ALLELIC VARIATIONS IN THE HUMAN T CELL RECEPTOR Vβ6.7 GENE PRODUCTS

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Two cell surface molecular structures participate in the specific recognition of a vast number of different peptide antigens by T lymphocytes. MHC-encoded molecules bind peptide fragments of antigenic material in an antigen binding groove (1). On T cells, TCR, predominantly of the α/β chain variety, specifically recognize the antigenic peptide presented by MHC-encoded molecules (2). MHC-encoded molecules are extremely polymorphic within a species. This variability probably serves to safeguard the species against microorganisms that mutate producing peptide antigens no longer bound by a given allelic product of a MHC molecule, thus threatening to evade immune recognition. Since TCRs may be subject to similar evolutionary pressures, it is of interest to determine whether polymorphic gene products are expressed.

Several RFLP of the various human TCR genes have been described (3–5). In addition, an expressed polymorphic TCR determinant recognized by a mAb, termed OT145 (6), suggested the presence of TCR allotypes. The mAb OT145 distinguishes random individuals on the basis of the percentage of T cells staining with the antibody. In one group of individuals (+/+), a mean of 4.5% of T cells are positive, in a second group (+/−), 2.04% are positive, and in a third group (−/−) positive T cells are difficult to detect by the methods used (6). Ethnic background correlates strongly with these groups, and in family studies the antibody-defined phenotypes are inherited consistent with autosomal co-dominant transmission of two different alleles: the "+" allele recognized by OT145, and the "–" allele not recognized by OT145. Possibilities for the absence of reactivity with OT145 included (*a*) a mutation leading to chain termination or a defective promoter both resulting in a pseudogene, (*b*) a mutation resulting in an altered epitope at the site recognized by OT145, (*c*) germline gene deletion, and (*d*) somatic events resulting in the absence of $OT145^+$ cells. In view of the small percentage of T cells staining with OT145, a V or J region determinant was suspected.

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Materials and Methods

Family Studies. Five RFLPs were used to obtain the α chain haplotypes: Bgl II with a Hind III/Eco RI fragment of the Y14 probe (3′ Cα region probe); Msp I and Taq I, both with a PvuII/Pvu II fragment of Y14 (Cα region probe); Taq I with an Eco RI/Ava I fragment of Y14 (Vα region probe); Msp I and Pvu II both with the VR5 Vα region probe (3). Six RFLPs were used to obtain the β chain haplotypes: Bgl II with a Cβ probe derived from YT35; Hind III and Bam HI with a murine C5V probe; Bam HI with a Vβ probe derived from YT35; Taq I and Pvu II both with the Vβ97 probe (7–9). The γ chain haplotypes were obtained with Pvu II and a Cγ probe as described (5). The γ chain haplotypes in family S have been published elsewhere (5).

Cloning Strategy. The OT cDNA library was prepared by modification of the method of Gubler and Hoffman (10). Poly(A)$^+$ RNA from OT leukemia cells was isolated by the guanidinium/CsCl method and passaged over an oligo(dT) cellulose column. First-strand cDNA synthesis was carried out with avian myeloblastosis virus (AMV) reverse transcriptase on 800 ng poly(A)$^+$ RNA. Second-strand synthesis was carried out with RNase H and *Escherichia coli* DNA polymerase I. Blunt ends were prepared with T4 DNA polymerase. Internal Eco RI sites were protected by Eco RI methylase. Kinased Eco RI linkers (New England Biolabs, Beverly, MA) were then ligated onto the cDNA, and the resulting product was digested with Eco RI to produce Eco RI cohesive ends. The cDNA was separated from free linkers by passage over a Sephadex G-50 column and then directly ligated to Eco RI cut, dephosphorylated λgt10 vector at a molar ratio of 1:2. The ligation products were packaged in vitro by standard techniques using *E. coli* C600 hfl strain as a bacterial host. The OT cDNA library contained 3.4×10^5 plaque-forming units and was screened with a probe consisting of a 140-bp Pvu II–Bgl II fragment derived from the constant region of a human TCR β chain cDNA clone (Jurkat 2) provided by Dr. T. Mak (Hospital for Sick Children, Toronto, Ontario, Canada). Positive plaques from the OT cDNA library were subcloned into Eco RI–digested, phosphatase-treated pIBI 31 vector (International Biotechnologies, Inc., New Haven, CT). The purified plasmid was prepared according to Holms and Quigley (11) with modifications. The sequencing reaction was carried out by the dideoxy chain termination method of Sanger et al. (12), using both T_3 and T_7 universal primers (Stratagene, Inc., La Jolla, CA) and a DNA sequencing kit (Pharmacia/LKB Biotechnology, Inc., Piscataway, NJ).

Polymerase Chain Reaction (PCR). Peripheral blood T cells from a normal OT145 +/+ individual were stimulated with OT145 mAb (1:20 dilution of supernatant) and 10% IL-2 was added twice a week over 2 wk as described (13). The resultant cell line consisted of >95% OT145$^+$ T cells as analyzed by immunofluorescence. RNA was isolated from 5×10^7 cells (yield 30 μg) and first-strand cDNA synthesis was accomplished by adding the C region primer (II) to 10 μM, dNTPs to 1 mM, sodium pyrophosphate to 4 mM, RNAsin 12 U, reverse transcriptase 50 U (Pharmacia Fine Chemicals) in a final reaction volume of 0.02 ml Tris/HCl 50 mM, $MgCl_2$ 10 mM at pH8.3. The reaction was incubated at 43°C for 30 min. One-fourth of this reaction was then used for the PCR reaction with dNTPs (200 μM each), primers I and II at 1 μM each, Taq polymerase 2.5 U, in a 0.1-ml reaction volume with 50 mM KCl, 10 mM Tris HCl, pH 8.3, 1.5 $MgCl_2$, 0.01% (wt/vol) gelatin. The amplified material obtained after 30 cycles of automated PCR hybridized strongly with a Vβ6.7 probe (Ph79) but not with a Vβ8.1 probe by Southern blotting, suggesting that the majority of the amplified material contained Vβ6 gene segments. In other experiments, RNA isolated from 5×10^7 fresh unselected T cells from two OT145 −/− individuals was used as template for first strand cDNA synthesis and PCR amplification of Vβ6 genes as described above. Similar PCR reactions were also performed with 100 ng germline DNA from an OT145 −/− individual as template and with DNA eluted from the 12-kb band of an agarose gel loaded with Bam HI–digested germline DNA from an OT145 −/− individual and using primers III and IV. PCR products were cloned into the polylinker of a sequencing vector (pIBI31) using restriction enzyme sites added to the 5′ ends of the primers used for the PCR as previously described (14). The primer sequences were: I: 5′ CGGAATTCAGGTGTGATCCAATTTC 3′ (sense), II: 5′ CGGTCGACCTCGGGTGGGAAC 3′ (antisense), III: 5′ GCGAATTCACCAGGCTCCTCTTCT 3′ (sense), IV: 5′ CTGAATTCACAGAGATACACGGCC 3′ (antisense).

Southern Blotting. DNA was isolated from EBV-transformed cell lines from each individual by standard methods (5). 10 μg DNA was digested with Bam HI or other restriction enzymes and loaded on a 0.8% agarose gel. The blot was hybridized with a ^{32}P-labeled probe corresponding to the 5' 108 nucleotides of the OT-1 clone (Eco RI-Bst XI fragment). After hybridization, the blot was washed twice in 0.1 × SSC, 0.1% SDS at 65°C for 30 min.

Results and Discussion

Initially we performed family studies with several RFLPs of the α, β, and γ chain TCR genes. Haplotypes for these genes could be assigned as previously described (3, 5, 7) and summarized in Fig. 1. TCR gene haplotypes were compared with HLA haplotypes, obtained by routine serologic typing of the family members and to the OT145-defined phenotype. In family A, only the β chain haplotypes correlate with OT145 phenotypes. For instance, the maternal β chain "D" haplotype matches with the "–" allele. This is consistent with an OT145 determinant encoded by a β chain gene. In family S, four siblings are HLA identical (S1, S2, S3, S6); yet all three phenotypes defined by OT145 are represented among these individuals, suggesting that HLA type does not influence expression of the OT145 TCR in this family. In murine systems, MHC-encoded products, probably in association with certain autoantigens, may determine TCR V gene usage by causing clonal deletion of T cells expressing a TCR with the autoreactive V gene product. Thus for example, IE$^+$ mouse strains have clonally deleted Vβ17a expressing T cells (15, 16). The variations in the percentages of OT145$^+$ cells are apparently due to a different mechanism.

To determine which gene encodes the OT145 polymorphic determinant, a cDNA library was constructed from the OT leukemia cells that had served as the immunogen to produce the mAb (6). The library was screened with a β chain probe containing C region sequences. A positive clone (OT-1) was selected for further study. It contained 252 nucleotides, of which 81 were in the C region, 65 in the D-J region, and 106 in the V region (Fig. 2). Using OT-1 as a probe, another clone, OT-2 (196 nucleotides), was obtained, which contained only V region sequences, partially overlapping with OT-1 (Fig. 2). From these sequences, it is apparent that the OT leukemic T cell has a functional rearrangement of Vβ6.7, Dβ1.1, and Jβ1.3 and uses the Cβ1 gene for its TCR β chain. The Vβ6.7 sequences of OT-1 and OT-2 are identical to

Family A	F	M	S1	S2	S3	S4	S5
OT145 phenotype:	+/+	+/-	+/+	+/-	+/+	+/-	+/-
OT145+ T cells (%):	3.3	2.8	3.7	2.7	3.3	2.6	1.7
	3.8		3.4	2.8	3.6	2.7	1.8
HLA	ab	cd	bc	b c/d	bd	bd	ad
TCR α-chain	ab	cd	bd	bc	ac	ac	bd
TCR β-chain	ab	cD	ac	aD	ac	aD	bD

Family S	F	M	S1	S2	S3	S4	S5	S6
OT145 phenotype	+/-	+/-	+/+	+/-	-/-	-/-	+/-	+/-
OT145+ T cells (%):	2.2	2.3	3.5	1.7	<0.1	<0.1	1.2	1.8
	2.3		3.5	1.9	<0.1	<0.1	1.2	
			3.0					
HLA	ab	cd	bc	bc	bc	bd	ac	bc
TCR α-chain	ab	cd	bd	ac	bc	bd	bd	bd
TCR β-chain	Ab	Cd	Ad	bC	AC	AC	bC	bC
TCR γ-chain	ab	cd	ac	ac	ac	ac	bc	bd

FIGURE 1. Results of RFLP typing of two families are summarized and compared with the OT145 defined phenotypes and HLA haplotypes obtained by routine serologic typing. By convention, "a" and "b" denote the paternal, and "c" and "d" the maternal haplotypes. The capitalized haplotypes indicate matching with the OT145 defined "–" allele. Individuals with <0.5% OT145$^+$ T cells are typed as –/–, those with up to 3.0% OT145$^+$ T cells as +/–, and those with over 3.0% OT145$^+$ as +/+ (6).

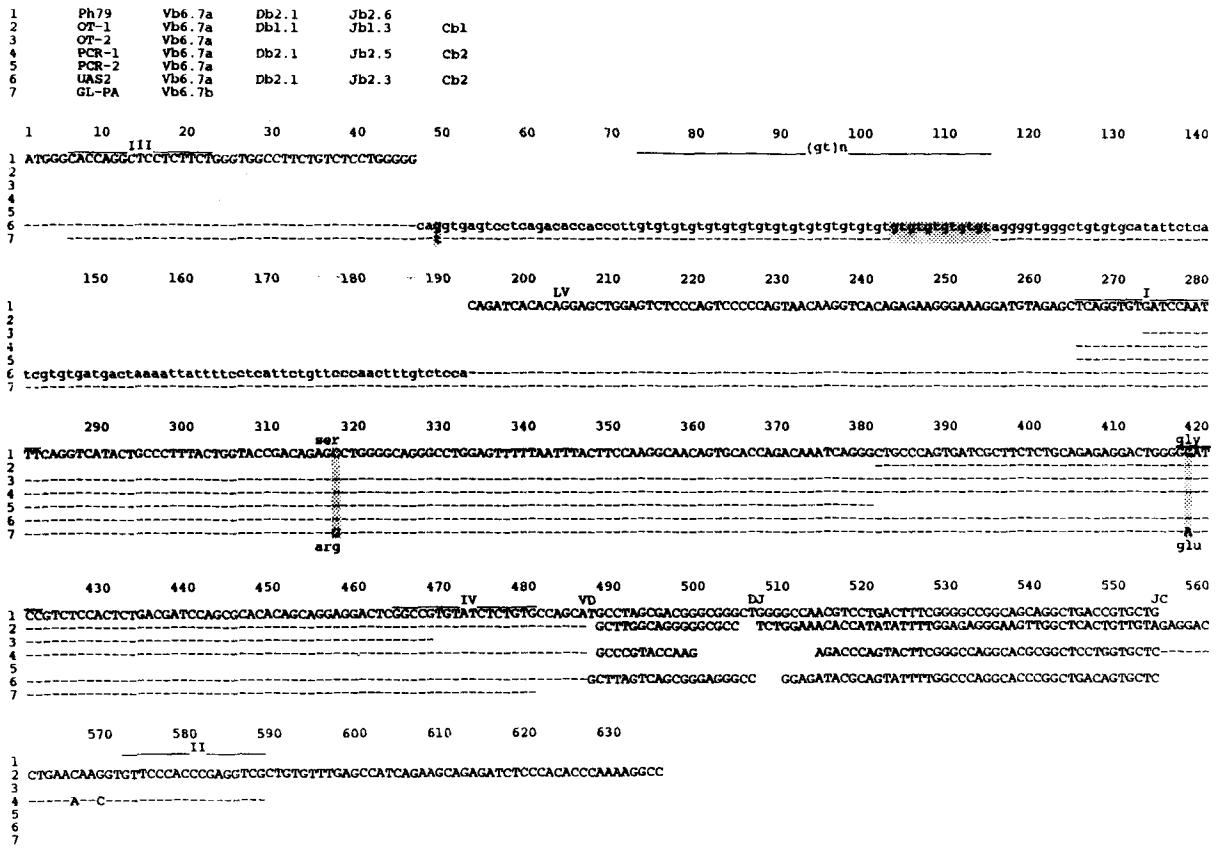

FIGURE 2. Comparison of seven TCR sequences. The names and the composition of the sequences are summarized above. Six sequences have identical Vβ6.7 regions, denoted Vβ6.7a. The last sequence, GL-PA, has three nucleotide changes, of which two are in the exon, a 12-bp deletion in the intron, and is denoted Vβ6.7b. Differences between Vβ6.7a and Vβ6.7b sequences are shaded. Ph79 (17) and UA-S2 (18) have previously been published. OT-1 and OT-2 are cDNA clones obtained from a cDNA library derived from the OT leukemia cells. PCR-clone 1 and PCR-clone 2 were obtained from PCR amplification of cDNA derived from RNA of a polyclonal OT145$^+$ T cell line. GL-PA is a clone derived from the germline of an OT145 -/- individual by PCR amplification. The primers used in the PCR for PCR-clones 1 and 2 were I and II, and for the clone GL-PA, they were primers III and IV. The primer sequences and the Bam HI site (position 417–422) are overligned. The limits of the leader (L), variable (V), diversity (D), joining (J), and constant (C) regions are indicated.

three reported Vβ6.7 sequences (17, 18), of which one represents the Vβ of an autoreactive T cell clone, UA-S2. This clone is positive with the mAb OT145 (Schlesier, M., personal communication).

Nine different V gene sequences have been classified as members of the Vβ6 gene subfamily (19). Some of these sequences differ by only a few nucleotides and could be allelic products of the same gene. To determine which products of these genes are recognized by OT145, $OT145^+$ cells were expanded from fresh T cells by adding the mAb with IL-2 to the cultures (13). After 3 wk, a cell line was obtained containing 95% OT145 T cells as assayed by immunofluorescence. Cytoplasmic RNA was isolated and a first strand cDNA copy was synthesized with reverse transcriptase and then amplified using the PCR. The primers used for the PCR (primers I and II, Fig. 2) represent a downstream C region sequence and an upstream Vβ6 sequence that is common to all known Vβ6 genes. Thus it was expected that any Vβ6 genes transcribed in this cell line would be amplified. Seven clones that were positive by hybridization with a Vβ6 probe were isolated. Five of these clones have been sequenced; four have Vβ6.7 sequences (Fig. 2), and one has a Vβ6.3 sequence (Fig. 2; data not shown). OT-1, PCR-clone 1, and UAS2, all derived from $OT145^+$ T cells, contain different D and J regions (Fig. 2), suggesting that these regions do not encode the OT145 determinant. Thus the combined data suggest that Vβ6.7 is the predominant (although possibly not the only) gene encoding the OT145 determinant.

To further confirm these results, Southern blots of genomic DNA from individuals typed with the OT145 antibody were performed. The OT-1 clone was digested with Bst XI, which cuts this clone in two fragments at the VD junction. The resultant 108-bp V region probe hybridizes to the downstream portion of Vβ6.7 genes, and crosshybridizes only minimally with other Vβ6 genes under high stringency conditions. Several restriction enzymes (Pvu II, Hind III, and Bam HI) revealed polymorphic sites in the vicinity of the Vβ6.7 gene using this probe. Bam HI, which has a site within the Vβ6.7 gene (Fig. 2) was chosen for further study. The OT-1-derived V region probe hybridizes predominantly to sequences downstream of this Bam HI site. From individuals typed as +/+ with the OT145 mAb a 4-kb band was consistently obtained, while −/− individuals had a 12-kb band and heterozygous individuals had both bands (Fig. 3). This analysis was extended to 36 unrelated individuals with similar results (Table I). Including the members of family S, 42 individuals were screened by Southern blotting with only two individuals in which the RFLP analysis did not match the phenotype obtained with OT145. One such case is sibling 1 in family S where the antibody typing suggested a +/+ genotype, although one value of 3.0% $OT145^+$ T cells was borderline (Fig. 1). The Bam HI RFLP, however, typed this individual as heterozygous (4- and 12-kb bands). TCR β chain haplotypes matched the OT145 phenotypes in all but sibling 1 in this family. Such cases could be explained by variations in the percentages of peripheral blood $OT145^+$ T cells due to extraneous influences such as an ongoing immune response.

Fig. 3 also demonstrates a second allelic system represented by 4.7-kb and 5.8-kb bands. Presumably these represent two alleles of another Vβ6 gene that is closely related to Vβ6.7 and crosshybridizes under the conditions used. This allelic system represents a fortuitous control since it is not related to the OT145-defined phenotypes (Table I). Homozygotes for the 5.8-kb band are most frequent, followed by heterozygotes (4.7- and 5.8-kb bands), and only two individuals were found to be

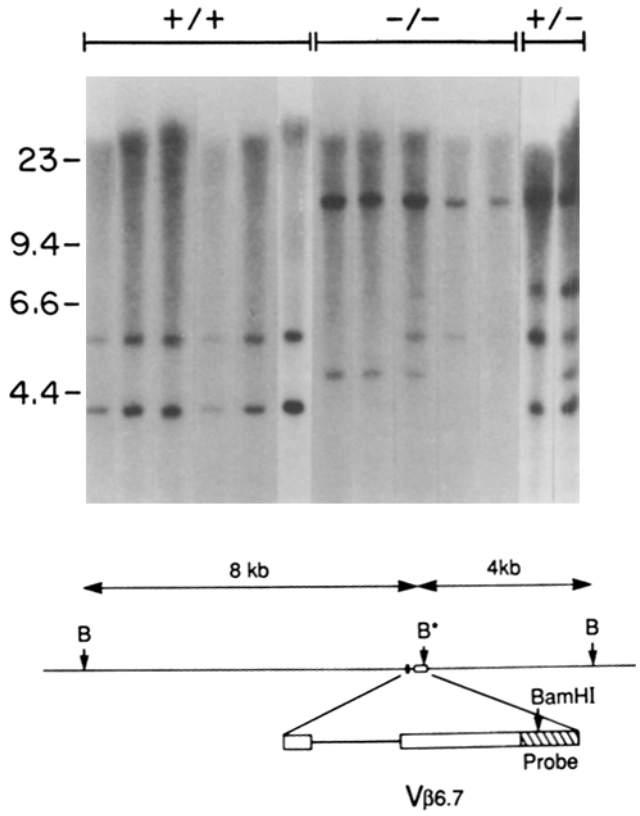

FIGURE 3. Southern blot of Bam HI-digested germline DNA from unrelated individuals with different OT145 defined phenotypes (+/+, -/-, or +/-). B, Bam HI site; B*, polymorphic Bam HI site responsible for 4-/12-kb RFLP.

homozygotes for the 4.7-kb band. The distribution of these bands is similar among OT145 +/+ and -/- individuals. In contrast the 12-/4-kb Bam HI RFLP matched closely with the OT145 phenotype (Table I). These data are also consistent with results demonstrating that RFLP-defined alleles of different genes within a TCR haplotype, such as the β chain locus, are not necessarily concordant in population studies (3, 7).

Southern blots of DNA digested with Bam HI as well as other enzymes showed equal numbers of bands in OT145 +/+ and -/- individuals. Thus a deletion of the Vβ6.7 gene cannot explain the negative staining with OT145. A mutation resulting in a nonexpressed pseudogene, as described in the mouse (20), remained a possibility. Therefore the Vβ6.7 gene was amplified by PCR directly from the germline

TABLE I

Distribution of Vβ6 Alleles in OT145-defined Groups

OT145 phenotype	*n*	Bam HI RFLPs					
		12/12 kb	12/4 kb	4/4 kb	5.8/5.8 kb	5.8/4.7 kb	4.7/4.7 kb
OT145 +/+	17	0/17	1/17	16/17	12/17	5/17	0/17
OT145 +/-	4	0/4	4/4	0/4	1/4	2/4	1/4
OT145 -/-	15	15/15	0/15	0/15	11/15	3/15	1/15

DNA of an OT145 −/− individual. The primers used (Fig. 2) represent a 3′ region of the Vβ6.7 gene (primer IV) and a 5′ region in the leader sequence (primer III). Three independent clones were sequenced and revealed identical nucleotide sequences (GL-PA in Fig. 2). We termed this sequence "Vβ6.7b," in contrast to the original sequence, now termed "Vβ6.7a." In Vβ6.7b, a G to A substitution results in abolition of the internal Bam HI restriction enzyme site (Fig. 2), which is the most probable explanation for the Bam HI 4-/12-kb RFLP shown in Fig. 3. In +/+ individuals, probes 5′ to this site hybridize to an 8- or a 12.5-kb fragment (data not shown). Together, the 4- and 8-kb fragments represent the 12-kb band of the "−" allele. The Bam HI site defining the 5′ margin of the 12- and 8-kb fragments appears itself to be polymorphic. To confirm this interpretation, the 12-kb band (derived from a "−/−" individual) was excised from an agarose gel and the eluted DNA was used as a template for PCR with primers I and IV. The resultant clone had a sequence identical to Vβ6.7b.

To determine whether Vβ6.7b is a functional gene from which mRNA is transcribed, cytoplasmic RNA was isolated from freshly prepared T cells of a "−/−" individual. cDNA was synthesized with reverse transcriptase and PCR amplification was performed with primers I and II to amplify any transcribed Vβ6 genes. 10 independent clones were sequenced: five had Vβ6.1 sequences, two had Vβ6.7b sequences, and one each had Vβ6.2, Vβ6.3, and Vβ6.4 sequences. Thus, in this individual Vβ6.7b represented the second most common Vβ6 gene transcribed. The two Vβ6.7b cDNA sequences and the Vβ6.7b germline sequence (GL-PA, Fig. 2) demonstrate no mutation that might result in premature chain termination. In view of the frequency of Vβ6.7b cDNA clones, Vβ6.7b appears to be capable of producing a functional message.

Two nucleotide differences between Vβ6.7a and Vβ6.7b result in nonconservative amino acid substitutions while the third is located in the intron (Fig. 2). In the V region encoded by Vβ6.7b residue 38 is changed from a serine to an asparagine and residue 72 is changed from a glycine to a glutamic acid. These residues both represent putative framework residues with high residue variability and high exposure to solvent (21, 22). This is consistent with the fact that both sites contain charged amino acids in the Vβ6.7b allele, which are unlikely to be buried residues. Position 38 is thought to be located at the margin of a β-pleated sheet and facing the α chain V domain (22). Position 72, however, represents the hairpin bend between β strands E and D and is probably located close to or at the antigen/MHC binding site (22). Therefore, substitution of a glutamic acid for a glycine at this site may influence Ag/MHC binding. Whether binding of the OT145 mAb is dependent on position 38, position 72, or both is not evident from our data. However, the epitope recognized by the mAb represents a true allotype of a TCR V region. OT145 is one of only four similar mAbs to different human TCR V regions that we have studied. If one-fourth of such antibodies to TCR V region determinants detect polymorphic determinants, allelic polymorphism of TCR V genes may be a frequent phenomenon. In support of this, evidence for polymorphic Vβ1 products has also been obtained (23).

MHC-encoded molecules exhibit polymorphisms with numerous alleles. In comparison, the description of two alleles of Vβ6.7 may seem modest. However, a high frequency of genetic recombination has been observed in TCR gene complexes (3).

In addition, our data do not exclude further allelic products of Vβ6.7. Microsatellite regions, such as the (GT)n repeat found in the Vβ6.7 intron, have been found to be associated with abundant polymorphism, 4–11 different alleles in one study (24, 25). These interesting (GT)n repeats are identical to zDNA forming sequences (26, 27), may be associated with hot spots for recombination (28–30), or may participate in gene regulation (31, 32). The Vβ6.7b (GL-PA) sequence has a deletion of 12 nucleotides from this region: i.e., $(gt)_{15}$ instead of the $(gt)_{21}$ observed in the Vβ6.7a sequence of UA-S2 (Fig. 2). This is consistent with observations on the polymorphic nature of these (gt)n repeat sequences (24, 25).

In view of the absence of somatic mutation in the TCR V genes, TCR V gene allelic products may play a role in increasing the repertoire for the species and for heterozygous individuals. In addition, the finding of TCR V region polymorphisms has two important predicted consequences. First, there should be immune responses under control of TCR V genes, an example of which has already been described (33). Second, disease associations with TCR V genes would be expected (34), possibly due to differences in key residues in the TCR antigen/MHC binding site.

Summary

Polymorphisms of human TCR gene products have been suggested by the description of a mAb, OT145, that identifies a subset of TCRs in some individuals but not in others (6). Here we demonstrate that this mAb detects a TCR allotype of the Vβ6.7 gene. Two allelic products of this V gene differ by two nonconservative amino acid substitutions. The mAb OT145 appears to react with Vβ6.7a gene products ("+" allele), but not with Vβ6.7b gene products ("–" allele). This represents the first direct demonstration that TCR V gene allotypes exist and provides a possible explanation for immune responses under the control of TCR V genes and for disease associations with TCR V genes.

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