3′
Vβ21: 5′ AAAGGAGTAGACTCCACTCTC 3′
Vβ22: 5′ CATCTCTAATCACTTATACT 3′
Vβ23: 5′ TATCCCTAGACACGACACTG 3′
Vβ24: 5′ GACTTTGAACCATAACGTACA 3′

PCR was performed starting with 2 min at 94°C, then 30 cycles of 30 s at 95°C, 30 s at 56°C and 60 s at 72°C, finishing with 5 min at 72°C. Amplification in a 50-μl volume used 2 U Taq polymerase (Pharmacia, Freiburg, Germany) and a Omnigene thermocycler (Hybaid, Teddington, UK) under tube control. DNA strands were separated using streptavidin-coupled magnetic parti-

[1] *Abbreviations used in this paper:* LCL, lymphoblastoid cell lines; MFI, mean fluorescence intensity.

cles (M-280; Dynal) and 0.1 M NaOH. Direct sequencing was performed with Sequenase 2.0 (Un. States Biochem. Corp., Cleveland, OH) and [^{32}P]dATP or using the A.L.F. DNA sequencer (Pharmacia) with the AutoRead sequencing kit (Pharmacia) and fluorescein-labeled primers. The repertoire of the T cell line was analyzed for expression of 30 TCRAV and 24 TCRBV gene families in separate PCR. To obtain semiquantitative estimates of gene usage and to monitor efficiency of amplification, primer pairs encompassing a stretch of the TCRAC and TCRBC gene segments, respectively, were included as internal controls in each reaction (5′ Cα-ST/3′Cα-ST for TCRA-PCR and 5′ Cβ-ST/3′Cβ-ST for TCRB-PCR).

P-5′αST: 5′ CTGTGCTAGACATGAGGTCT 3′
P-3′αST: 5′ CCTGCCTCTGCCGTGAATGT 3′
P-5′βST: 5′ AAGCAGAGATCTCCCACAC 3′
P-3′βST: 5′ GAGGTAAAGCCACAGTTGCT 3′

The PCR protocol was as above except that 35 cycles were performed. Amplification products were separated on a 4% NuSieve 3:1 agarose gel (FMC Corp. BioProducts, Rockland, ME) and photographed (Film Type 55; Polaroid, Cambridge, MA). The TCRAV and TCRBV amplicons of the T cell line were directly sequenced as described above.

Sequencing of TCR Junctional Regions from PBL. The cDNA prepared from unstimulated PBL samples of donor GM was amplified with oligonucleotides Vα2.3 and Jα36 to isolate TCRAV2S3J36 junctional regions and oligonucleotides Vβ4-Sal and Jβ2.7 for TCRBV4J2S7 junctional regions.

Vα2.3: 5′ TGTTCTAGAGGGAGCCACTGTCG 3′
Jα36: 5′ GGGAATTCGGGAATAACGGTGAGTCTC 3′
Vβ4-Sal: 5′ CCGTCGACATATGAGAGTGGATTTGTC 3′
Jβ2.7: 5′ CCGAATTCTTTTTCAGGTCCTCTGTG 3′

Both PCR were optimized for specific amplification via primer se-

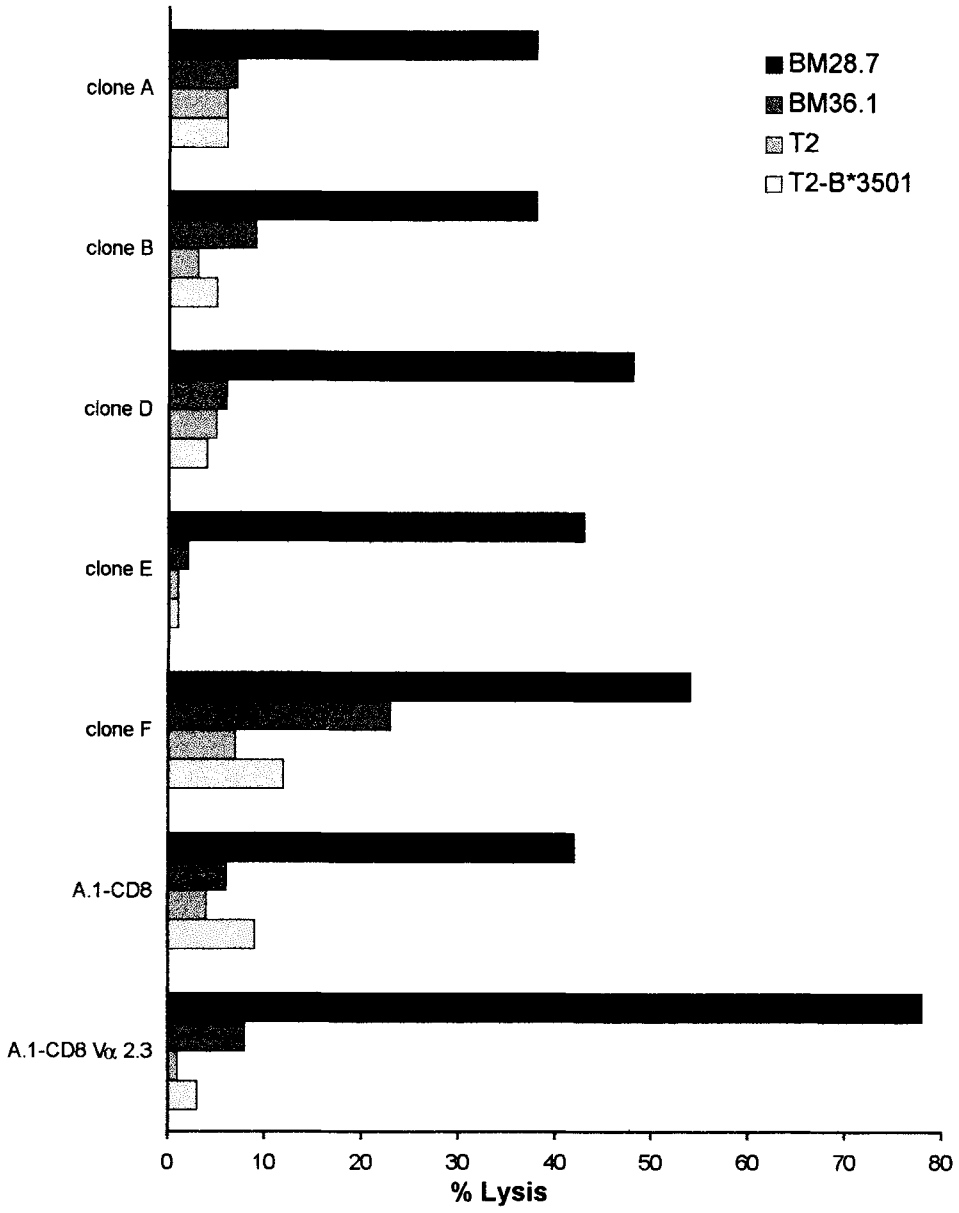

Figure 1. Cytotoxic activity of alloreactive T cells against TAP-deficient mutant cells. The T cell clones, the T cell line A.1-CD8, and the TCR-Vα2.3–positive cells selected from this line were tested for lysis of cell lines T2, T2-B*3501, BM36.1 and its functional TAP-expressing precursor cell line BM28.7. Data are given as % lysis, calculated according to the formula given in Materials and Methods.

lection and application of stringent annealing temperatures. PCR started with 2 min at 94°C, then 35 cycles of 30 s at 95°C, 30 s at 62°C (Vα2.3), and 58°C (Vβ4), respectively; 60 s at 72°C; finishing with 5 min at 72°C. Amplification in a 50-μl volume used 2 U Taq polymerase. VA2S3J36 amplicons were cloned into pUC18 via XbaI and EcoRI and VB4J2S7 amplicons via SalI and EcoRI restriction sites built into the PCR primers. The cloned junctional regions were sequenced with an A.L.F. DNA sequencer using the fluorescein-labeled reverse primer.

Results

Peptide-dependency of HLA-B35–specific, Alloreactive T Cells. The serological specificity HLA-B35 encompasses at least eight different alleles (29). Previously, we described several B35-specific, alloreactive T cell clones derived from donor GM that exhibited different lysis patterns for target cells expressing molecular subtypes of HLA-B35 (18). An additional T cell clone (clone F) not initially characterized is included here. LCL of B*3501 donors were well recognized by all clones, recognition of LCL of B*3508 donors varied among the clones and none of the clones lysed LCL of a B*3503 donor (18 and data not shown). B*3503 and B*3508 differ from B*3501 by single amino acid substitutions at positions thought to interact with the peptide but not to be accessible to a TCR (18). The differences in allorecognition of cells expressing B35 variants suggested that the T cells were specific for particular B35 allo-self complexes. Recognition of EBV peptides could be excluded since all clones lysed activated T cells of donor DS with equal efficiency (data not shown). All clones also lysed the HLA-A, -B, -C null cell line LCL721.221 after transfection with HLA-B*3501, but not untransfected LCL721.221 cells, nor did they lyse murine P815 cells transfected with HLA-B*3501 (30). These results confirmed that HLA-B*3501 molecules played a direct role in determining the specificity of the T cells; the difference with human versus mouse transfectants expressing HLA-B35 suggested that species variations in endogenous peptides might influence allorecognition.

To substantiate the assumption that these T cells were peptide-dependent we studied their lysis of two different B*3501 positive target cells having defects in the transport of endogenous peptides (Fig. 1). BM36.1 cells are TAP2 mutant derivatives of BM28.7 whose surface expression of en-

A

clone A	Y F C A	Y R S T E	T S Y D K V I F
	TATTTCTGTGCT	TATAGGAGCACGG	AAACCTCCTACGACAAGGTGATATTT
	AV14S1		AJ50
clone B	Y L C V V	I R	T G A N N L F F
	TACCTCTGTGTGGTGA	TACG	AACTGGGGCAAACAACCTCTTCTTT
	AV2S3		AJ36
clone C	Y F C A L	I P	S G N T P L V F
	TACTTCTGTGCTCTGA	TTCC	TTCAGGAAACACACCTCTTGTCTTT
	AV12S1		AJ29
clone D	Y L C V V	I K A	G A N N L F F
	TACCTCTGTGTGGTGA	TCAAGG	CTGGGGCAAACAACCTCTTCTTT
	AV2S3		AJ36
clone E	Y L C A V	S N G W	N N N D M R F
	TACCTCTGTGCTGTG	AGTAACGGATGG	AATAACAATGACATGCGCTTT
	AV23S1		AJ43
clone F	Y F C	A P	S G T Y K Y I F
	TACTTCTGTGC	TC	CCTCAGGAACCTACAAATACATCTTT
	AV8S1		AJ40
A.1-CD8	Y L C V V	I K	T G A N N L F F
	TACCTCTGTGTGGTGA	TTA	AAACTGGGGCAAACAACCTCTTCTTT
	AV2S3		AJ36
	Y L C A L	V	
	TACCTCTGTGCTCTAG	TGCG	GGTGGTACTAGCTATGGAAAGCTGACATTT
	AV5S1		AJ52
	Y L C A V	R G P P	G A G S Y Q L T F
	TACCTCTGTGCTGTGAG	GGGACCTC	CTGGGGCTGGGAGTTACCAACTCACTTTC
	AV7S2		AJ28
	Y F C A L S	G L	G G Y Q K V T F
	TACTTCTGTGCTCTGAGT	GGGCTC	GGGGGTTACCAGAAAGTTACCTTT
	AV12S1		AJ13
	Y F C A	Y W G A	Y N F N K F Y F
	TACTTCTGTGCG	TACTGGGGGGCA	TACAACTTCAACAAATTTTACTTT
	AV32S1		AJ21

dogenous HLA-B*3501 molecules is reduced 10-fold (24 and data not shown). All T cell clones lysed BM28.7 cells at levels similar to B*3501 cells of donor DS; whereas BM36.1 cells were not recognized, except by clone F that showed weak lysis compared to BM28.7 cells. T2 cells have no TAP1 and 2 genes and consequently their peptide transport is heavily impaired (22). The endogenous HLA-A2 molecules of T2 are expressed at 20–25% of wild-type level (31); expression of other HLA-class I molecules, such as endogenous HLA-B51, was barely detected (32). T2 cells transfected with B*3501 DNA bound an anti-Bw6 antibody showing that they expressed B*3501 molecules (T2: negative control [mean fluorescence intensity (MFI) = 127], anti-Bw6 [MFI = 208]; T2-B*3501: negative control [MFI = 190], anti-Bw6 [MFI = 341]). Untransfected T2 cells as well as T2-B*3501 transfectants were poorly recognized by all CTL (Fig. 1).

After several months of culture the $CD8^+$ T cell fraction from which the T cell clones were derived exhibited a pattern of lysis similar to those of clones B, C, and D: it lysed 721.221 cells transfected with HLA-B*3501, recognized B*3501 and B*3508, but not B*3503 cells (not shown), and hardly recognized the B*3501 transporter-defective cell lines (Fig. 1). These data supported the conclusion that the clones as well as this line were B35 specific and peptide dependent.

TCR Sequences of HLA-B35 Alloreactive T Cells. The TCRα and TCRβ variable regions of the alloreactive T cell clones were sequenced after PCR amplification using family-specific oligonucleotides (Fig. 2, *A* and *B*). Interestingly, clones B and D utilized nearly identical TCR: both had recombined TCRAV2S3 with TCRAJ36 and TCRBV4S1 with TCRBJ2S7 gene segments. The CDR3 sequences of both TCR chains were also strikingly similar; they were of identical overall length and composed of the same or chemically related amino acids located at the same positions (Fig. 3). The other four T cell clones used different TCRAV and TCRAJ gene segments. Clones A and C expressed TCRβ chains encoded from TCRBV7 gene segments that had CDR3β regions of identical length with homologous carboxyterminal halves. Five of the six clones used either TCRBJ2S3 or TCRBJ2S7 gene segments that are closely

B

clone A	C A S S Q	E W L G Q G I S	D T Q Y F
	TGCGCCAGCAGCCAAG	AGTGGTTGGGACAGGGCATTTCG	GATACGCAGTATTTT
	BV7S1	BD1	BJ2S3
clone B	C S V E	G G V F	Y E Q Y F
	TGCAGCGTTGAA	GGCGGGGTCTT	CTACGAGCAGTACTTC
	BV4S1	BD2	BJ2S7
clone C	C A S S Q	A L P L P G M T	Y E Q Y F
	TGTGCCAGCAGCCAAG	CTCTCCCGTTACCGGGAATGA	CCTACGAGCAGTACTTC
	BV7S2	BD2	BJ2S7
clone D	C S V E	G G T I	Y E Q Y F
	TGCAGCGTTGAA	GGGGGGACGAT	CTACGAGCAGTACTTC
	BV4S1	BD1/BD2	BJ2S7
clone E	C A S S	Y Q T V	T D T Q Y F
	TGTGCCAGCAGT	TACCAGACAGT	CACAGATACGCAGTATTTT
	BV13S2	BD1	BJ2S3
clone F	C A S S	E I L Q G	N E K L F F
	TGTGCCAGCAGT	GAAATCCTCCAGGG	TAATGAAAAACTGTTTTTT
	BV22S1	BD1	BJ1S4
A.1-CD8	C S V E	G G S T	Y E Q Y F
	TGCAGCGTTGAA	GGGGGGTCTA	CCTACGAGCAGTACTTC
	BV4S1	BD1	BJ2S7
	C A S S L	Y I R V P	Y E Q Y F
	TGTGCCAGCAGCTTA	TATATCAGGGTGC	CCTACGAGCAGTACTTC
	BV6S2	BD1	BJ2S7
	C A S S	T A G G T	Y E Q Y F
	TGTGCCAGCAGCA	CCGCAGGGGGCA	CCTACGAGCAGTACTTC
	BV13S1	BD1	BJ2S7
	C A S S Q	D R G	T E A F F
	TGTGCCAGCAGCCAA	GACAGGGG	CACTGAAGCTTTCTTT
	BV16S1	BD1	BJ1S1
	C A T S S	D R G R	N Q P Q H F
	TGTGCCACCAGCAGC	GACAGGGGGC	GCAATCAGCCCCAGCATTTT
	BV24S1	BD1	BJ1S5

Figure 2. Sequences of the TCRA (*A*) and TCRB (*B*) junctional regions of the T cell clones A–F and the five TCRA and TCRB amplicons obtained from the T cell line. Amino acid residues are indicated with the single letter code. Since the germline sequences for most of the TCRAV and TCRBV gene segments are unknown their borders are inferred by comparison with other published cDNA sequences. The BV7S2 gene segment used by clone C differs from the published sequence BV7S2 (clone PL4.19) by a silent C to T substitution in the codon for the carboxy-terminal cysteine. Nucleotide stretches corresponding to TCRBD1 and TCRBD2 sequences are underlined. Boxed nucleotides indicate a frameshift at the N/J junction leading to an out of frame transcript. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers Z46961 (*A*) and Z46963 (*B*).

A

	AV2S3	N	AJ36
A.1-CD8+ PBL seq. 1	L C V V	I K	T G A N N L F F G
clone D PBL seq. 2	L C V V	I K A	G A N N L F F G
clone B	L C V V	I R	T G A N N L F F G
PBL seq. 3	L C V V	I R T	G A N N L F F G
PBL seq. 4	L C V V	T R	T G A N N L F F G
PBL seq. 5	L C V V	I V R G	T G A N N L F F G

B

	BV4S1	NDN	BJ2S7
A.1-CD8+	C S V E	G G S T	Y E Q Y F G P G
clone D	C S V E	G G T I	Y E Q Y F G P G
clone B	C S V E	G G V F	Y E Q Y F G P G

Figure 3. Comparison of the homologous CDR3 sequences of the TCRAV2S3J36C1 (*A*) and TCRBV4S1J2S7C2 junctional regions (*B*) characterized in the allostimulated T cell clones and line and the unstimulated PBL for donor GM.

related in sequence. In addition we found an out of frame rearranged TCRBV2JS3C1 for clone D, TCRAV17J40C1 for clone E, and TCRBV1J2S4C2 for clone F.

TCR Usage and Sequences of the HLA-B35–specific, Alloreactive T Cell Line. We also evaluated the TCR usage of the $CD8^+$-positive T cell line that was initiated from the same primary MLC as the clones. Repertoire analysis detected five TCRAV (data not shown) and five TCRBV gene segments (Fig. 4 *C*), revealing the oligoclonal composition of the line. Direct sequencing of each amplicon resulted in a single, clearly readable sequence even in the V(D)J junctional region (Fig. 2, *A* and *B*), thus the vast majority of sequences comprising each amplicon was derived from a clonal TCR. The T cell line also included TCR composed of VA2S3J36 and VB4S1J2S7 rearrangements like those identified in clones B and D. The CDR3 sequences of each receptor chain exhibited close homology, in terms of length and amino acid composition, to CDR3 of clones B and D (Fig. 3). Therefore it seemed likely that the Vα2.3 and Vβ4.1 chains were expressed by the same T cell population. To demonstrate this directly we isolated Vα2.3-bearing T cells from the line via immunomagnetic bead separation. FACScan analysis revealed that 33% of unselected cells bound the Vα2.3-specific mAb (Fig. 4 *A*); 98% of the positively selected cells were Vα2.3 positive (Fig. 4 *B*). When tested in CML these T cells showed a lysis pattern like the unseparated line and clones B and D (Fig. 1 and data not shown). PCR analysis of cDNA prepared from the selected $V\alpha2.3^+$ T cells showed that only amplicons for TCRAV2 (data not shown) and TCRBV4 were visible after 35 cycles (Fig. 4 *D*), demonstrating that they coexpressed Vβ4 receptor chains.

Further TCRA sequences detected in the unselected line were an out of frame V5S1J52C1 transcript, a V7S2J28 sequence with a CDR3 homologous to VA2S3J36 sequences (i.e., an aliphatic residue, followed by a basic aliphatic residue, followed by three small nonpolar residues), a sequence using V12S1 like clone C, and a V32S1J21C1 sequence (Fig. 2 *A*). Additional TCRB sequences were defined as V6S2J2S7C2, V13S1J2S7C2, V16S1J1S1C1, and V24S1J1S5C1 (Fig. 2 *B*). Three of five TCRB sequences characterized in the T cell line used the J2S7 gene segment which is also present in three T cell clones. The VB16S1 and VB24S1 sequences exhibited a unique, common CDR3 that shared the amino acid sequence "DRG" at the same position exclusively encoded by the TCRBD1 gene segment. In both cases BD1 was joined to the TCRBV and TCRBJ gene segments without insertion of additional nucleotides, thus representing so-called fetal-type TCR (33).

In Vivo Expansion of HLA-B35 Alloreactive T Cells. TCR with rearranged VA2S3J36 and VB4S2J2S7 gene segments and highly related CDR3 were found in three of seven HLA-B35–specific, alloreactive T cells and one of these was prevalent in the unselected line. To understand how preferential receptor usage arose following allogeneic stimulation we analyzed the diversity of VA2S3J36 TCR in the 1979 PBL sample of donor GM that was used for establishing the alloreactive T cells and in a PBL sample from 1988. Two color immunofluorescence of the PBL samples revealed that 3.4 and 2.0% of the $CD8^+$ cells expressed Vα2.3 TCRα chains, respectively (Fig. 4, *E* and *F*). Frequencies of 2–6% were described for $V\alpha2.3/CD8^+$ T cells in PBL of healthy individuals (34). For analysis of the VA2S3J36 TCR repertoire we designed a PCR specific for VA2S3J36 rearranged TCR. Single strand chain polymorphism analysis of the PCR products of the two GM samples showed strong distinct bands whereas those of seven other unrelated individuals gave weak bands or smears, as expected for heterogeneous mixtures of TCR having different CDR3 (data not shown). The strong bands of the GM samples indicated a clonal expansion of VA2S3J36 TCR-expressing T cells; therefore ~50 cloned VAS3J36 junctional regions were sequenced from each PBL sample. In the 1979 isolate a single sequence identical to the junctional sequence of the $CD8^+$ line was found in the majority of clones (32 of 55) (Fig. 5). Sequence 2 is identical to that of clone D. No PCR clone was found with the junctional sequence of T cell clone B; however one PCR clone identical in amino acid sequence but differing by five nucleotides in the junctional region was found twice (sequence 3). Four additional sequences encoded identical or highly related CDR3. Since two of them differed from sequence 1 by only one nucleotide they may represent Taq incorporation error variants. Altogether 41 of 55 junctional sequences (75%) could be grouped into one homology class.

In the second PBL sample, 27 of the 50 clones (54%) had junctional regions identical to clone D (Fig. 5). Sequence 1, which was dominant in the 1979 sample was found six times (12%). Again, the junctional sequence of clone B was not

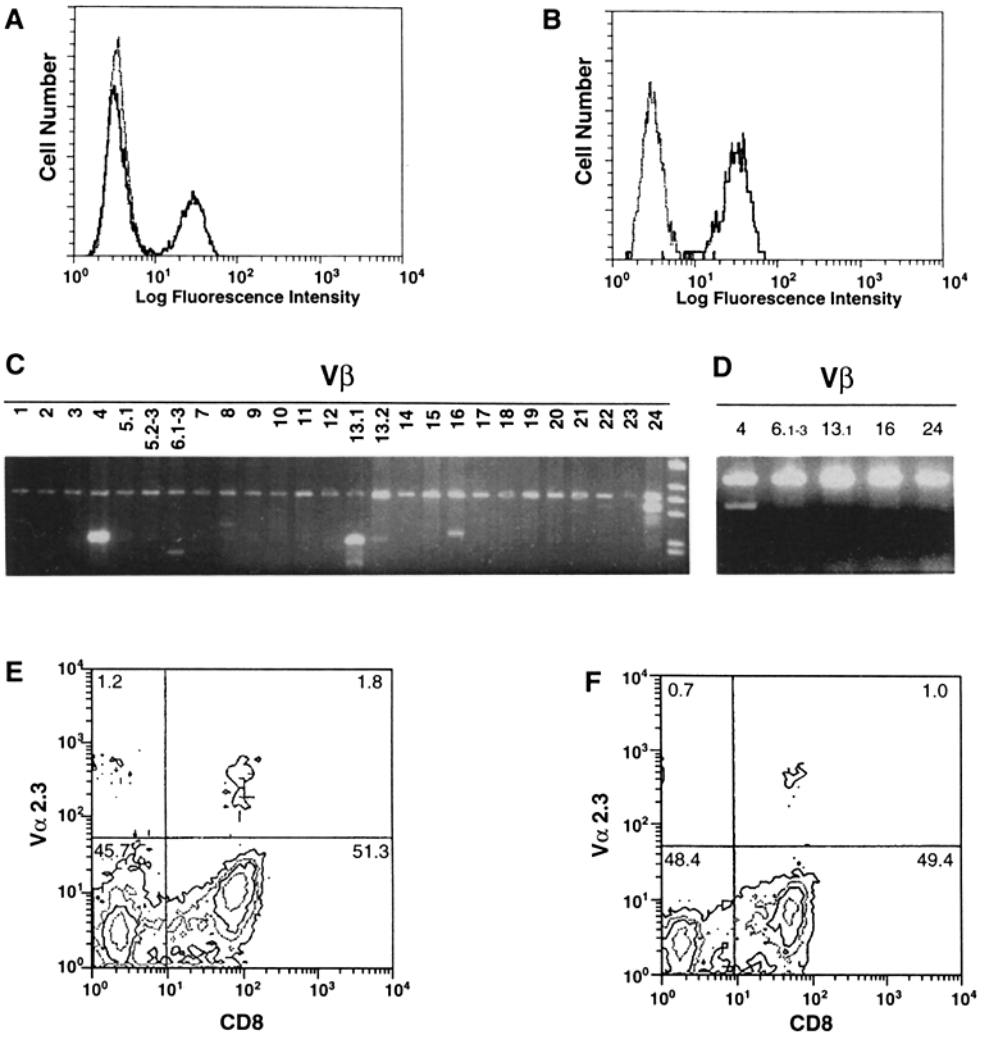

Figure 4. TCRV gene segment expression of the T cell line A.1-CD8. FACScan® analysis of the A.1-CD8 line (*A*) and the TCR-Vα2.3 positively selected cells of this line (*B*) with the Vα2.3 specific mAb F1 (*hatched line*, isotype control). TCRBV repertoire (*C*) of the T cell line A.1-CD8 determined with 26 TCRBV family-specific primers and coamplification of TCRBC as an internal control. Analysis of the TCR-Vα2.3 positively selected cells for the TCRBV gene segments detected in the line (*D*). Frequency of TCR Vα2.3/CD8 positive cells in PBL samples of donor GM from 1979 (*E*) and 1988 (*F*). The Vα2.3-specific mAb F1 was indirectly labeled with PE and used in combination with the FITC labeled anti-CD8 mAb. The percentages of cells are indicated in each quadrant.

found but a TCR identical in amino acid but differing in nucleotide sequence (sequence 3) was detected five times (10%). Therefore after nine years the repertoire of VA2S3J36 TCR was still dominated by a particular homology class that represented 76% (38 of 50 clones) of the junctional sequences.

Junctional sequences not belonging to this homology group (sequences 6–23) were diverse in length and amino acid composition. None was found more than three times or in both PBL samples. 10 started with codon AAC encoding asparagine (but none with AAT that also encodes asparagine) indicating that the AAC codon represented the 3′ end of the germline TCRAV2S3 sequence. None of these sequences started with a codon for isoleucine, suggesting that the generation of the homologous sequences did not represent a frequent recombinational event but that their dominance in the PBL of GM was due to selection through antigenic stimulation.

Sequencing 50 cloned VB4S1J2S7 junctional regions from each PBL sample supported the results of the VA2S3J36 repertoire analysis. In the 1979 isolate we found the VB4S1J2S7 sequence of the T cell line once (Fig. 2 *B*); in the 1988 isolate the sequence of clone D was found four times. Both sequences correspond to the clonally expanded VA2S3J36 sequences found at the two time points. The remaining VB4S1J2S7 sequences were neither identical nor homologous to those found in the alloreactive T cells (not shown). TCRBJ2S7 is utilized by 15–20% of all TCRB sequences in peripheral blood (35) whereas TCRAJ36 is rearranged by ~0.7% of TCRA sequences (36). Therefore the peripheral VB4S1J2S7 repertoire probably encompasses at least 10-fold more TCR than the VA2S3J36 repertoire. This could explain why we only found the TCRB sequence corresponding to the expanded T cell clone prevalent at each time and that the TCRB sequences occurred at much lower frequencies than the TCRA sequences. Although several hundred clones would need to be sequenced to obtain an accurate frequency distribution in the VB4J2S7 repertoire, these initial findings are in agreement with the preferential expansion of the same T cells identified via their VA2S3J36 sequences.

Discussion

The currently favored model of alloreactivity suggests that peptides associated with allogeneic MHC molecules play a critical role in dictating specificity (6, 7). Functional evidence

	AV2S3		AJ36	n clones '79	'88
sequence 1	Y L C V V TACCTCTGTGTGGTGA	I K TTA	T G A N N L F F AAACTGGGGCAAACAACCTCTTCTTT	32	6
sequence 1a	Y L C V V TACCTCTGTGTGGTGA	I K TcA	T G A N N L F F AAACTGGGGCAAACAACCTCTTCTTT	1	0
sequence 1b	Y L C V V TACCTCTGTGTGGTGA	I r TTA	T G A N N L F F gAACTGGGGCAAACAACCTCTTCTTT	1	0
sequence 2	Y L C V V TACCTCTGTGTGGTGA	I K A TCAAGG	G A N N L F F CTGGGGCAAACAACCTCTTCTTT	1	25
sequence 2a	Y L C V a TACCTCTGTGTGGcGA	I K A TCAAGG	G A N N L F F CTGGGGCAAACAACCTCTTCTTT	0	1
sequence 2b	Y L C V m TACCTCTGTGTGaTGA	I K A TCAAGG	G A N N L F F CTGGGGCAAACAACCTCTTCTTT	0	1
sequence 3	Y L C V V TACCTCTGTGTGGTGA	I R T TCAGGACC	G A N N L F F GGGGCAAACAACCTCTTCTTT	2	5
sequence 4	Y L C V V TACCTCTGTGTGGTGA	T R CTCG	T G A N N L F F AACTGGGGCAAACAACCTCTTCTTT	1	0
sequence 5	Y L C V V TACCTCTGTGTGGTGA	I V R G TTGTCCGGGGG	T G A N N L F F ACTGGGGCAAACAACCTCTTCTTT	3	0
sequence 6	Y L C V V TACCTCTGTGTGGTGA	K	T G A N N L F F AAACTGGGGCAAACAACCTCTTCTTT	2	0
sequence 7	Y L C TACCTCTGT	L TTC	G A N N L F F GGGGCAAACAACCTCTTCTTT	2	0
sequence 7a	Y L C TACCTCTGT	L TTC	G A s N L F F GGGGCAAgCAACCTCTTCTTT	1	0
sequence 8	Y L C V V TACCTCTGTGTGGTGA	N R K ACAGAAAA	G A N N L F F GGGGCAAACAACCTCTTCTTT	3	0
sequence 9	Y L C V V TACCTCTGTGTGGTGA	N G G V E ACGGGGGAGTCG	T G A N N L F F AAACTGGGGCAAACAACCTCTTCTTT	2	0
sequence 10	Y L C V V TACCTCTGTGTGGTGA	N M K G K ACATGAAGGGGA	T G A N N L F F AAACTGGGGCAAACAACCTCTTCTTT	1	0
sequence 11	Y L C V V TACCTCTGTGTGGTGA	N S ACTCC	Q T G A N N L F F CAAACTGGGGCAAACAACCTCTTCTTT	1	0
sequence 12	Y L C V V TACCTCTGTGTGGTGA	N K G G E ACAAGGGGGGTGAA[GA]	ACTGGGGCAAACAACCTCTTCTTT	2	0
sequence 13	Y L C V V TACCTCTGTGTGGTG	R Y L L G G CGCTATCTCCTGGGGGG	N L F F CAACCTCTTCTTT	0	2
sequence 14	Y L C V V TACCTCTGTGTGGTGA	S GC	Q T G A N N L F F CAAACTGGGGCAAACAACCTCTTCTTT	0	1
sequence 15	Y L C V V TACCTCTGTGTGGTGA	N R R D ACAGGCGAG	N L F F ACAACCTCTTCTTT	0	1
sequence 16	Y L C V V TACCTCTGTGTGGTGA	N G A ACGGGG	G A N N L F F CTGGGGCAAACAACCTCTTCTTT	0	1
sequence 17	Y L C V TACCTCTGTGTG	T K M G ACTAAAATGGG	N N L F F AAACAACCTCTTCTTT	0	1
sequence 18	Y L C V V TACCTCTGTGTGGTGA	N G Y ACGGCTA	Q T G A N N L F F TCAAACTGGGGCAAACAACCTCTTCTTT	0	1
sequence 19	Y L C V V TACCTCTGTGTGGTGA	N L F P ACTTGTTTCCG	T G A N N L F F ACTGGGGCAAACAACCTCTTCTTT	0	1
sequence 20	Y L C V TACCTCTGTGTGG	A C	Q T G A N N L F F TCAAACTGGGGCAAACAACCTCTTCTTT	0	1
sequence 21	Y L C V TACCTCTGTGTG	P R D CCTCGAGA	Q T G A N N L F F TCAAACTGGGGCAAACAACCTCTTCTTT	0	1
sequence 22	Y L C V V TACCTCTGTGTGGTGA	N AC[AG]	CTGGGGCAAACAACCTCTTCTTT	0	1
sequence 23	Y L C V TACCTCTGTGTGG	A * CCTAA	TCAAACTGGGGCAAACAACCTCTTCTTT	0	1

Figure 5. Junctional regions of cloned VA2S3J36 amplicons from unstimulated PBL of donor GM from 1979 and 1988. Amino acid residues are shown as a single letter code. The asterisk (*) indicates a stop codon. Sequences differing by single nucleotide substitutions in TCRAV2S3 and TCRAJ36 gene segments are likely due to nucleotide misincorporations by Taq polymerase (sequences *2a*, *2b*, and *7a*). Single nucleotide differences in the N region can be either due to Taq errors or actual junctional diversity (sequences *1a* and *b*). Boxed nucleotides indicate a frameshift at the N/J junction leading to an out of frame transcript. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z46962.

supports the peptide-dependency of the HLA-B35–allospecific T cells studied here: first, 721.221 LCL transfected with B*3501 were well lysed, whereas murine mastocytoma cells (P815-B*3501) were not recognized (30). Secondly, these alloreactive T cells did not recognize two HLA-B*3501–expressing cell lines defective in TAP gene expression which fail to present the broad spectra of peptides found in normal cells. Thirdly, these clones distinguished cells expressing allelic variants of B35 that differ by single amino acid exchanges located at positions thought not to directly interact with the TCR but rather with bound peptide. Particularly the amino acid exchange, Ser116 to Phe116, in the β pleated sheet of B*3503

drastically impaired allorecognition, probably through its effect on determining the carboxyterminal peptide anchor side chains (18). The sources of endogenous peptides recognized by the alloreactive T cells in the context of HLA-B*3501 remain to be defined. Although they could be derived from viral proteins, several viruses of the Herpes family (EBV, CMV, HHV6, HHV7) that persist in lymphoid cells can be ruled out because they show strong tropism for B or T lymphocytes and are normally harbored by very low numbers of lymphocytes. LCL and PHA-stimulated PBL were lysed at very high levels and purified CD4 or CD8 cells were also found to be well recognized in experiments not shown here.

From mass spectrometry studies thousands of different peptides are known to associate with any MHC molecule (37); this extensive diversity would generate suitable ligands for many TCR and could account for the high frequency of alloreactive T cells. Several functional studies of murine and human alloresponses corroborate this model (6–9, 38–40). Less experimental data exists on TCR usage in alloresponses. Sequences of MHC class II specific, alloreactive T cell clones were found to be heterogeneous in usage of TCR V and J gene segments and in CDR3 sequence motifs and no simple correlation between sequence and allospecificity could be drawn (14–16). One study demonstrated biased usage of TCRBV elements and a nonrandom, but fairly heterogeneous composition of CDR3β sequences for HLA-B27 specific, alloreactive T cells; whereas TCRα chains exhibited no common features (17). This type of heterogeneity is expected if many different allo-self complexes induce allogeneic responses.

Our TCR analysis of B35-allospecific T cell clones revealed a contrasting picture. Although usage of different V and J gene segments was detected in this small set of cells, two of six clones were found to express highly related receptors and a third highly related sequence was found as a dominant population in a long-term B35-allospecific T cell line established from the same primary culture from which the T cell clones were derived. These results revealed that a very restricted TCR usage can dominate an alloresponse. This dominance could be explained either by a strong immunogenicity of the peptide/B35 ligand or by a selective in vivo expansion of these particular T cells. Sequencing of VA2S3J36 transcripts of the PBL sample used for generation of these B35 alloreactive T cells revealed that more than 75% of the transcripts belonged to one homologous TCR family including the TCRA sequences of clone D and the $CD8^+$ T cell line.

Although this pattern of TCR usage contrasts with reports of other alloreactive T cells, conserved TCR usage was described for a xenogenic response of mice to HLA-Cw3 (41) and for antigen specific T cells. For example, TCR specific for influenza peptides presented by HLA class I molecules exhibited dominant usage of certain TCRAV and TCRBV gene segments and highly homologous CDR3 sequences (42, 43). Strong evidence exists that the CDR3 interact with protruded side chains of the antigenic peptide presented by the MHC molecule (3, 44, 45). Accordingly, only one or two amino acids of the CDR3, are critically involved in the interaction with one of the peptide side chains. The CDR3α of these B35-associated TCR all have an isoleucine followed by a basic, aliphatic residue and an amino acid with a small noncharged side chain; similarly the CDR3β show a stretch of three conserved amino acids with glutamic acid followed by two glycine residues. These particular arrangements in the CDR3 and similar functional patterns support the contention that these TCR recognize the same or highly related peptides and that their specificity can be attributed to CDR3 sequences in a manner analogous to self MHC-restricted T cells. For these alloreactive T cells the specificity of the TCR would appear to result from a direct interaction with a peptide presented by B35 and not from an indirect conformational effect of peptide on the MHC molecule. The role of peptide in driving this selection is best exemplified by the finding that from an in vivo pool of VA2S3J36 and VB4J2S7 TCR with rather different CDR3 only those with homologous CDR3 were found among the B35-alloreactive T cells.

The nature of the original stimulus that led to in vivo expansion of these particular T cells remains unknown: the donor was not directly exposed to a B35-associated allogeneic stimulus through transplantation, blood transfusion, or pregnancy. Even stronger clonal expansions of particular $CD8^+$ T cells that dominate a given Vβ family were described for elderly individuals, but their specificity was not characterized (46). A recent study of Burrows et al. demonstrated that the alloresponse to HLA-B*4402 of HLA-B8, EBV-seropositive individuals is dominated by CTL that recognize an EBNA3-derived peptide presented by HLA-B8; thus a pathogen driven response in vivo can result in a cross-reactive allogeneic response in vitro (47). EBV transformed B cells of donor GM were not recognized by the HLA-B35-alloreactive T cells (data not shown), although EBV would provide a persistent stimulus in vivo.

The maintenance of oligoclonally expanded, alloreactive T cell populations over long periods could have important implications for transplantation. Acute graft rejection occurring from one week to several months after transplantation is usually considered to be initiated by interactions of T helper and CTL; a restricted Vβ usage was shown for alloreactive T cell lines established from renal allografts during an active phase of rejection (48). The immunological basis of accelerated (early acute) graft rejection that occurs within days of engraftment is unclear, although it is also thought to be cell mediated (49). The prevalence of presensitized CTL that mediate alloresponses against a class I mismatched graft may contribute to this practically untreatable form of rejection. If preexpanded T cell populations sharing homologous TCR cause early acute allograft rejection it may be possible to develop strategies to achieve TCR-specific immunosuppression similar to those utilized in experimental animal models (50).

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