T CELL RECEPTOR Vβ GENE USAGE IN A HUMAN ALLOREACTIVE RESPONSE

Shared Structural Features among HLA-B27-specific T Cell Clones

By RAFAEL BRAGADO, PILAR LAUZURICA, DANIEL LÓPEZ, AND JOSÉ A. LÓPEZ DE CASTRO

From the Department of Immunology, Fundación Jiménez Díaz, 28040 Madrid, Spain

TCRs are cell surface heterodimers of variable α and β chains that mediate T cell recognition of peptide antigens as presented by MHC proteins. The structural features of this interaction are still poorly understood. It is likely that amino acid residues from both the peptide and the MHC molecule contact with the TCR (1–3). Thus, MHC protein polymorphism may contribute to the specificity of T cell recognition by modulating direct interaction with the TCR and peptide binding.

TCR variability is generated, in both α and β chains, by the combinatorial rearrangement of multiple germline-encoded V, D (for the β chain), and J gene segments, through mechanisms that allow introduction of great diversity at the junctional regions (4, 5).

The TCR might adopt an Ig-like folding. The equivalents, in the TCR, to the complementarity-determining regions (CDR)[1] 1 and 2 from Igs would be encoded in the Vα and Vβ gene segments and would presumably contact with residues in the α helices of the MHC molecule; the CDR3-equivalents would correspond to the junctional regions and would interact mainly with bound peptide (3, 6).

The clonal heterogeneity of T cell alloreactive responses is highly complex (7). In humans, an extremely diverse spectrum of specificities has been revealed by analyses of CTL clones with structurally defined HLA mutants (8–10). A possible interpretation of such diversity is that alloreactive T cells might recognize alloantigen-bound peptides. This is supported by mounting evidence (11–15). Indeed, availability of peptide ligands may be required for correct folding of class I MHC molecules and for their transport to the cell surface (16). The question remains as to whether the complexity of allospecific responses can be understood in terms of sets of epitopes sharing some structural features. If so, one may ask whether such epitopes

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Address correspondence to José A. López de Castro, Department of Immunology, Fundación Jiménez Díaz, Avenida Reyes Católicos, 2, 28040 Madrid, Spain.

[1] *Abbreviations used in this paper:* CDR, complementarity-determining region; LCL, lymphoblastoid cell line; PCR, polymerase chain reaction; T_m, melting temperature.

are recognized by related TCR structures and what is the molecular basis of this relatedness.

To address these questions, we have examined the structure of the Vβ genes used by a series of alloreactive CTL clones against HLA-B27, whose fine specificity was previously established. A strategy, based on specific amplification and direct sequencing of Vβ genes, was used. The results suggest a nonrandom Vβ gene diversity in the alloreactive CTL response against HLA-B27.

Materials and Methods

T Cell Clones. 12 human HLA-B27-specific CTL clones were used in this study. 11 of these clones were raised from four different HLA-B27$^-$ individuals against the B*2705$^+$ lymphoblastoid cell lines (LCL) LG15 (HLA-A32; B*2705) or R69 (HLA-A3, 24; B*2705, 7). The following anti-B*2705 CTL clones were derived from each responder: from donor PA (HLA-A24, w33; B35,39), CTL 28 and 40 (17); from donor DL (HLA-A29, 31; B39, 44), CTL 64DRF, 67DRF, 102DRF, 172DRF, and 212DRD; from donor GM (HLA-A1, 24; B7, 8), CTL GM7, 5A2, and 17A2; from donor BG (HLA-A2; B5, 7), CTL G36 (10). One additional clone, CTL 64.8P, was obtained from yet another HLA-B27$^-$ individual against a B*2704$^+$ LCL (18). In addition, eight CTL clones not reactive with HLA-B27, but otherwise uncharacterized, were used. These were CTL 1DRD, 14DRD, 17DRD, 223DRD, 55DRF, 166DRF (all from donor DL), M42 (from donor GM), and G21 (from donor BG).

All clones were derived by limiting dilution as described (19). Cells were cloned after primary, secondary, or tertiary (for 64.8P) MLC and were selected, among those with lytic activity against the stimulator LCL, for their capacity to lyse B*2705$^+$ HMy2.CIR transfectant cells, but not the same cells transfected only with pSV2neo, at an E/T ratio of 4:1 (10). HMy2CIR (a gift of Dr. P. Creswell, Duke University, Durham, NC) is a class I MHC-deficient mutant derived from the human plasma cell leukemia line LICR.LON.HMy2 (20).

The fine specificity of the anti-HLA-B27 CTL clones was established: (*a*) by panel analysis with LCL expressing all six structurally characterized HLA-B27 subtypes, B*2701 to B*2706 (21), and multiple HLA-B27$^-$ LCL; (*b*) by using a panel of HMy2.CIR transfectants expressing HLA-B*2705, B*2702 and nine site-specific B*2705 mutants, most of them mimicking, at one or at two positions, changes occurring in the HLA-B27 subtypes (10). A standard ^{51}Cr release cytotoxicity assay was used (19).

The anti-B*2705 CTL clones could be classified into three groups (Table I) on the basis of their reactivity with HLA-B27 subtypes (10, 17). Group A included seven CTL clones reacting only with B*2705. Group B included two CTL clones, reacting only with B*2705 and B*2703. Group C included two clones that reacted only with B*2705 and B*2702. Thus, none of the 11 anti-B*2705 CTL clones recognized B*2701, B*2704, or B*2706. The anti-B*2704 CTL clone 64.8P reacted with all B27 subtypes except B*2703 (18). Most CTL clones were different from one another when tested with the mutants (10). The single exceptions were CTL 67DRF (unpublished data) and 102 DRF, which were indistinguishable by this criterium.

Polymerase Chain Reaction (PCR) and Sequencing. TCR β chain mRNA from each CTL clone was converted to cDNA, amplified, and reconverted to single-stranded templates for direct DNA sequencing. This strategy required the steps described below.

The first step was specific PCR amplification of Vβ cDNA. For each of the 20 described Vβ families (5), an oligonucleotide with a sequence common to all known members from that family, and different from available Vβ sequences from other families, was selected. The only exceptions were the Vβ13-specific oligonucleotide, which showed one mismatch with one Vβ13 member, and the Vβ8-specific primer, which presented one and two 5′ end-located mismatches, respectively, with two Vβ8 members. An oligonucleotide, designated as 12a, whose sequence was shared by Vβ3, Vβ12, Vβ13, Vβ14, and Vβ15, was also selected. A compilation of 49 different Vβ sequences (5) was used. The search was done in an HP Vectra RS/25C computer using the Local (22; Molecular Biology Computer Research resource, Harvard School of Public Health, Boston, MA) and Pattern Matching (DNA and Protein Se-

quence Analysis Programs, version 4.3; Department of Molecular and Cellular Biology, University of Arizona, Tucson, AR) programs. A value of four mismatches, including gaps, was the minimum allowed disparity when comparing each family-specific oligonucleotide with sequences from other families. As the only exception, the Vβ6-specific oligonucleotide possessed three mismatches with Vβ16 members. In addition, a Cβ-specific oligonucleotide was chosen to match a common sequence near the 5′ ends of the Cβ1 and Cβ2 genes. The Vβ- and Cβ-derived oligonucleotides were used as 5′ sense and antisense primers, respectively, for specific PCR amplification of Vβ cDNA. These oligonucleotides were designated as "external" (E) and their sequences are given in Table II.

Total cytoplasmic RNA was isolated from each CTL clone by a simplification of a standard technique (23). Briefly, pellets from 3×10^4 to 3×10^5 cells were lysed in 200 μl 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM $MgCl_2$ containing 0.5% NP-40 and 10 mM vanadyl ribonucleoside complexes (Bethesda Research Laboratories, Gaithersburg, MD). After vortexing for 10 s, lysates were stored on ice for 5 min and then microfuged for 1 min at 4°C to pellet nuclei. Supernatants were added to an equal volume of 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA containing 1% SDS. Proteins were then removed by extracting several times with phenol/chloroform and once with chloroform, and the RNA was precipitated by adding 3 M sodium acetate, pH 5.2, to a final concentration of 0.15 M and 2.2 Vol of ethanol. After storing at −70°C for 15 min, tubes were microfuged for 30 min at 4°C. Pellets were allowed to dry at room temperature and a second precipitation was carried out in 400 μl of 0.15 M sodium acetate, pH 5.2, by adding 880 μl ethanol.

To synthesize cDNA, the RNA was incubated at 42°C for 1 h with 50 μl of a reaction mixture containing 1 μM CβE oligonucleotide, dNTPs (Cetus Corp., Emeryville, CA), 200 μM each, 2 mM DTT, 2 U human placental ribonuclease inhibitor, and 2 U avian myeloblastosis virus reverse transcriptase (both from Boeringer Mannheim Biochemicals, Mannheim, FRG) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin (Taq buffer; Cetus Corp.).

After incubation, the reaction mixture was heated at 95°C for 5 min, to denature the DNA/RNA complexes, cooled to 4°C, and 850 μl of a solution containing CβE oligonucleotide and dNTPs at final concentrations of 1.1 μM and 220 μM each, in Taq buffer, respectively, was added. 15 U of Taq polymerase (Cetus Corp.) was then added, and 45-μl aliquots of this solution were transferred to 20 Eppendorf tubes, each containing 5 μl of 1 of the 20 VβE family-specific oligonucleotides at 10-μM initial concentration. Each reaction mixture was overlaid with five drops of mineral oil and then subjected to 30 amplification cycles of 2 min at 95°C, 3.5 min at 39°C, and 2 min at 72°C, using a thermocycler (Hybaid Ltd., Middlesex, UK). After removing the layer of mineral oil, an aliquot of each amplification reaction was loaded in a 2% agarose gel and run for 1 h at 100 V. Due to the small number of cells used for RNA isolation, ethidium bromide–stained bands were not always visualized. To detect specific amplification products, alkaline Southern blots were carried out using Z probe membrane (Bio-Rad Laboratories, Richmond, CA) as indicated by the manufacturer. The membrane was then washed for 2 min in 2× SSC (1× SSC = 0.15 M NaCl/0.013 M sodium citrate) and prehybridized directly in 5× SSPE/10× Denhardt's solution/0.1% SDS/40 μg/ml salmon sperm DNA, for 45 min, at 5°C below the melting temperature (T_m) of the CβI oligonucleotide (see below), to be used in the hybridization (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA and 1× Denhardt's solution = 0.02% polyvinyl pyrrolidone/0.02% Ficoll/0.02% BSA). This oligonucleotide was labeled with (γ-[^{32}P]dATP (5,000 Ci/mmol; Amersham International, Amersham, UK) by using polynucleotide kinase (New England Biolabs, Beverly, MA). Unincorporated nucleotide was removed by impregnating a Whatman DE81 paper with the labeling reaction mixture and washing it with low-salt buffer (20 mM Tris-HCl, pH 8, 1 mM EDTA, 0.1 M NaCl). Labeled material was recovered by eluting with high-salt buffer (20 mM Tris-HCl, pH 8, 1 mM EDTA, 1 M NaCl). Blots were hybridized in the same solution as that used for prehybridization, except that 5× Denhardt's was used. Labeled oligonucleotide was added at a concentration of 1 nM and incubated for 3 h at T_m −5°C. Blots were then washed three times in 6× SSC at room temperature for 20 min, once in 6× SSC for 1 min at the hybridization temperature, and exposed to Kodak X-Omat film.

The second step was generation of single-stranded DNA and direct sequencing. New sets of Vβ- and Cβ-derived oligonucleotides were selected as sense and antisense primers, respectively, to perform asymmetric PCR on each strand of the PCR-amplified cDNA, so that an excess of single-stranded DNA of a chosen strand was produced. This second set of oligonucleotides was designated as "internal" (I), as they were matched to sequences located 3′ and 5′, respectively, from those of the VβE and CβE primers used for the first PCR amplification. Each of the VβI primers was chosen to match with all members of a given family, but mismatch with other Vβ families was not required at this stage. Asymmetric PCR amplification was done as previously described (24, 25), using 10 nM VβE plus 1 μM CβI oligonucleotides for the generation of the minus strand, and 1 μM VβI plus 10 nM CβE oligonucleotides for the generation of the positive strand. Direct sequencing of amplified DNA strands was carried out as described (25), using the corresponding VβI oligonucleotide or the CβI oligonucleotide as primers for sequencing the minus or the positive strand, respectively. The CβI oligonucleotide was matched to a sequence common to Cβ1 and Cβ2. The nucleotide sequences of all "internal" primers used in this study are given in Table II.

Results

Limited Vβ Gene Segment Usage among HLA-B27-specific Alloreactive CTL Clones. The Vβ gene segments used by 11 human CTL clones obtained, from various responder individuals, against B*2705 and one clone raised against B*2704 were examined by PCR. The particular strategy was based on using a set of Vβ family-specific and one Cβ-specific oligonucleotide as primers. With this strategy, specific amplification was expected only in the aliquot of cDNA from each clone that was incubated with the appropriate family-specific primer. The specificity of the Vβ primers was assured by the mismatching with other families and was indicated by the following criteria: (*a*) amplification was obtained with all tested T cell clones; (*b*) the amplified cDNA was always of the appropriate size, on the basis of the expected priming locations of the Vβ and Cβ primers used; (*c*) for each clone, bands were only obtained with one of the Vβ primers (Fig. 1); and (*d*) sequencing of the amplified material confirmed in each case that the amplified Vβ gene segment corresponded to the expected specificity of the primer used (see below).

Fig. 1 shows the PCR amplification of TCR Vβ cDNA from the anti-B27 CTL clones analyzed in this study. Among those clones reactive only with the stimulating B*2705 subtype (group A), amplification in CTL 67DRF, 212DRD, and 40 was observed with the Vβ14E primer. CTL 17A2 cDNA was amplified with Vβ13E. CTL G36 cDNA was amplified with the crossreactive 12aE oligonucleotide but not with Vβ12E or any other Vβ family-specific primer. Sequencing of the amplified material indicated that this CTL clone also expressed a Vβ13 gene segment (see below). Amplification of cDNA from CTL 64DRF and 102DRF was obtained with Vβ4E. By this criterium the two CTL clones from group B, CTL 5A2 and GM7, expressed Vβ15 and Vβ3 gene segments, respectively, and those from group C, CTL 28 and 172 DRF, both expressed Vβ4. cDNA from the anti-B*2704 CTL clone 64.8P was amplified with Vβ7E.

The Vβ families detected among the anti-B*2705 CTL clones fall clearly into two groups: (*a*) Vβ4 was observed in 4 of the 11 CTL clones; (*b*) Vβ3, Vβ13, Vβ14, and Vβ15, detected in the remaining seven clones, belong to a group of particularly homologous families (see Discussion). Interestingly, CTL 64.8P, whose reaction pattern with HLA-B27 subtypes is quite different from those of the anti-B*2705 CTL clones (Table I), uses a Vβ family that shares little amino acid sequence homology

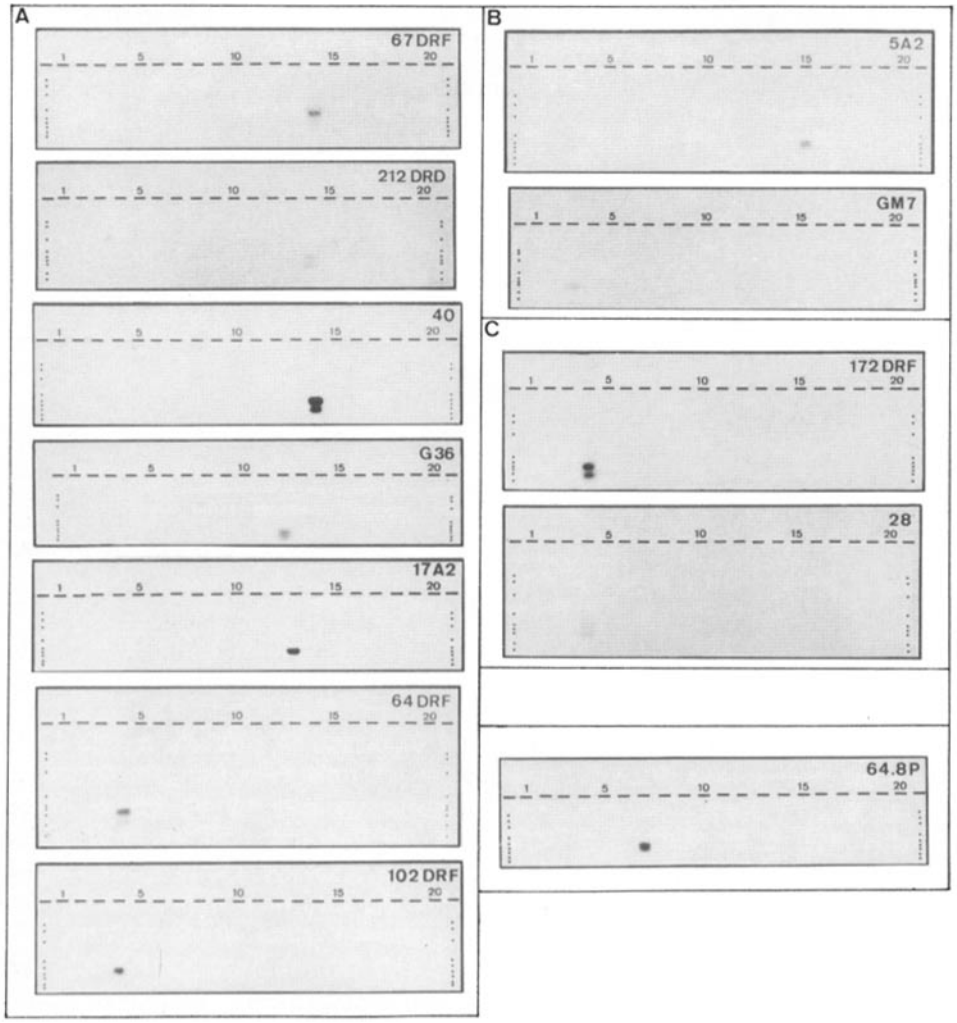

FIGURE 1. Vβ gene segment usage among human HLA-B27-specific alloreactive CTL clones, as detected by Southern blot analysis of PCR-amplified cDNA. For each clone, cDNA aliquots were subjected to PCR using, in parallel reactions, each of 20 TCR VβE family-specific and a common CβE primer (Table II). Lanes *1–20* in each autoradiogram contain cDNA samples assayed for amplification with Vβ1E to Vβ20E, respectively, and hybridized with ^{32}P-labeled CβI oligonucleotide. Thus, the location of radioactive spots indicates the Vβ family amplified from each T cell clone. For CTL G36, the crossreactive 12aE primer (see Materials and Methods) was used instead of Vβ12E; as in previous experiments (not shown), no amplification was obtained with any of the family-specific primers. This clone expresses a new Vβ13 segment (see text). The double bands seen in some cases probably correspond to amplified double- and single-stranded cDNA. This could arise from unbalance in the two primers used in the PCR, as a result of inaccuracies in the spectrophotometric quantitation of oligonucleotides differing widely in base content. Dots at both sides of each autoradiogram correspond to molecular weight markers (1-kb DNA ladder; Bethesda Research Laboratories) of 2,036, 1,635, 1,016, 516/506, 394, 344, 298, 220, and 200 bp, respectively. Autoradiograms are grouped according to the reaction patterns of the CTL clones with HLA-B27 subtypes (Table I).

TABLE I

Reaction Patterns of Alloreactive CTL Clones with HLA-B27 Subtypes

Reaction pattern	CTL clone	B*2705	B*2701 (74,77, 81)*	B*2702 (77,80, 81)	B*2703 (59)	B*2704 (77,152)	B*2706 (77,114, 116,152)
A	67DRF	+‡	–	–	–	–	–
	212DRD	+	–	–	–	–	–
	40	+	–	–	–	–	–
	G36	+	–	–	–	–	–
	17A2	+	–	–	–	–	–
	64DRF	+	–	–	–	–	–
	102DRF§	+	–	–	–	–	–
B	5A2	+	–	–	+	–	–
	GM7	+	–	–	+	–	–
C	172DRF	+	–	+	–	–	–
	28	+	–	+	–	–	–
	64.8P	+	+	+	–	+	+

* The amino acid sequence positions in which each B27 subtype differs from B*2705 (20) are given in parentheses.

‡ (+) > 25% specific cytotoxicity at an ET ratio of 4:1; (–) absence of cytotoxicity (<10%) at the same E/T ratio.

§ This clone crossreacted weakly with HLA-B40*(B*4002).

(~20–30%) with those from the anti-B*2705 CTL clones. In addition, in eight CTL clones not reactive with HLA-B27, which were obtained from the same donors as the anti-B*2705 clones, a different set of Vβ families was detected (not shown). These included Vβ2 (CTL M42), Vβ5 (CTL G21, 14DRD and 17DRD), Vβ8 (CTL 166DRF and 1DRD), and Vβ13 (CTL 55DRF and 223DRD), which are not related to each other by specially high homology. Taken together, these results indicate that the anti-B*2705 CTL clones analyzed use a selective subset of Vβ segments.

Sequence Analysis of Amplified Vβ cDNA Reveals Only Correctly Rearranged Vβ Genes and Detects New Vβ Members. Double-stranded cDNA amplified from each anti-B27 CTL clone was subjected to asymmetric PCR. Both DNA strands were separately amplified and sequenced in all cases. The span of each sequence was determined by the location of the corresponding Vβ primers used, and this was different for different families. However, partial Vβ segment sequences, spanning approximately their second half in most cases, as well as complete junctional and Jβ segment sequences, were obtained from all CTL clones (Fig. 2 *A*). The Vβ segment sequences allowed us to unambiguously establish the Vβ family used in all cases. In addition, the priming location of the Cβ-derived oligonucleotide allowed us to establish the precise Cβ gene used on the basis of the partial Cβ sequence obtained (Fig. 2).

All sequences correspond to Vβ gene material and were always translatable to amino acid sequences. This indicates that: (*a*) the PCR-amplified material was Vβ cDNA; and (*b*) no evidence for amplification of aberrant transcripts was obtained. Whether this is due to the fact that these clones only produced correct rearrangements, or to the inability of this technique to detect aberrant Vβ transcripts, was not examined.

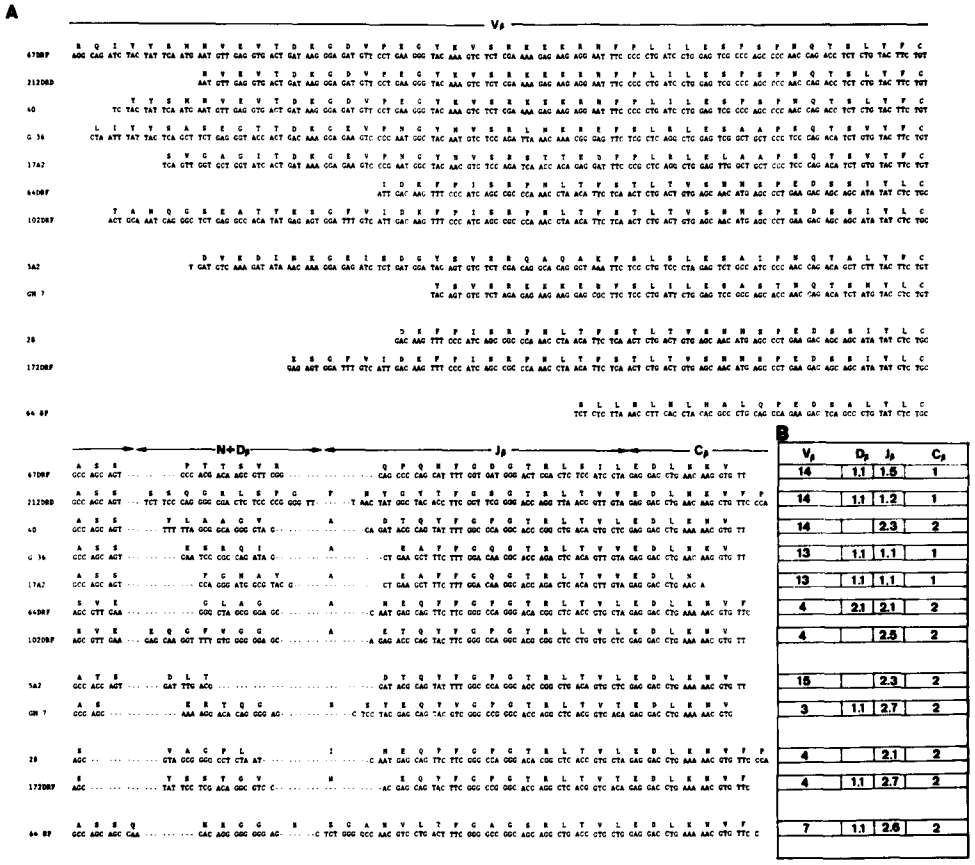

FIGURE 2. Sequence of TCR Vβ genes used by 12 human HLA-B27-specific CTL clones. (*A*) Nucleotide and deduced amino acid sequences of specifically amplified Vβ cDNA from each CTL clone. Partial Vβ segment and complete N + D region and Jβ segment sequences are given. The breaks in the nucleotide sequence indicate junctional boundaries. V segment boundaries are assigned as in Wilson et al. (5). Jβ segment boundaries are considered to be the breaking point of identity with the germ-line sequence. The CDR2 equivalent spans codons 4–16, referred to as the determined sequence of CTL 67DRF; the CDR3 equivalent spans the junctional region, which varies in size among T cell clones (for assignment of CDR equivalents, see reference 6). Sequences are grouped according to the reaction patterns of the corresponding T cell clones with HLA-B27 subtypes. (*B*) Assignment of the Vβ, Jβ, and Cβ gene segments used by each T cell clone was made on the basis of homology with known sequences. The fourth and fifth Cβ triplets allow distinguishing between Cβ1 and Cβ2. Dβ1.1 segments were assigned to all clones using Jβ1 segments on the basis of the requirements for gene rearrangement. All other Dβ assignments were based, when possible, on sufficient homology with germ-line sequences (30). These sequence data have been submitted to the EMBL/GenBank Data Libraries under the following accession numbers: X51785(CTL 102DRF); X51786(CTL 172DRF); X51787(CTL 17A2); X51788(CTL 212DRD); X51789(CTL 28); X51790(CTL 40); X51791(CTL 5A2); X51792(CTL 64.8P); X51793(CTL 64DRF); X51794(CTL 67DRF); X51795(CTL G36); X51796(CTL GM7).

The Vβ gene segment sequences obtained were identical in most, but not all, cases to previously reported sequences. This allowed formal confirmation of the Vβ family used by each CTL clone, as assigned on the basis of hybridization of the PCR-amplified

material (Fig. 1). However, for CTL G36 and 17A2, the sequences obtained were different from those of known Vβ segments. Both were assigned as Vβ13 members on the basis of their homology with other sequences from this family (26–28). The Vβ segment from CTL G36 was sequenced in its last 50 triplets. It differed from known Vβ13 members by 12–14% of the nucleotide sequence in this region. Such disparity suggests that failure to amplify Vβ cDNA from this clone with the Vβ13E primer could be due to sequence differences in the relevant priming site. This was not formally established because the sequence obtained did not include the priming location of Vβ13E. Similarly, the last 46 triplets of the Vβ segment from CTL 17A2 were determined and shown to differ from known Vβ13 sequences by at least 5% and as much as 17% over this region (Fig. 3). Thus, homology between the partial Vβ segment sequences from CTL G36 and 17A2 with other Vβ13 sequences is comparable with the homology with each other, which is 88% (Fig. 3 and Table III). These data indicate that both clones express new, hitherto undescribed, Vβ segments. Most likely, these are new Vβ13 members, although their definitive family assignment would require determining their complete sequences. Homology of these Vβ segments with members of the highly homologous Vβ12 family ranged from 72 to 75% over the determined regions. We have adopted a convention of 20 Vβ families (5), but fusing Vβ12 and Vβ13 has been proposed (29).

The nucleotide and amino acid sequence homology among the Vβ segments from the anti-B*2705 CTL clones analyzed was compared in Table III through the regions whose sequence was determined. The data show that the Vβ3, Vβ13, Vβ14, and Vβ15 segments expressed by these clones share nucleotide sequence homologies ranging from 58 to 100% over the sequenced regions, as the Vβ14 sequences obtained were identical (Fig. 2 *A*). This is in agreement with the overall homology among members of these related families (5). The four identical Vβ4 sequences obtained share a clearly lower homology (Table III) with members of the above mentioned Vβ families, also in agreement with global homology figures. The Vβ7 sequence from CTL 64.8P is not particularly related to any of the Vβ sequences from anti-B*2705 CTL clones.

HLA-B27-specific CTL Clones Display Wide, but not Unrestricted, Vβ Junctional and Jβ Segment Diversity. As shown in Fig. 2 *A*, the rearranged β genes differ both in length, ranging from three to nine codons, and sequence at the V-D-J junctions. The exact identification of the Dβ segments used was not always possible (Fig. 2 *B*), due to difficulties arising from the combined effects of junctional and N-region diversity with the small number of bases making up the germ-line Dβ segments (30). In general, no common structural motives are apparent in this region, except that five of the seven clones from group A (B*2705 specific) use Ala codons at the D-J junction. No CTL clones from other groups use such codons at this point (Fig. 2 *A*).

Jβ segment usage is also diverse (Fig. 2). However, some clones share identical Jβ segments. First, CTL 64DRF and 28 express the same Vβ4 and identically rearranged Jβ2.1 segments. Second, CTL G36 and 17A2 express highly homologous Vβ13 and identically rearranged Jβ1.1 segments. Third, CTL 40 and 5A2, which express significantly homologous Vβ14 and Vβ15 segments (Table III), both express Jβ2.3 segments. In this case, both Jβ segments start at the same Asp codon, thus having identical amino acid sequences, but that from CTL 40 was apparently rearranged with two additional bases at its 5′ end (Fig. 2 *A*). CTL 172DRF and GM7, which possess the little homologous Vβ4 and Vβ3 segments, respectively (Table III),

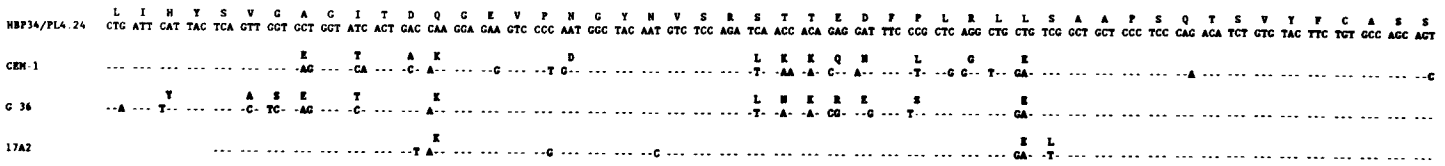

FIGURE 3. The Vβ gene segments from CTL G36 and 17A2 are new Vβ13 members. The nucleotide and inferred amino acid sequences from the Vβ gene segments used by these two clones are compared with the known Vβ13 segments (HBP34, PL-4.24 and CEM-1 are from references 28, 26, and 27, respectively). A partial Vβ13 segment, PL 5.3 (28), differing from CEM-1 only by two nucleotides in the 3′ end codon, has not been included.

TABLE II

Oligonucleotide Primers for Specific PCR Amplification of TCR Vβ cDNA

External		Internal	
Name	Sequence	Name	Sequence
	5′ 3′		
Vβ1E	TCC CCT AGG TCT GGA GAC CTC TCT		
2E	TTT CAG GCC ACA ACT ATG TTT TG		5′ 3′
3E	GAT ATG GAC CAT GAA AAT ATG TTC	Vβ3I	AAA ATG AAA GAA AAA GGA GAT
4E	ACG ATC CAG TGT CAA GTC GA	4I	CTG ATC GCA ACT GCA AAT CAG
5E	CTG ATC AAA ACG AGA GGA CAG CA		
6E	TCA GGT GTG ATC CAA TTT C		
7E	CAA CAT ATG GGG CAC AGG GCA ATG	7I	TTC TCA CCT GAA TGC CCC AAC
8E	GAG GTC ACA GAG ATG GGA CA		
9E	GAA CAA AAT CTG GGC CAT GAT ACT		
10E	GTT CCT ATA AAA GCA CAT AGT TAT		
11E	TCT CAA ACC ATG GGC CAT GAC AAA		
12E	CTG AGA TGT CAC CAG ACT GAG		
12aE	CTG GTA TCG ACA AGA CCC		
13E	GCA TGA CAC TGC AGT GTG CCC	13I	TCA TTA CTC AGT TGG TGC TGG
14E	ACC CAA GAT ACC TCA TCA CAG	14I	AAC AGT GAC TTG TTC TCA GA
15E	TCT CAG ACT AAG GGT CAT GAT AGA	15I	TTT GAT GTC AAA GAT ATA AAC
16E	GAC CCA ATT TCT GGA CAT GAT AAT		
17E	GAA CAG AAT TTG AAC CAC GAT GCC		
18E	AGC CCA ATG AAA GGA CAC AGT CAT		
19E	ACC CCC GAA AAA GGA CAT ACT TTT		
20E	GAG GGA ACA TCA AAC CCC AAC CTA		
Cβ E	TTT TGG GTG TGG GAG ATC TC	Cβ I	TTC TGA TGG CTC AAA CAC

both express Jβ2.7 segments, but they are differently rearranged, so that the one from CTL GM7 is two codons larger. Furthermore, this Jβ2.7 segment has a T to G nucleotide change in its sixth codon (GTC instead of TTC), resulting in a Phe to Val change (Fig. 2 *A*), as compared with the published germ-line Jβ2.7 sequence (30). This substitution was confirmed in an independent RNA preparation and specific PCR amplification from the same CTL clone, suggesting that an artifactual introduction of this change during sample processing was unlikely. It might reflect a genetic polymorphism, as also suggested for the Dβ2.1 segment (26), since the two clones expressing Jβ2.7 came from different individuals.

It is interesting that the Jβ2 segments used by the anti-B*2705 CTL clones are either Jβ2.1, Jβ2.3, Jβ2.5, or Jβ2.7 (Fig. 2). These have a Pro codon at a polymorphic position around their middle, which is different in all other Jβ2 segments (30). In contrast, the anti-B*2704 CTL clone 64.8P expressed Jβ2.6, lacking this Pro codon. The possible significance of this structural motif for the specificity of the anti-HLA-B27 clones is unclear, as the combined usage of Jβ2.1/2.3/2.5/2.7 in nonspecific human T cell populations may be more frequent than that of the remaining three Jβ2 segments (29).

Discussion

Analyses of epitope structure and TCR usage in alloantigen-specific T cell recognition are faced with the extraordinary diversity of alloreactive responses. For in-

TABLE III
*Homology Matrix of the Vβ Gene Segments from Anti-HLA-B*2705 CTL Clones*

	67DRF 212DRD 40 (Vβ14)	G36 (Vβ13)	17A2 (Vβ13)	5A2 (Vβ15)	GM7 (Vβ3)	64DRF 102DRF 28 172DRF (Vβ4)
67DRF 212DRD 40 (Vβ14)	-	67	69	70	79	38
G36 (Vβ13)	64	-	88	61	71	33
17A2 (Vβ13)	59	76	-	58	68	35
5A2 (Vβ15)	52	52	48	-	75	33
GM7 (Vβ3)	67	63	57	60	-	38
64DRF 102DRF 28 172DRF (Vβ4)	24	23	24	21	27	-

The partial Vβ segment sequences obtained in this study were compared with each other at both the nucleotide and the amino acid levels. Numbers above and below the diagonal indicate percentage nucleotide and amino acid sequence homology, respectively, from any two Vβ segments. In comparisons involving identical Vβ sequences determined at various lengths from different clones (Vβ4 and Vβ14), all combinations were calculated and the means are given. Figures do not reflect exactly overall Vβ segment homology, as partial sequences are compared.

stance, virtually all CTL clones amenable to analysis, among those generated against HLA-A2 (8, 9) or HLA-B27 (10), possess different fine specificities. In addition, in the human system, difficulties in growing sufficient cells from most CTL clones seriously hamper studies at the clonal level. The existence of multiple members in many TCR Vα and Vβ families adds further complication to the system. We have attempted to circumvent these difficulties in addressing TCR usage in alloreactive responses against HLA-B27 by using: (*a*) CTL clones of well characterized fine specificity; and (*b*) a PCR strategy that enabled us to establish the use of Vβ gene segments and the complete structure of the V-D-J junctions and Jβ segments, starting from very low cell numbers.

Most of the 11 anti-B*2705 clones analyzed were different in panel analyses with site-specific HLA-B27 mutants, but they showed only three reaction patterns with HLA-B27 subtypes (Table I). Thus, they represented a defined subset of the clonal allospecificities generated against B*2705.

The PCR strategy used has some advantages over that of anchored PCR (31). First, it simplifies manipulation, as it avoids restriction enzyme and ligase treatments, as well as transformation and selection of bacterial colonies. Second, the specificity of the Vβ primers allows a fast and reliable assignment of the Vβ segment used, before sequencing. Third, since direct sequencing eliminates the requirement to clone amplified cDNA, the probability of detecting misincorporated nucleotides is very much decreased, as the entire amplified product is sequenced (24). A disadvan-

tage with respect to anchored PCR is that, because of the specificity requirements of the Vβ family-specific primers, only partial Vβ segment sequences are obtained. However, these are sufficient for unambiguous Vβ family assignment and, as shown in this study, for the definition of new Vβ sequences.

Three Vβ13 sequences have been determined and four Vβ13 members were suggested by sequence and Southern blot analysis (26–28). In contrast to all other Vβ segments in this study, the two Vβ13 segments show hitherto undescribed sequences. This indicates that the Vβ13 family includes at least five members and suggests that it may be larger.

The structures of the Vβ genes expressed by the CTL clones examined present three main features: (*a*) limited use of Vβ segments; (*b*) great junctional diversity; and (*c*) wide, but not totally unrestricted, use of Jβ segments.

Amino acid sequence homology among members of different Vβ families ranges from 15 to 60%, with homologies of ~25–35% being most frequent (26, 28). However, virtually all known members of the Vβ3, Vβ12 to Vβ15, and Vβ19 families share >50% amino acid sequence homology. Indeed, merging Vβ13 and Vβ14 with Vβ12 and Vβ3, respectively, has been proposed (29). In contrast with unrelated T cell clones, 7 of the 11 anti-B*2705 CTL clones examined express one member of this group of related families, whereas the remaining four clones express apparently identical Vβ4 segments. This clearly indicates a nonrandom restriction in Vβ segment usage among these clones. This cannot be ascribed to limitations in the repertoire of CTL precursors from a particular individual because the CTL clones were derived from various unrelated donors. It is likely that the restriction is imposed by the stimulator alloantigen, either directly by its structure, or indirectly, through putative peptides that HLA-B27 might present to alloreactive T cells. Hypothetical models predict that hypervariable regions encoded in the V gene segments would interact mainly with residues from the MHC molecule (3). This does not imply a strict correlation between particular V segments and MHC molecules, but it explains that a given alloantigen might show some preference for V segment subsets, as suggested by this study. In addition, it is conceivable that only some of the putative peptides bound to HLA-B27 may be recognized by the clones that we have examined. Such peptides could also be involved in the restricted Vβ segment expression observed, because the last residues of these segments actually are part of the CDR3-equivalent region, predicted to interact with peptide. Preferential use of certain V segments has been repeatedly reported in antigen-specific, self-MHC-restricted responses (32–36), suggesting that particular combinations of MHC plus peptide may bias V segment usage.

The relatively strict limitation of Vβ segments observed among the selected set of clones examined may not apply to the whole anti-HLA-B27 CTL response. Nevertheless, it appears that some correlation could exist between the fine specificity of alloreactive CTL clones and the Vβ segments that they express. The precise terms of such correlation require further definition. For instance, CTL clones within groups B and C share either identical or related Vβ gene segments, but those in group A include both related Vβ13 and Vβ14 segments, and the more distinct Vβ4 (Fig. 2). In their reactivity with site-specific mutants (10), no more similarity was apparent among CTL clones with the same or related Vβ segments than with those of structurally more distant ones. Further characterization of their fine specificity might

reveal new similarities among some of these clones. The contribution of other TCR elements should also be borne in mind.

This is, to our knowledge, the first structural analysis of rearranged TCR genes among human T cell clones in specific responses. Skewed V segment use among alloreactive T cells has also been observed in the mouse (25, 37–39), but very few V gene sequences were reported in these studies (25).

The great variability at the Vβ-Dβ-Jβ junction among the CTL clones examined is not surprising, as much TCR β chain diversity accumulates in this region. This diversity could be correlated with the variability of epitopes recognized by these clones, as detected with site-specific mutants, by assuming that different TCRs bind the same MHC molecule, or the same MHC-peptide complex, in different ways. Alternatively, it could be related to a corresponding multiplicity of bound peptides. Both alternatives are not mutually exclusive, as the predicted surface of the MHC-peptide complex is large enough to allow TCR binding in different registers (3). Subtle similarities are sometimes found in the Vβ-Dβ-Jβ junction among related T cell clones (40, 41). A conserved residue in this area from cytochrome *c*–specific T cell clones is critical for specificity (42). Thus, the fact that five of the seven anti-B*2705 CTL clones in group A (Table I) possess Ala codons at the D-J junction might be significant regarding the fine specificity of these clones.

The observed associations of identical or highly homologous Vβ segments with the same Jβ segments could also reflect similar specificity, as they occur frequently among related T cell clones (40, 41, 43). However, such putative similarity has not been revealed by the fine specificity analysis of these clones. Alternatively, these associations, and the presence of only certain Jβ2 segments among the T cell clones examined, might reflect preferential expression of certain V-J combinations due to selection occurring before antigen stimulation (44). Further analysis is required to establish this point.

In conclusion, the data suggest that T cell clones activated in the alloreactive CTL response against HLA-B27 use a nonrandom subset of TCR Vβ segments, coupled to great variability at the junctional regions. This variability, however, is somewhat limited by the existence of a common structural motif at the D-J junction among some related CTL clones and by the association of the same Jβ segment to identical or particularly related Vβ segments in some instances. Besides the preferential use of certain individual V gene families, emphasized in most previous studies, our results suggest that a more subtle selectivity may operate in recognition of MHC molecules, namely, that of groups of particularly homologous Vβ families. It is tempting to speculate that this additional level of selectivity could also influence intrathymic modulation of the T cell repertoire by self-MHC antigens.

This study suggests that diversity in alloreactive responses can be rationalized by molecular analysis. Further understanding of the extent and rationale of V gene usage in these responses would require examining V gene structures from allospecific T cell clones with a wide range of well characterized fine specificities. It is likely that such correlation will enlighten our concepts on alloreactivity.

Summary

A strategy, based on using Vβ family-specific oligonucleotides, was developed for specific amplification and direct sequencing of human TCR Vβ genes. With this

strategy, it was possible to undertake a structural analysis of TCRs from human T cell clones in specific responses. 12 HLA-B27-specific cytotoxic clones were examined. The results reveal a nonrandom use of Vβ gene diversity in this alloreactive response in that: (*a*) the clones express a restricted number of Vβ segments, including a subset of Vβ families that are significantly more related to one another than to most other Vβ families; (*b*) five of seven clones having a particular reaction pattern with HLA-B27 subtypes possess Alanine at the D-J junction; and (*c*) identical Jβ segments are found associated in several instances with identical or highly homologous Vβ gene segments. In addition, two new Vβ13 members are reported.

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