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Heimo Breiteneder · Otto Scheiner · Roswitha Hajek
Wolfgang Hulla · Robert Hüttinger · Gottfried Fischer
Dietrich Kraft · Christof Ebner

Diversity of TCRAV and TCRBV sequences used by human T-cell clones specific for a minimal epitope of Bet v 1, the major birch pollen allergen

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Abstract T-cell clones (TCC) were raised from the peripheral blood of patients suffering from tree pollen allergy. All TCC were restricted by HLA-DR molecules. In order to investigate possible intervention targets in Type I allergic diseases, we examined T-cell receptor (TCR) α and β chain nucleotide sequences of five allergen-reactive human CD4⁺ TCC specific for a C-terminal epitope (BV 144) of Bet v 1, the major birch pollen allergen. Proliferation assays using synthetic peptides revealed the 10-mer LRAVESYLLA as minimal epitope for three TCC; two TCC also displayed reactivity with the nonapeptide LRAVESYLL. Two TCC expressed *TCRBV2S3*, all other BV144-specific TCC used diverse *TCRAV* and *TCRBV* gene segments. Moreover, the junctional regions encoding the third complementary determining regions (CDR3) of the TCR showed a striking heterogeneity in length and amino acid composition. Nevertheless, all TCC showed an arginine residue in the N-terminal region of their *TCRBV* CDR3 loops. Therefore, therapeutic strategies aimed at the clonal deletion of allergen-specific T-cell clones, providing help for IgE synthesis, will not be feasible. Our results cast a doubt on the theory that the CDR3 exclusively provides the primary contact with the peptide bound in the major histocompatibility (MHC) groove, and suggest additional interaction with MHC class II.

The nucleotide sequence data reported in this paper have been submitted to the EMBL nucleotide sequence database and have been assigned the accession numbers Z47366–Z47376

H. Breiteneder · O. Scheiner · R. Hajek · W. Hulla · R. Hüttinger · D. Kraft · C. Ebner (✉)
Institute of General and Experimental Pathology, AKH-EBO,
University of Vienna, Währinger Gürtel 18–20, A-1090 Vienna,
Austria

G. Fischer
Institute of Blood Group Serology, AKH, Währinger Gürtel 18–20,
A-1090 Vienna, Austria

Introduction

The T-cell antigen receptor (TCR) of mature T lymphocytes recognizes antigens in the form of short peptide fragments bound to major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APC; Schwartz 1985). Over 90% of peripheral blood T lymphocytes express the TCR in the form of a disulfide-linked heterodimer composed of a TCR α and TCR β chain (Haskins et al. 1983; Meuer et al. 1983). Each chain consists of a variable region which confers the specificity of the TCR for the peptide/MHC-complex, and a constant region that attaches this variable region to the cell surface. During thymic maturation, unique variable region genes are created by recombination of germ-line encoded variable (V), diversity (D, for the β chain only) and joining (J) segments. Combinatorial diversity is generated by the joining of unique combinations of these segments as well as the random association of any α chain with any β chain. Further diversity of the variable domain is contributed by the loss (through nucleotide “nibbling”) or template-independent addition of N-region nucleotides at V-(D)-J junctions (Davis and Bjorkman 1988). After rearrangement, thymic selection leads to abortion or rescue from abortion of developing lymphocytes (von Boehmer 1992). Unlike in immunoglobulin (Ig)-rearrangement, somatic hypermutation does not seem to play a significant role in generating TCR diversity (Ikuta et al. 1985). However, the structure of the TCR is similar to that of Ig, containing three or four hypervariable loops that correspond to the complementarity-determining regions [(CDR) (Clothia et al. 1988)]. The CDR1 and CDR2 loops of the TCR are encoded by germ-line V-segment sequences and are believed to interact with the α helices forming the side walls of the binding groove of the MHC molecules. In contrast, the CDR3 loops are encoded by the V-(D)-J junctional regions and are believed to provide the primary contact with the peptide bound in the MHC binding groove (Davis and Bjorkman 1988; Clothia et al. 1988).

The precise involvement of specific TCR gene products in the recognition of particular MHC molecules or peptide/MHC complexes is still unclear. The same holds true for the extent of diversity of TCR in TCC recognizing an identical peptide presented by the same or different MHC molecules. Previous studies have frequently shown a limited usage of germline-encoded *V* gene segments – but ignoring CDR3 forming hypervariable regions – in TCR recognizing defined peptide/MHC complexes, which in some cases was correlated with MHC restriction (Moss et al. 1992; Casanova and Maryanski 1993).

In this work, we examined the TCR usage of human CD4⁺ T-cell clones specific for a minimal T-cell epitope of the major birch pollen allergen, Bet v 1 (residues 144–154). This epitope can be considered as a major epitope of Bet v 1 (Ebner et al. 1993a, b, c). The aim of the study was to characterize TCR variable α and variable β regions of TCC established from different allergic individuals, but specific for the same T-cell epitope. A detailed characterization of TCC recognizing important allergen-derived T-cell epitopes is a prerequisite for the development of therapy measures aimed at the deletion of specific TCC. Sequence data of the junctional regions are presented.

Materials and methods

Birch pollen-allergic volunteers

The TCC studied in this paper were obtained from five birch pollen-allergic patients (1 female, 4 males). They all showed typical case histories, positive skin tests, and their sera displayed IgE-binding to Bet v 1, the major birch pollen allergen.

Bet v 1 and Bet v 1-peptides

Recombinant Bet v 1 was expressed in *Escherichia coli* as a non-fusion protein and purified as described (Ferreira et al. 1993). A panel of 75 overlapping peptides was synthesized according to the Bet v 1 sequence by Cambridge Research Biochemicals Limited (Cambridge, UK) and used for epitope mapping of TCC as described (Ebner et al. 1993a). Peptides for analysis of the minimal epitope (Sandoz, Vienna, Austria) were >95% pure according to high pressure liquid chromatography (HPLC) analysis. Their sequences were proved by sequencing. In order to make direct comparison possible, in all experiments peptide concentrations of 0.75 μ M were applied.

Allergen-specific T-cell clones

Short term T-cell lines (TCL) were generated by incubating peripheral blood mononuclear cells (PBMC) of birch pollen-allergic volunteers with Bet v 1 (10 μ g/ml; Ebner et al. 1993a). T-cell blasts from Bet v 1-specific TCL were seeded in limiting dilution (0.3 cells/well) in 96-well round-bottom plates (Nuncclone, Nunc, Denmark) in the presence of 10^5 -irradiated (5000 rad) allogeneic PBMC as “feeder cells”, 1% PHA v/v (Gibco, Grand Island, NY) and rIL-2 (4 units/well) in the above-described medium. Growing microcultures were then expanded at weekly intervals with fresh feeder cells and rIL-2. The specificity of TCC was assessed as previously described. Briefly, $2-5 \times 10^4$ T-cell blasts were incubated in triplicate cultures in 96-well plates in the presence of 10^5 autologous irradiated PBMC, with whole birch pollen extract, purified nBet v 1, rBet v 1 and overlapping Bet v 1 peptides. Microtiter plates were then incubated for 48 h at 37 °C, 5% CO₂, in a

humidified atmosphere. After being pulsed for 16 h with [³H] TdR, cells were harvested and the incorporated radioactivity was measured by scintillation counting. When the stimulation index (ratio between cpm obtained in cultures with TCC plus autologous irradiated PBMC plus antigen and cpm obtained in cultures containing TCC and PBMC alone) exceeded 10, responses were considered positive. Epitope mapping was performed as described (Ebner et al. 1993a).

Analysis of TCC phenotype

The phenotype of the TCC was analyzed by flow cytometry using a FACScan (Becton-Dickinson, Mountain View, CA). For analyses the FITC-labeled mAbs anti-Leu 4/CD3 anti-Leu 3a/CD4, anti-Leu 2a/CD8, anti-TCR α/β WT 31, and anti-TCR γ/δ I (Becton-Dickinson) were used as described (Ebner et al. 1993a).

HLA-typing and restriction

HLA typing of patients was performed according to methods described (Fischer et al. 1992). In order to identify HLA molecules involved in antigen presentation, mouse monoclonal antibodies against human MHC class II determinants L 243 [anti-HLA-DR (IgG2a)], Leu 10 [anti-HLA-DQ (IgG1)], and B7/21 [anti-HLA-DP (IgG1)] were purchased from Becton-Dickinson. Proliferation assays were performed in 96-well plates as described [O’Hehir et al. 1988]. TCC (2×10^4 cells/ml) were cultured with autologous irradiated PBMC (5×10^4 cells/ml) in the presence of rBet v 1 (5 μ g/ml). The antibodies directed against framework antigens of HLA-D-region molecules were used in a final concentrations of 25, 10, and 5 μ g/ml. Proliferation assays revealing blocking of more than 50% of antigen-dependent proliferation were considered positive. Moreover, proliferation assays using a panel of matched and mismatched PBMC of HLA-typed individuals were performed in order to reveal restriction elements.

First-strand cDNA synthesis and polymerase chain reaction (PCR) amplification

Total RNA was prepared from 1×10^6 cells according to a single-step guanidinium-isothiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). Single-stranded cDNA synthesis was carried out on 1.2–1.8 μ g of total RNA with oligo(dT) (12–18), and avian myeloblastosis virus reverse transcriptase. PCR was performed by amplification of TCR α and β cDNA with oligonucleotide primers complementary to TCR V and C region sequences (Panzara et al. 1992; Genevée et al. 1992) at a final concentration of 0.25 μ M. Reactions were incubated in a DNA thermal cycler (Perkin Elmer Corporation, Norwalk, CT). Reaction mixtures were subjected to the following conditions: denaturation at 93 °C for 1 min, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min with a further 5 s extension after each cycle.

Cloning and sequence analysis of TCR α and β chains

The PCR reaction products were cloned into the TA cloning vector pCRII (Invitrogen Corporation, San Diego, CA) and sequenced by the enzymatic chain termination method (Sanger et al. 1977), with Sequenase Version 2.0 (US Biochemicals, Cleveland, OH) in the presence of α -³⁵S-labeled dATP (Amersham, Little Chalfont, UK). For each of the TCR α and β chains studied, three independent pCRII subclones were sequenced to identify possible sequence errors caused by nucleotide misincorporation by *Taq* polymerase. Sequences were compared with gene databank entries (Genbank, Los Alamos, NM; EMBL, Heidelberg, Germany) and to available published TCR *J* segments (Klein et al. 1987; Yoshikai et al. 1986; Kimura et al. 1987; Roman-Roman et al. 1991; Concannon et al. 1986; Kimura et al. 1986; Toyonaga et al. 1985).

[illegible]

T-cell clones and epitope mapping

DQB*01,02. All clones revealed the helper cell phenotype (CD3⁺, CD4⁺) and expressed the TCR $\alpha\beta$. For these clones, using peptides overlapping for two residues and spanning the entire amino acid sequence of Bet v 1, an epitope near the C-terminus of the protein could be mapped. Using the antibody L243, which recognizes a determinant common to all DR molecules, proliferation in response to stimulation with Bet v 1 could be blocked in all five TCC in a dose-dependent manner, indicating restriction by HLA-DR molecules. Antigen presentation experiments using matched and mismatched APC revealed different restriction elements for TCC. In spite of the fact that all donors

T-cell clone	HLA-DRβ	Gene segments					
		<i>TCRAV</i>	<i>TCRAJ</i>	<i>TCRBV</i>	<i>TCRBD</i>	<i>TCRBJ</i>	<i>TCRBC</i>
XPE15	DRB1*07, DRB1*07, DRB4	<i>AV11SI</i> ^a	<i>AJ6</i> = <i>AJ15S3</i> ^b = <i>JαT</i> ^a	<i>BV24S1</i> ^c	<i>BD1</i>	<i>BJ2S2</i>	<i>BC2</i>
XPZ10	DRB1*07, DRB1*15, DRB4, DRB5	<i>AV2S3</i> ^c	<i>AJ44</i> = <i>AJ17S7</i> ^b = <i>JαAA17</i> ^d	<i>BV17S1</i> ^e	<i>BD1</i>	<i>BJ2S3</i>	<i>BC2</i>
XPD25	DRB1*07, DRB1*07, DRB4	<i>AV5SI</i> ^a	<i>AJ21</i> = <i>AJ16S7</i> ^b = <i>JαAC24</i> ^d	<i>BV2S3</i> ^c	<i>BD1</i>	<i>BJ1S5</i>	<i>BC1</i>
XPR04	DRB1*07, DRB1*16, DRB4, DRB5	<i>AV8S1</i> ^a	<i>AJ15</i> = <i>AJ9S5</i> ^b = <i>JαS</i> ^a	<i>BV15S1</i> ^f	<i>BD1</i>	<i>BJ2S5</i>	<i>BC2</i>
XPF10	DRB1*07, DRB1*15, DRB4, DRB5	<i>AV8S2</i> ^a	<i>AJ11</i> = <i>AJ9S15</i> ^b = <i>JαAD17</i> ^d	<i>BV2S3</i> ^c	<i>BD2</i>	<i>BJ2S4</i>	<i>BC2</i>
XPF10		<i>AV9SI</i> ^a	<i>AJ53</i> = <i>AJ9S3</i> ^b = <i>JαK</i> ^c				

A

T cell clone	TCRA gene segments	TCRAV	CDR3	TCRAJ	TCRAC
XPE15	AV11S1J6C	GAGGCAGATGCTGCTTTACTACTGTCT E A D A A V Y Y C A	GTGGGGCTGCTCAGGAGGAGCTACATACCTACA V G A A S G G S Y I P T	TTTGGAGAGGAGCTAGCTTTATGTTGATCCG F G R G T S L I V R P	TATATCCAG Y I Q
XPZ10	AV2S3J46C	CTCAGTCAATTCAGCCACTTCTCTGTG L S D S A T Y L C V	GTGAACACTGCGAGTAACCTCACC V N T A S X L E	TTTGGAGCTGGACAGAGCTTACGTCAGCTC F G F G T R L Q V R L	GATATCCAG D I Q
XPD25	AV5S1J21C	AATGCAGACTCAGCTACCTACTCTGTCT N A D S A T Y L C A	CTAGTGGGAACTTCAACAAATTTTAC L V G N F N X P Y	TTTGGATCTGGGACCAACTCAATGTAACCA P G S G T K L N Y X P	AATATCCAG N I Q
XPR04	AV8S1J15C	CCTGAAGACTCGGCTCTACTTCTGTGCA F E D S A V Y F C A	GCAAGTAACGGCCAGCAGGAATGCTCTGATC A S N G Q A G T A L I	TTTGGAGAGGAGCACTTATCACTGAGTCC F G R G T T L S V S S	AATATCCAG N I Q
XPF10	AV8S2J11C	CCTGGAGACTCAGCTGTCTACTTTTGCA F G D S A V Y F C A	GASATGATGAATTCAGGATACAGCACTCTCACC E M H N S G Y S T L F	TTTGGAGAGGAGCTATGTTCTAGTCTCTCA F G R G T M L L V S P	GATATCCAG D I Q
XPF10	AV9S1J53C	GAGGAAGACTCAGCCATGTAATCTGTCC E S D S A H Y Y C A	TCFAAGAGTAGTGAGGTAGCACTAATAACTGACA S K S S G G S N Y X L F	TTTGGAGAGGAGCTCTCTTAAGCTGAATCA F G R G T L L L T V N P	AATATCCAG N I Q

B

T cell clone	TCRB gene segments	TCRBV	TCRBD	CDR3	TCRBJ	TCRBC
XP215	BV24S1D1J2S2C2	GCCATGTACCTGTCTGCCACTAGC A M Y L C A T S	AGTCGGCAACCCACCGGAGCCTCTT S R A A N T G E L F	TTTGGAGAGGCTTACGCTGACGCTACTG E G P G S R L T V L	GAGGACCTG E D L	
XP210	BV17S1D1J2S3C2	GCTTCTATCTCTGTGCCAGTAGT A F Y L C A S S	AGGATCCAGATACGAGTAT R D P D T Q Y F	TTTGGCCAGGCACTCCGCTGACAGTCTC F G P G T R L L V L	GAGGACCTG E D L	
XP225	BV2S3D1J1S5C1	AGCTTCTACATCTCAGTCTAGA S F Y I C S A R	GGAATGCGGAGACCGCTTAATAAGCAATCAGCCCTCAGCAT G N G G Q G L I S N Q P D H F	TTTGGTATGGGACTCGACTTCATCTTA F G D G T R L S L I	GAGGACCTG E D L	
XP204	BV15S1D1J2S5C2	GCTCTTACTTCTGTGCCACTAGT A L Y F C A T S	GTCACAGGCGGAGGAGACCTCAGTAG V T R G R K T Q Y F	TTTGGCCAGGCACTCCGCTTCTGAGTCTC F G P G T R L L V L	GAGGACCTG E D L	
XP210	BV2S3D2J2S4C2	AGCTTCTACATCTCAGTCTAGA S F Y I C S A R	GAGGAGAGATTCAGTAG E G Q I Q Y F	TTTGGCCAGGCACTCCGCTTCTGAGTCTC F G P G T R L S V L	GAGGACCTG E D L	

revealed the *DRB1**07 haplotype, only in one case (XPR04) T-cell reactivity with Bet v 1 was restricted by this HLA molecule. XPF10 recognized BV144 only in association with APC expressing *DRB1**15. Three TCC displayed degenerate recognition of BV144 they also recognized Bet v 1 in the context of a panel of nonheterologous APC (not shown).

Minimal epitope

Truncation of the Bet v 1 residues 144–156 peptide revealed decreased immunogenicity when lacking the N-terminal leucine (Bet v 1 position 144). Proliferation assays using a peptide deficient of the N-terminal arginine (Bet v 1 position 145) showed non-reactivity in all TCC investigated, indicating a particular importance of this positively charged amino acid for either peptide-MHC or peptide-TCR recognition. A peptide displaying leucine at position 144 (from now on referred to as BV144) could be reduced from the C-terminal end down to 10 amino acids in length (144–153), still inducing proliferation in every TCC investigated. TCC XPR04 and XPD25 even displayed strong reactivity with the 9-mer LRAVESYLL (Fig. 1). Within this peptide a five-amino-acid motif (RAVES) characteristic for T-cell epitopes could be observed (Rothbart and Taylor 1988).

Epitope-specific TCCs use different TCR V and J genes

Sequence analyses of TCC reactive with BV144 revealed the usage of *TCRBV2S3* in two (XPD25 and XPF10) of the five TCC (Table 1). Each of the other three TCC (XPE15, XPZ10, and XPR04) expressed a different *TCRBV* family

Fig. 2 A TCR α and B TCR β cDNA junctional nucleotide sequences of independent BV144-specific TCC. The deduced amino acid sequence is shown under each corresponding nucleotide sequence. V gene segments were classified according to family designations outlined by Wilson and co-workers (1988). *TCRAJ* germline sequences were assigned as described by Koop and co-workers [(1994) (*underline*)]. In the case of TCC XPR04 the *AJ* sequence found differed from the reported *AJ15* sequence (Koop et al. 1994) in the two codons following the conserved FGXGT motif (i.e., ACC TTA instead of CAC CTA). However, it was identical to the reported J α S (Yoshikai et al. 1986) and AJ9S5 (Moss et al. 1993) sequences. *TCRBD*, *BJ*, and *BC* elements were assigned according to Toyonaga and co-workers (1985). For each clone, only the last 7–10 V gene residues are shown, followed by the presumed *Ig*-like loops (CDR3) defined according to Clothia and co-workers (1988) and by the first three residues of the C region

member (Table 1). Two of the TCC expressed the closely related *TCRAV8S1* and *AV8S2* gene segments (XPR04 and XPF10, respectively). All other *TCRAV* gene segments were drawn from different families.

TCRAJ and *TCRBJ* segment usage was highly diverse. Each TCC used an *AJ* and *BJ* segment different from the ones found in the other TCC (Table 1). Therefore, no particular selection of *AJ* and *BJ* sequences could be observed. Two functional α chains were detected for clone XPF10, indicating that there were two discrete TCR expressed by this TCC. It is not clear which α and β chain-pair constitute the TCR that reacts with the particular peptide/MHC II complex (Padovan et al. 1993). A second β chain was not detected for XPF10. It is therefore unlikely that this phenomenon is due to biclonality of the culture analyzed.

The diverse *J* region gene usage and variability in sequences used was reflected in the heterogeneity of

CDR3 regions of both α and β chain. Some common residues within the junctional regions could be identified. A positively charged basic residue (K) was found in the TCRAV CDR3 loops of three of six TCC (ZPZ10, XPD25, and XPF10) three residues from the carboxyl terminus of each CDR3 (Fig. 2A). As this residue is conserved in 34 of 61 *TCRAJ* gene segments (Koop et al. 1994), its occurrence may not be due to selection. A positively charged basic residue (R) was observed in the amino-terminal portion of the TCRBV CDR3 loops of all TCC investigated (Fig. 2B). There was no conservation in length of the CDR3 in either α or β chain of the TCR (ranging from 8 to 12 residues and from 6 to 14 residues, respectively). All TCRAJ sequences were identical to sequences of TCRAJ segments published by Koop and co-workers [(1994) (Fig. 2A)] with the exception of the TCRAJ sequence of TCC XPR04 which differed in one amino acid residue (Fig. 2A).

Discussion

Five CD4⁺ TCC, isolated from the peripheral blood of five different birch pollen-allergic individuals were shown to be specific for a major epitope (BV144: Ebner et al. 1993 a, b, c) located near the C-terminus on Bet v 1, the major allergen of birch pollen. In all TCC the recognition of this peptide was restricted by HLA-DR molecules, though no association between particular restriction molecules and reactivity with BV144 could be observed. This is in accordance with the fact that allergic and non-allergic individuals with different HLA types harbor T cells specific for this – in the case of birch pollen allergy – important epitope. Type I allergy is a disorder where particular Th subsets play a crucial role. In recent years, a pathway of IgE regulation, essentially based on the reciprocal activities of IL-4 and IFN- γ , has been described for mice and humans (Mosmann and Coffman 1989; Wierenga et al. 1990; Romagnani 1991). IL-4 was identified as an IgE-switch factor, whereas IFN- γ was characterized as the most important antagonist of most biological activities of IL-4. It has been proven that TCC, established from atopic individuals, with a specificity for allergens characteristically could be attributed to the Th2 subset, whereas TCC specific for bacterial antigens produced high levels of IFN- γ and IL-2 upon stimulation, thus designating them members of the Th1 subset. The study of the TCR usage of house dust mite-specific CD4⁺ TCC revealed dominant expression of certain *TCRAV* and *TCRBV* genes and also suggested the presence of dominant long-lived TCC in vivo (Wedderburn et al. 1993). In order to investigate allergen-specific T lymphocytes as possible targets for therapeutical intervention, we investigated the molecular structure of BV144-specific TCC.

Analysis of TCR α and β chain expression revealed diversity in *V* (*D*) and *J* gene segments in both chains. The junctional regions of both chains appeared to be quite diverse in both length and amino acid composition, although some restriction was observed in the TCRBV CDR3, where a basic charged residue (R) was found in

all five clones. This amino acid was present exclusively at the BV – BJ junction of the clones. Although this arginine residue was contributed by the *BV2* gene segments in clones XPD25 and XPF10, it may well be selected for recognition of the BV 144 – MHC complex. The BV 144 peptide contains a basic positively charged and an acidic negatively charged residue separated by two hydrophobic residues. These charged amino acid residues certainly play an important role in the interaction with the TCR and the MHC II molecule. They are also part of a five-amino-acid motif found in T-cell epitopes as described by Rothbart and Taylor (1988).

CDR3 regions of the α and β chain are believed to be rearranged independently of thymic MHC molecules and to represent the particular parts of the TCR which come in contact with the antigenic peptide. It was therefore tempting to speculate that this region might display a certain degree of homology in TCC recognizing identical peptides. However, again no similarity, either in length or in the amino acid composition, could be observed in the sequences.

In accordance with our findings, a study examining the T-cell response to an influenza virus hemagglutinin determinant, HA 255–270, in the context of HLA-DQ and -DR molecules, also showed a diverse selection of *TCRAV* and *J* gene segments that contributed to the junctional heterogeneity of the TCR. *TCRBV9* and *BV13* gene segments seemed to be preferentially used by these TCC. CDR3 sequences of both the α and β chain were heterogenous (Jones et al. 1994). TCC reactive with a tetanus toxin-derived peptide (tt830–844) presented by HLA-DR molecules showed a preferential *TCRBV* gene usage. *TCRAV* gene use was more heterogenous. Again, a striking heterogeneity was found in the junctional regions of both α and β chains (Boitel et al. 1992). TCC specific for mycobacterial 65000 *M_r* heat shock proteins showed restricted TCR expression, also in this case CDR3 sequence conservation was not apparent (Henwood et al. 1993). TCC involved in the human autoreactivity to myelin basic protein with a defined peptide specificity and HLA-DR restriction also expressed multiple TCR (Giegerich et al. 1992).

Although it may be speculated that charged residues (arginine can be found at the N-terminal ends of every CDR3 β region) may play a role in recognition of BV144, we came to the conclusion that the homology of the hypervariable regions of these TCC is not sufficient to serve as a target for the elimination of BV144-specific TCC.

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