Dominant Selection of an Invariant T Cell Antigen Receptor in Response to Persistent Infection by Epstein-Barr Virus

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Summary

To examine T cell receptor (TCR) diversity involved in the memory response to a persistent human pathogen, we determined nucleotide sequences encoding TCR-α and β chains from HLA-B8-restricted, CD8+ cytotoxic T cell clones specific for an immunodominant epitope (FLRGRAYGL) in Epstein-Barr virus (EBV) nuclear antigen 3. Herein, we show that identical TCR protein sequences are used by donors from each of four healthy unrelated virus carriers; a clone from a fifth varied conservatively at only two residues. This dominant selection of α and β chain rearrangements suggests that a persistent viral infection can select for a highly focused memory response and indicates a strong bias in gene segment usage and recombination. A novel double-step semiquantitative polymerase chain reaction (PCR) procedure and direct sequencing of amplified TCR cDNA from fresh lymphocytes derived from three HLA-B8 individuals detected transcripts specific for the conserved β chain in an EBV-seropositive donor but not in two seronegative donors. This report describes an unprecedented degree of conservation in TCR selected in response to a natural persistent infection.

The TCR-α/β is a clonally distributed heterodimeric glycoprotein that interacts with immunogenic peptides presented by class I or class II MHC molecules. TCR-α and β chains are derived through the rearrangement of variable (TCRAV and TCRBV), diversity (TCRBD), and joining (TCRAJ and TCRBJ) gene segments. These segments, in combination with N region diversity in the junctional regions, provide the variation necessary to recognize the wide range of peptide/MHC combinations (1). The hypervariable complementarity determining region 3 (CDR3) spans the junctional regions and can interact directly with peptide epitopes (2). Despite the vast potential diversity of the TCR repertoire, receptors specific for certain epitopes preferentially use particular gene segments (3, 4) although several receptors specific for a given epitope usually exist, even within a single individual. The fine specificity pattern of a TCR toward peptide/MHC varies with differences in TCR primary structure, particularly in the CDR3 hypervariable region (5). Thus, different receptors that recognize a particular peptide/MHC will vary in affinity and in antigenic crossreactivity. This suggests the possibility of in vivo selection of a particular TCR subset with optimal proliferative reactivity to pathogen and environmental antigens.

The present study was designed to define the TCR diversity in a long-term persistent infection, where the opportunity for selection of T cell clones of maximal affinity might be optimal. Epstein-Barr virus (EBV) is a γ herpesvirus that persists in humans as a latent infection of B cells under the control of class I-restricted CD8+ cytotoxic T lymphocytes (CTL) (6). Here, we report the TCR-α and β chain sequences of six CTL clones specific for an immunodominant HLA-B8-restricted epitope within EBV nuclear antigen (EBNA) 3 (7). These clones, from five unrelated donors, show an unprecedented degree of conservation for a specific TCR heterodimer. A degenerate primer complementary to transcripts encoding the conserved TCR-β chain CDR3 region was used to monitor these EBV-specific CTL ex vivo. The conserved TCR-β chain could be detected in peripheral blood mononuclear cells (PBMC) from the EBV-seropositive, but not from the two seronegative HLA-B8 donors tested.
Materials and Methods

**CTL Clones.** To generate EBV-specific CTL clones, PBMC from five healthy unrelated EBV seropositive donors, IM (HLA A1, A11; B8, B51), LC (HLA A1, B8, B18), SC (HLA A1, A31; B8, B51), AS (HLA A1; B8), or DD (HLA A1, A3; B8, B40), were stimulated in vitro with autologous EBV-transformed lymphoblastoid cell lines, and the activated T cells clonally sorted in agarose (8). CTL clones specific for an immunodominant HLA-B8–restricted epitope within EBNA 3 (FLRGRAYCL, residues 339-347; hereafter referred to as FLR) (7) were defined by CTL-CTL killing (9) in the presence of peptide or by their capacity to specifically lyse autologous T cell blasts pulsed with exogenous peptide.

**Amplification of Rearranged TCRA and TCRB Sequences.** Poly A+ RNA was extracted from 1-5 x 10⁶ CTL using a QuickPrep Micro mRNA purification kit (Pharmacia Biotech AB, Uppsala, Sweden). First strand cDNA was synthesized from 500 ng of poly A+ RNA in a 20 μl volume using 10 pmol each of antisense TCRA-(Cα1; 5'-GTCGTCGACGCCCCAGAGCCTGACGCTC-3') and TCRBC-(Cβ1; 5'-GTCATGCTAGTCAGCACAGGGCGGCCGCGA-3') gene-specific primers, 200 U of reverse transcriptase (MMLV RT RNaseH-, Superscript II; GIBCO BRL, Gaithersburg, MD) according to the reaction conditions recommended by the manufacturer. After 60 min at 45°C, 10 μl of H2O was added and the RNA template hydrolyzed by the addition of 2 μl 6 M NaOH and incubation at 65°C for 30 min. The cDNA was subsequently neutralized by the addition of 2 μl 6 M acetic acid and excess primer removed using a QiAquick-spin PCR purification kit (Qiagen Inc., Chatsworth, CA). The cDNA was concentrated by ethanol precipitation and 4 pmol of the anchor (5'-CAGGATTTCTACTGATCTGGAAACCTCTCAGAGG-NH3') was ligated to the 3' end of the cDNA in a 10 μl reaction volume according to the conditions described by Troutt et al. (10). After anchor ligation, 50% of the cDNA was used as a template for PCR amplification using 10 pmol each of one primer complementary to the anchor (5'-CCCTCGAGAAGGTTCCAAGTAATGCATGAG-3') and nested TCRAC-(Cα1; 5'-GTCGTCGACGCCCCAGAGCCTGACGCTC-3') and TCRBC-(Cβ1; 5'-GTCATGCTAGTCAGCACAGGGCGGCCGCGA-3') gene-specific primers. Amplifications were performed in 50 μl reaction volumes consisting of 200 μM dNTPs and 2.5 U of Taq polymerase (Ampli Taq) using a GeneAmp PCR 9600 system (all from Perkin-Elmer Cetus Corp., Norwalk, CT). Reaction mixtures were subjected to 35 cycles of amplification followed by a 5 min final extension at 72°C. Each cycle included denaturation at 95°C for 20 s, annealing at 60°C for 40 s, and extension at 72°C for 40 s. PCR products were excised from 2.5% (1/2 x Tris-buffered EDTA) Nusieve GTG agarose gels (FMC BioProducts, Rockland, ME) and purified using β-agarase I (New England Biolabs, Inc., Beverly, MA).

**Nucleotide Sequencing.** Nucleotide sequence analysis of recovered DNA fragments was performed using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and a 373A DNA sequencer (Applied Biosystems Inc., Foster City, CA). The nucleotide sequences published in this report were also confirmed by direct PCR amplifications from 1st strand cDNA using specific TCRAV and TCRBV family-specific primers (11).

**Semiquantitative PCR Analysis of FLR-specific TCR-β Gene Expression in Fresh Lymphocytes.** Total RNA was extracted from 5 x 10⁶ PBMC derived from EBV-seronegative donors PP (HLA A1, A24; B8, B14) and WH (HLA A1; B8, B57) and sero-positive donors LC using an RNagents total RNA isolation kit (Promega Corp., Madison, WI). First strand cDNA was synthesized from 2 μg of total RNA according to the abovementioned procedure. 200 ng of 1st strand cDNA was used as a template for PCR amplification using Cα1 and a TCRBV6 family-specific primer (5'-GGCGGTTAGGGATCCGTCTC-3'). The amplified transcripts were resolved on 4% Nusieve GTG agarose gels and purified using β-agarase I as described above. 10 ng of this material was used for a subsequent round of PCR using 5 pmol each of a TCRBV6 family-specific primer and either a degenerate primer complementary to the CDR3 region of the conserved β chain (βCDR3; 5'-GTATTGCTGCTGCAAGT(A,G)-TA(A,G,C,T)GC-3') or C30 for use as a standard for the calculation of the relative abundance of the product obtained with the TCRBV6-βCDR3 primer set. The amplification schedule was 94°C for 20 s, 60°C for 20 s, and 72°C for 20 s. Amplifications were performed in 25-μl reaction volumes and samples were removed after 5, 10, 15, 20, and 25 cycles. The amplified products were separated on a 4% agarose gel and transferred to nylon membranes (Hybond N', Amersham International, Little Chalfont, UK). Specific sequences were identified by hybridization with 32P-labeled CB and TCRBV6 probes at a concentration of 1-5 x 10⁶ cpm/ml in a mixture containing 6 x SSC, 5 x Denhardt's solution, 0.5% SDS, and 500 μg/ml herring sperm DNA. Hybridizations were carried out at 42°C overnight. The membranes were washed using a protocol consisting sequentially of two washes with 2 x SSC, 0.1% SDS and two washes with 0.1 x SSC, 0.1% SDS. All washes were performed at 42°C. Specific DNA sequences were visualized by autoradiography using Kodak XAR-films. Relative quantification of the amplified products was performed using a Computing Densitometer and ImageQuant software Version 3.3 (Molecular Dynamics, Inc., Sunnyvale, CA).

**Results and Discussion**

TCRA(V-J-C) and TCRBV(V-D-J-C) rearrangements expressed by FLR-specific CTL clones from IM (IM6), SC (SC17), AS (AS1 and AS7), LC (LC13), and DD (DD1) were identified using a modification of the single strand ligation to single-stranded cDNA technique (10, 12) (Fig. 1; see reference 13 for nomenclature). All clones expressed TCRAV4S1J14S3 (Fig. 1A) and TCRBV6S3*bJ2S7 (Fig. 1B). Comparison of these sequences with known or interpreted germline V and J segment sequences (13-16) showed that the α chain junctional CDR3 regions of IM6, SC17, AS1, AS7, and LC13 encoded identical proteins and that codon usage varied only in N regions. Analysis of nucleotide sequences encoding the β chain CDR3 regions showed that clones IM6, SC17, and AS1 arrived at the same junctional sequence by loss of nucleotides from TCRBD1. The glycine codons in clones AS7 and LC13 were formed either by a portion of TCRBD2 rearranged to TCRBD1 or by N region addition. These clones have the same α and β chains generated by different rearrangement events. The junctional regions of the α and β chains of clone DD1 were encoded mostly by N region additions and differed from the other five by a single amino acid change in the CDR3 region of each chain: α110 (Ala → Ser) and β115 (Leu → Ile) (Fig. 1).

To determine how these differences related to target recognition, the fine specificity of FLR-specific CTL clones LC13, IM6, and DD1 was determined by using a replacement set of peptides in which each residue within FLR was sequentially replaced with all other genetically coded amino acids (7). The three clones had identical reactivity patterns (data not shown), except that DD1 did not tolerate the substitution of serine for glycine at position 8 (P8) of the parent pep...
puting densitometer revealed that a DNA band with the ex-

The conservative changes in the TCR sequence of clone DD1 may account for the intolerance of this clone to the P8 (Gly → Ser) substitution in the replacement analysis.

We took advantage of the conserved FLR-specific TCR-α/β heterodimer to synthesize a degenerate primer complementary to the TCRB CDR3 region, to monitor the FLR-specific CTL ex vivo. Fresh PBMC were isolated from one HLA-B8 EBV-seropositive (LC) and two HLA-B8 seronegative donors (PP and WH). A double-step PCR, performed in which all TCRBV6 sequences were amplified in the first step. Semi-quantitative PCR was employed in the second step using a TCRBV6 family primer (TCRBV6) and a degenerate primer complementary to the 3' end of the CDR3 region of the conserved FLR-specific TCRB gene (βCDR3) coding for the amino acid sequence GA→EQY (degenerate codon specification underlined). Total TCRBV6 sequences were coamplified in the first step. Semi-quantitative PCR was employed in the second step using a TCRBV6 family primer (TCRBV6) and a degenerate primer complementary to the 3' end of the CDR3 region of the conserved FLR-specific TCRB gene (βCDR3) coding for the amino acid sequence QAYEQY (degenerate codon specification underlined). Total TCRBV6 sequences were coamplified in the first step. Semi-quantitative PCR was employed in the second step using a TCRBV6 family primer (TCRBV6) and a degenerate primer complementary to the 3' end of the CDR3 region of the conserved FLR-specific TCRB gene (βCDR3) coding for the amino acid sequence QAYEQY (degenerate codon specification underlined). Total TCRBV6 sequences were coamplified as

Comparison of the normalized PCR profiles using a complementary to the TCRB CDR3 region, to monitor the FLR-specific TCRB gene (BCDvJ primer coding for the CDR3 sequence GQAYE (data not shown). Further extensions of the βCDR3 primer were not used because of the high level of degeneracy (12 x 4 x 2 x 4 for I/L x G x Q x A) involved. Direct sequencing of the PCR products showed that only one nucleotide sequence was amplified from donor LC, identical to that found in the CTL clone LC13 (Fig. 1). This is in accord with a preliminary study to this report in which 17 FLR-specific CTL clones from donor LC, derived from three separate experiments using different stimulation protocols, coded for identical TCRB nucleotide sequences (our unpublished data). Further amplification (35 cycles) of the TCRBV6-βCDR3 products from the EBV-seronegative donors PP and WH allowed direct sequencing of the corresponding bands. In neither case was a coherent sequence derived for the N-(D)-N region. A coherent sequence was obtained downstream of this region from donor WH, but not from donor PP, indicating a dominant CDR3 length component in the amplified product, one amino acid longer than the conserved β chain. The high level of specific product amplified in the EBV-seropositive donor is consistent with the high precursor frequency (up to 1/2,000; Burrows, S. R., D. J. Moss, R.

Figure 1. Sequence analysis of TCR variable regions. The nucleotide sequences are presented, and the one-letter code designating the translated amino acid is shown above the first nucleotide in each codon: (A) α chain sequences; (B) β chain sequences. The borders between TCR V, N, (D+N), and J regions are based on known or interpreted germline sequences (13-16) and are shown by gaps in the sequence. Framework cysteines are numbered according to published TCRAV4S1 and TCRBV63* (13) deduced amino acid sequences. TCRBD-matching sequences are underlined. These α/β chain sequence data are available from EMBL/GenBank/DDBJ under accession numbers L26453/L26454 (IM6); L26457/L26458 (SC17); L29035/L29037 (AS1); L29036/L29038 (AS7); L26455/L26456 (LC13); and L26451/L26452 (DD1), respectively.

Figure 2. Semiquantitative analysis of the conserved FLR-specific β chain in HLA-B8* donors PP, WH, and LC. (A) Amplification of total TCRBV6 sequences. (B) Amplification of conserved FLR-specific β chain. Reactions were terminated at the completion of 5, 10, 15, 20, and 25 cycles. (+) denotes control reactions excluding template that were terminated after 25 cycles of amplification.
Khanna, I. S. Misko, and V. P. Argaet, manuscript in preparation) of FLR-specific CTL in HLA-B8 donors, and provides strong evidence that these clones are reflective of the circulating CTL population. The detection of the particular $\beta$ chain employed by FLR-specific CTL in PBL from an EBV-seropositive donor, but not in two seronegative donors provides an exciting possibility of using PCR to directly monitor FLR-specific CTL in individuals during primary infection or after vaccination with the peptide FLR.

This report describes an unprecedented degree of conservation in TCR selected in response to a natural persistent infection. We interpret these results in a model that invokes restriction on the TCR repertoire not only (a) at the protein level, shaped by positive and negative selection in the thymus and by events occurring in the periphery as a result of chronic immune stimulation, but also, (b) at the nucleic acid level before thymic selection as indicated by the remarkable bias among unrelated individuals toward identical dominant TCR rearrangements.

Peripheral restimulation of CTL is thought to maintain a life-long potent memory T cell compartment, a feature of healthy virus carriers. In a persistent infection, dominant CTL clones could arise from their preferential restimulation due to higher affinity antigen receptors. Thus, EBV-specific CTL with a high affinity TCR could respond to marginal antigen concentrations on infected B cells, an important advantage post convalescence when antigen is presumably limiting. Our rearrangements.

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We have recently shown that CTL (such as those examined here) from $B^*4402^-$ donors specific for FLR/HLA-B8 recognize non-EBV-infected cells from HLA-B$^*4402$ donors, presumably through a self-peptide in association with that allele (22). In a subsequent study to the one presented here, we have shown that CTL recognizing FLR/HLA-B8 can be raised from EBV seropositive HLA-B$^*4402$ donors, but these do not employ the same TCR sequences described in this report (Burrows et al., manuscript in preparation). These data indicate that the conserved TCR heterodimer defined herein has not dominated simply because it is the only $\alpha/\beta$ combination capable to recognizing FLR/HLA-B8 due, for example, to structural constraints (23).

Our results differ with other studies that have shown variable CDR3 regions or the usage of different $\alpha$ or $\beta$ chain rearrangements in T cells recognizing specific epitopes derived from nonpersisting antigens (4). For instance, the human TCR repertoire against defined influenza nuclear protein epitopes has been shown to be oligoclonal (23). Moreover, TCR repertoires in mice are often examined in the short-term and in response to high challenge doses of antigen (4). The former precludes selection by chronic restimulation, while the latter may encourage the growth of clones of lesser affinity. In terms of the EBV model, such protocols are more likely to be relevant to the primary response seen in acute infectious mononucleosis, where a range of TCR may respond to the same epitope. Indeed, a longitudinal study is in progress that is designed to monitor the FLR-specific TCR repertoire in HLA-B8 donors from an acute to a persistent phase of the disease.

Estimates of the potential TCR-$\alpha/\beta$ repertoire range from $10^{15}$ to $10^{20}$ based on random employment of $N$ region nucleotides and $V$, $D$ and $J$ elements (24, 25). Since the adult human has $\sim10^{13}$ lymphocytes, the finding of clones with identical TCR in unrelated individuals must also involve a bias in gene segment rearrangement. Contributions to bias at this level have been demonstrated by nonrandomness in $V$-$J$ pairing, $N$ region addition and CDR3 region length (26). Also, the expression of a rearranged $\beta$ chain before $\alpha$ chain rearrangement may allow a process of "$\beta$ selection" (27) to favor the survival of certain $\beta$ chain sequences. We further note that the five clones expressing identical TCR are almost exclusively germline encoded in the $\beta$ chain CDR3 region indicating that these may be especially favored rearrangements. TCRBV63S and TCRBJ2S7 gene segments have been shown to be particularly common in adult peripheral blood (28, 29) and based on these data, the TCRBV63J2S7 rearrangements may account for up to 4% of $\beta$ chains. As there are potentially $5 \times 10^7$ different $\alpha$ chains (24), the finding of identical TCRV sequences in several individuals also strongly suggests a bias in $\alpha$ chain rearrangement.

EBV is ubiquitous and related $\gamma$ herpesviruses are found in all old-world primate species (30). Moreover, infection is common prior to the onset of sexual maturity and is potentially fatal in individuals with a defective CTL response who are typically at higher risk of developing lymphoblastoid cell line-like lymphomas. We favor, therefore, that a bias in the production of specific TCR rearrangements has coevolved with EBV and the MHC to facilitate a balanced virus-host relationship.
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