

Identification of a highly promiscuous and an HLA allele-specific T-cell epitope in the birch major allergen Bet v 1: HLA restriction, epitope mapping and TCR sequence comparisons

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Summary

Background Allergen-specific CD4⁺ T cells play an important regulatory role in atopic allergy.

Objective To investigate the human leucocyte antigen (HLA) restriction and T-cell receptor (TCR) usage of allergen-specific T-cell clones (TCCs) that react with defined epitopes of Bet v 1, the major birch pollen allergen.

Methods Five Bet v 1-specific TCCs derived from two birch pollen-allergic individuals and specific for Bet v 1, were epitope-mapped with overlapping synthetic peptides. In addition, HLA-restriction and TCR CDR3 sequences were determined.

Results Three TCCs reacted with a Bet v 1 peptide containing amino acid residues 21–33 (BP21), the other two TCCs reacted with a minimal peptide comprising residues 37–45 (BP37). Studies using neutralizing anti-HLA-monoclonal antibodies and HLA-typed APCs showed that the BP37-specific TCCs were restricted by a HLA-DQA1*0301/DQB1*0603 heterodimer. In contrast, BP21 was recognized in a highly promiscuous manner. TCCs recognizing this sequence were restricted by HLA-DPB1*0201, a HLA-DQA1*0201/DQB1*0201 heterodimer, or HLA-DRB3*0101. Reverse transcription-polymerase chain reaction with primers for all known TCRAV and TCRBV gene segments, followed by CDR3 region sequencing, revealed the usage of five different TCRAV and four different TCRBV gene segments by the TCCs, as well as diversity in the joining region. All BP21-specific TCCs contained a negatively charged residue in their CDR3 α regions, the CDR3 β regions showed a high concentration of polar and OH-group bearing residues. BP37-specific TCCs shared the amino acid combination LY in the middle of their CDR3 α regions, the CDR3 β regions showed high concentration of OH-group bearing or charged residues.

Conclusions This study shows the existence of a highly promiscuous T-cell epitope in Bet v 1. The presence of additional T-cell epitopes in Bet v 1 may, however, hamper the clinical applicability of the epitope. Likewise, the diversity in TCR usage by T cells recognizing the epitope does not support the development of TCR-directed immunotherapy for birch pollen allergy.

Keywords: T lymphocyte, MHC, immunotherapy, T-cell receptors

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Introduction

Allergen-specific CD4⁺ T lymphocytes with a bias towards

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the production of TH2 cytokines have been shown to play a crucial role in the regulation of type I allergies [1]. Upon activation by allergen-derived peptides presented on major histocompatibility complex (MHC) class II molecules, they simultaneously produce interleukin (IL)-4, which induces B

cells to switch to the production of immunoglobulin (Ig)E, and IL-5, which plays an important role in the differentiation and maturation of eosinophils. The presence of these allergen-specific TH2-like cells in affected tissues together with the cytokines they produce strongly indicate that they are involved in the pathophysiology of atopic disease [2–4].

Antigenic peptides, presented by MHC class II molecules are recognized via T-cell receptors (TCRs) [5]. X-ray structures of individual TCR chains and $\alpha\beta$ TCRs supported the notion that the TCR contains Ig-like domains [6]. Both the alpha and the beta chains comprise three hypervariable loops, the complementarity determining regions (CDRs) [7]. The CDR1 and CDR2 in the TCR V region seem to be involved in making contact with the polymorphic regions of the MHC II molecule. The CDR3 in the junctional (NDNJ) region is positioned over the antigenic fragments placed in the groove of the MHC II molecule [6].

Restriction of gene usage for both the TCRs and the MHC molecules may be a crucial factor for triggering an aberrant immune response. T-cell clones (TCCs) derived from the synovial fluid of patients with rheumatoid arthritis [8] and TCCs specific for a mycobacterial 65-kDa heat shock protein [9] showed conservation of either the V α or V β gene repertoire. Several investigators found that TCCs specific for myelin basic protein (MBP) showed a non-random V gene usage [10,11]. In contrast, Meinel *et al.* [12] described a rather heterogeneous T-cell response with respect to TCR gene usage and human leucocyte antigen (HLA) restriction, but a restricted epitope profile of MBP. In the context of type I allergy, studies of epitope-mapped Bet v 1-specific TCCs from birch pollen-allergic patients showed a rather diverse V gene usage [13], whereas Wedderburn *et al.* [14] described a limited TCR usage for house dust mite specific TCCs.

Characterization of the epitopes that are recognized by allergen-specific CD4⁺ T cells, as well as their HLA restriction pattern and TCR usage, is essential for the understanding of atopic disease and may help in defining novel approaches for specific immunotherapy. Several studies have shown that multiple T-cell activating epitopes are found along the sequence of major allergens of house dust mite, grass pollen, cat, tree pollen, and bee venom proteins [15]. In addition, HLA restriction-specificity of allergen-specific T cells has previously been shown to be very diverse [15].

Type I allergy to tree pollens of the Fagales order, of which birch is the most important representative, is widespread in the western world. Bet v 1, a protein with a molecular mass of 17 kDa, is the major allergen of white birch (*Betula verrucosa*), to which approximately 95% of all birch pollen-allergic patients are sensitized, underlining its importance for tree pollen allergy [16]. Bet v 1-specific IgE has been shown to be highly cross-reactive with group 1

pollen allergens of related Fagales trees such as alder, hazel, hornbeam and oak [17]. This could be explained by a high degree of sequence homology based on N-terminal sequences [17], confirmed by the cloning of cDNAs encoding Bet v 1, Car b 1, Aln g 1, and Cor a 1 [18–21]. Interestingly, all these group 1 allergens consist of many isoforms containing one to several amino acid substitutions, adding to the complexity of natural tree pollen allergens. The recent demonstration of the three-dimensional structure of Bet v 1 by NMR and X-ray crystallography has revealed the structural basis of cross-reactivity of Bet v 1-specific IgE with group I allergens of related Fagales allergens [22].

In a previous study, we described five T-cell clones that had an identical reactivity pattern with overlapping recombinant Bet v 1 peptides [23]. Here we report a detailed characterization of the recognition of allergen-derived peptides by allergen-specific T cells by analysing cognate HLA-peptide–TCR interaction of these T-cell clones.

Materials and methods

Patients

Peripheral blood mononuclear cells (PBMCs) were obtained from donors JN (HLA-DRB1*0701,0801; DRB4*0101; DQA1*0201,0401; DQB1*0201,0301) and AF (HLA-DRB1*0404,1301; DRB4*0103; DQA1*0103,0301; DQB1*0603,0302), who were clinically allergic to birch pollen. Type I allergy was further demonstrated by positive skin-prick test (Soluprick, ALK, Denmark) and by the presence of birch (*B. verrucosa*)-specific serum IgE (Magic Lite SQ specific IgE, ALK). The study was reviewed and approved by the local ethics committee.

Affinity purification of Bet v 1

Natural Bet v 1 was purified by immunosorbent affinity chromatography. Briefly, the murine monoclonal Bet v 1-specific antibody BV16 was covalently immobilized on HiTrap N-hydroxysuccinimide activated columns (Pharmacia, Uppsala, Sweden) according to the manufacturers instructions. Some 6 mg crude birch pollen extract per mL immunosorbent gel were applied on the column in phosphate-buffered saline (PBS), 0.5 M NaCl pH 7.2, the column was washed with five column volumes of PBS, 0.5 M NaCl pH 7.2 and the allergen was eluted with 0.1 M glycine HCl pH 2.8. The fractions containing Bet v 1 were neutralized immediately with 1.0 M NaHCO₃ pH 8.3, dialysed against 0.005 M NH₄HCO₃ pH 8.3 and lyophilized.

Synthetic peptides

Synthetic peptides corresponding with Bet v 1 residues 21–41, 31–51, 21–35, 23–37, 25–39, and 27–41 were purchased

from Schafer-N (Copenhagen, Denmark). Peptides 31–42, 34–45, 37–48, and 40–51, were a kind gift of C. Ebner, Vienna, Austria. Peptides were synthesized on a MK-III fully automatic synthesizer using 9-fluorenyl-methoxycarbonyl (Fmoc)-chemistry and checked for purity by high-pressure liquid chromatography. All peptides showed a single peak and were >80% pure. Lyophilized peptides were reconstituted in PBS with 50% glycerol, sterilised by passage through a 0.2 µm filter, and further diluted for use in T-cell assays.

HLA-specific monoclonal antibodies (MoAbs)

Monoclonal hybridoma lines producing HLA-DR specific mAb L-243, and HLA-A,B,C-specific MoAb W6–32 were purchased from ATCC (Rockville, MD, USA), and hybridomas producing HLA-DQ-specific MoAb SPV-L3 and HLA-DP-specific hybridoma B7/21 were used with kind permission of Dr H. Spits (Amsterdam, The Netherlands), and Dr I. S. Trowbridge (San Diego, CA, USA).

Culture conditions

T cells were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 2 mM L-glutamine (Gibco), 100 IU/mL of Penicillin-Streptomycin (Gibco) and 5% heat-inactivated, screened human AB⁺ serum (Harlan, UK) (later referred to as complete medium). All cultures were grown under sterile conditions at 37°C in a humidified atmosphere of 5% CO₂. HLA-typed EBV-LCL were cultured in RPMI, 2 mM L-glutamine, 100 IU penicillin-streptomycin, and 10% heat-inactivated fetal calf serum (Gibco, Paisley, UK) under the same culture conditions as for the T cells.

Generation of Bet v 1 reactive T-cell clones

Bet v 1-specific T-cell clones were generated from the peripheral blood of two birch allergic patients, as described elsewhere [23]. T-cell clones derived from donor AF were AF19, AF24, AF28, and AF31; T-cell clone JN26 was derived from patient JN. In short, PBMC (106/mL) were stimulated with 10 µg/mL Bet v 1 in complete medium. Bet v 1 reactive T-cell lines were established by adding IL-2, and Bet v 1 reactive T-cell clones were generated from such short-term lines by limiting dilution in the presence of Bet v 1, irradiated autologous Epstein–Barr virus (EBV), allogeneic PBMC, 10% IL-2, and 0.5 µg/mL phenylalanine. After 7–14 days growing clones were expanded in 96-well plates and afterwards in 24-well plates. Fresh feeder cells, antigen, and phenylalanine were added every 10 days.

Proliferative responses of Bet v 1-specific T-cell clones

Cloned T cells were washed twice in RPMI 1640 medium to remove excess phenylalanine and IL-2. Antigen-specific

proliferation was assessed by stimulation of the T cells, 2–4 × 10⁴ cells/well, by antigen using 5000 rad-irradiated autologous EBV-transformed B-cells as APC (10⁴ cells/well). The cells were cultured for 72 h, followed by a 16 h pulse in the presence of tritiated methyl thymidine (³H-TdR) (NEN DuPont, Wilmington, DE, USA), 0.5 mCi/well. Proliferation was expressed as mean c.p.m. of ³H-TdR incorporation of triplicate cultures.

Determination of HLA restriction

Proliferation assays were performed as described above. For inhibition studies, EBV-B cells were preincubated with 50 µg/mL HLA-specific MoAb for 1 h at 37°C, followed by the addition of the TCC and Bet v 1. When the HLA restriction of the TCC was analysed using panels of HLA typed EBV-LCL, the B cells were incubated for 2 h at 37°C in 20 µg/mL synthetic peptide, washed to prevent direct T–T cell antigen presentation, and added to the TCC. HLA typing of all EBV-LCL described in this study, including JY was carried out by Dr H. Madsen, Copenhagen, Denmark, and by Dr L. Gebuhrer, Lyon, France using standard PCR-single strand confirmation polymorphism techniques.

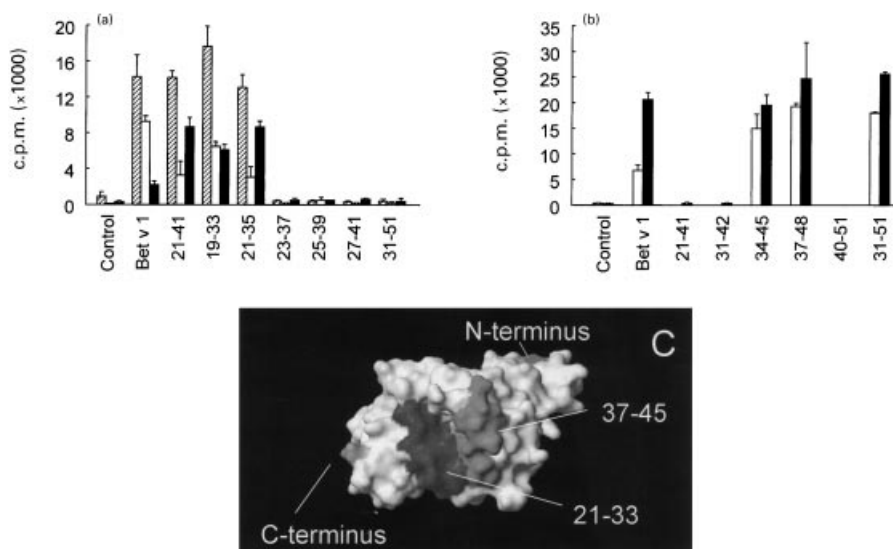
First strand cDNA synthesis and PCR amplification

Total RNA of the TCC was isolated from 5 × 10⁶ cells by the single step guanidinium-isothiocyanate-phenol-chloroform extraction method. Single stranded cDNA synthesis was carried out on 1.2–1.8 µg of total RNA with oligo (dT)_{12–18} and avian myeloblastosis virus reverse transcriptase. TCRα and TCRβ cDNAs were directly subjected to DNA amplification by PCR using Amplitaq DNA polymerase (Perkin Elmer, Norwalk, CT, USA) with oligonucleotide primers complementary to TCR V and C region sequences (T-cell receptor typing Amplimer kit, Clontech, Palo Alto, CA, USA) at a final concentration of 0.5 µM. Reaction mixtures were incubated in a PE 2400 DNA thermal cycler (Perkin Elmer Corporation). Reaction mixtures were subjected to the following conditions: denaturation at 93°C for 45 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, with a final extension time of 7 min at the end of cycling.

Cloning and sequence analysis of TCRα and TCRβ chains

The PCR reaction products were cloned into the TA cloning vector pCRII (Invitrogen Corporation, San Diego, CA, USA) and sequenced by the enzymatic chain termination method using a Sequenase version 2.0 kit (United States Biochemical Corporation, San Diego, CA, USA). For each of the TCRα and TCRβ chains studied, three independent pCRII clones were sequenced to identify possible sequence errors caused by nucleotide misincorporation by the Taq

Fig. 1. Epitope mapping of Bet v 1-specific TCC. The Bet v 1 TCC ($2-4 \times 10^4$ /well) were incubated with Bet v 1 ($1 \mu\text{g/mL}$) or synthetic peptides ($10 \mu\text{g/mL}$) in the presence of autologous EBV-LCL ($1-2 \times 10^4$ /well). (a) Shows reactivity pattern of Bet v 1-specific TCC that react with BP21 AF28 (grey bars), AF31 (white bars), JN26 (black bars). (b) Shows the two TCC AF19 (white bars), and AF24 (black bars) that react with BP37. Values shown represent mean c.p.m. values of triplicate cultures. Standard deviations are indicated by error bars. One of two experiments shown. (c) Space filling model of Bet v 1 with the minimal T-cell epitopes highlighted.



polymerase. Sequences were compared with gene data bank entries (Genbank, Los Alamos, NM, USA and EMBL, Heidelberg, Germany) and assigned to available published TCR V and J segments. Virtually all TCRV transcripts have the amino acid sequence Y-(L/F)-C-A (α chain) or Y-(L/F)-C-A with two additional serine residues in most cases at the 3' end of the V gene segment and the motif F-G-X-G-T within the J segment [24]. The CDR3 length was taken as the number of amino acids between the last germline encoded residue of the V segment and the conserved phenylalanine residue.

Results

Reactivity of Bet v 1-specific TCCs with synthetic peptides

All Bet v 1-specific TCCs used in this study reacted with the same truncated recombinant Bet v 1 peptides in the region of residues 21–51 [23]. To define the minimal T-cell activation-inducing epitopes that are recognized by these TCCs, the cells were screened for reactivity with short, overlapping peptides, derived from the two 21-mer peptides BP21–41 and BP31–51. Three of the TCCs (JN26, AF28 and AF31) proliferated in response to stimulation with BP21–41, BP19–33 and BP21–35, indicating that they recognized an epitope comprising amino acid residues 21–33 (BP21: AFILDGDNLFPAKV) (Fig. 1a). This epitope contains parts of two small α -helices that form an interface between the central β -sheet and the long c-terminal α -helix (Fig. 1c) and furthermore, the epitope has a highly polar and negatively charged core region (DGDN) flanked by hydrophobic stretches (four hydrophobic residues on the N-terminus and two in the C-terminal portion). Finally, a

single positively charged residue (K) follows a proline at the C-terminus of the epitope. TCCs AF19 and AF24 reacted with peptides BP31–51, BP37–48, and BP34–45, which identified the minimal T-cell epitope of amino acid residues 37–45 (BP37) recognized by these cells (Fig. 1b). This epitope mainly consists of β -strand 2. This peptide fragment shows negatively charged and polar residues separated by single hydrophobic residues.

HLA class II alleles involved in antigen presentation to Bet v 1-specific TCC

To determine the HLA restriction elements of the Bet v 1-specific TCC, inhibition studies with neutralizing HLA-specific MoAb were performed. As is shown in Table 1, proliferative activity of TCCs AF19, AF24 and JN26 was inhibited by an anti-HLA-DQ MoAb. In contrast, the proliferation of TCC AF28 and AF31 was inhibited by an anti-HLA-DP MoAb or an anti-HLA-DR MoAb, respectively. The addition of a neutralizing MoAb directed at a framework determinant of HLA class I molecules had no effect on the proliferative activity of any of the TCC. Analysis of the HLA molecules involved in the presentation of Bet v 1 to the TCCs, using a panel of EBV-LCL that were HLA typed for various HLA-DR and HLA-DQ gene products (Table 2) showed that the recognition of Bet v 1 by TCCs AF19 and AF24 was restricted by an HLA-DQA1*0103/DQB1*0603 $\alpha\beta$ dimer, whereas TCC AF31 reacted exclusively when DRB3*0101 expressing EBV-LCL were used as antigen presenting cells (Table 3). Restriction-specificity of TCC AF28 could not be determined directly, since no B-cell lines typed for the HLA-DP haplotype were available, with the exception of the DPB1*0201 homozygous EBV-LCL JY

Table 1. Inhibition of Bet v 1-specific proliferation of Bet v 1-specific TCC by α HLA-MoAb^a

TCC (specificity)	Medium HLA-restriction ^b (% inhibition)	Bet v 1				HLA-ABC
		+MoAb α –	+MoAb α HLA-DP	+MoAb α HLA-DQ	+MoAb α HLA-DR	
AF19 (37–45)	631 HLA-DQ (88.5%)	52211	36038	6575	51900	54975
AF24 (37–45)	455 HLA-DQ (75.9%)	25424	20558	6471	30120	28880
AF28 (21–33)	876 HLA-DP (87.3%)	24891	4796	19652	27201	26833
AF31 (21–33)	414 HLA-DR (98.5%)	2525	2679	3065	446	2738
JN26 (21–33)	139 HLA-DQ (100%)	4411	3615	122	3127	4401

^a Bet v 1-specific T-cell clones (TCC) ($2-4 \times 10^4$ /well) were stimulated with Bet v 1 in the presence of autologous Epstein–Barr virus-LCL (10^4 /well) as antigen-presenting cells (APC). Anti HLA-class II and human leucocyte antigen (HLA)-class I monoclonal antibody (MoAb) (all 50 μ g/mL) were added to suppress Ag presentation to the TCC. Values shown are mean c.p.m. of triplicate cultures and are representative for three experiments. ^b The suppressive effect of the MoAb is expressed as: % inhibition = [(c.p.m. without MoAb – background c.p.m.) – (c.p.m. with MoAb – background c.p.m.)]/(c.p.m. without MoAb – background c.p.m.) $\times$ 100%.

(12th international histocompatibility workshop cell panel), as HLA-DP restriction is rare among human CD4⁺ TCC. However, both the inhibition of the proliferative activity AF28 by a neutralizing antiHLA-DP MoAb and the observation that it recognized BP21–41 presented by the

DPB1*0201 homozygous EBV-LCL JY, strongly suggests that the recognition of this TCC is HLA-DPB1*0201-restricted.

TCC JN26 proliferated only to BP21–41 in the presence of EBV-LCL that expressed both HLA-DQB1*0201 and

Table 2. Haplotypes of HLA class II typed EBV-LCL^a

EBV-LCL	HLA-DR alleles				HLA-DQ alleles	
	DRB1	DRB3	DRB4	DRB5	DQA1	DQB1
AF ^b	1301, 0404	0101	0103	–	0103, 0301	0603, 0302
JN	0701, 0801	–	0101	–	0201, 0401	0201, 0301
ATG	0301, 0408	0101	–	–	0303, 0501	0201, 0301
ATO	1301, 1501	0101	–	0101	0101, 0103	0603, 0602
BTA	7, 1102	0201/2	–	–	0201, 0501	0201, 0301
BTC	1501, 0301	0101	0101	0102, 0501	0602, 0201	
BTH	0802, 1501	–	–	0101	0102, 0401	0402, 0602
KRF/KOF	1301, 0401	0101	–	–	0103, 0301	0603, 0302
LF	1501, 0401	–	0103	0101	0102, 0302	0301, 0602
MK	0301, 0401	0101	0103	–	0302, 0501	0201, 0301
AGC	1501, 1302	0301	–	0101	0102	0602, 0604
TL	0101, 0801	–	–	–	0101, 0401	0501, 0402
JY	0404, 1301	0101	0103	–	0103, 0301	0302, 0603
BTI	0402	–	0103	–	0301	0302

^a Epstein–Barr virus (EBV)-LCL were typed for human leucocyte antigen (HLA)-DR- and HLA-DQ haplotype by polymerase chain reaction-single strand confirmation polymorphism. Cells were not typed for HLA-DP alleles. ^b AF and JN are autologous EBV-LCL for AF and JN T-cell clones (TCC), respectively.

Table 3. Reactivity of Bet v 1-specific TCC with human leucocyte antigen (HLA) class II typed EBV-LCL as APC^a.

	AF19	AF24	JN26	AF28	AF31
EBV-LCL	(37–45)	(37–45)	(21–33)	(21–33)	(21–33)
AF	+++ ^b	+++	–	+++	+
JN	–	–	+	–	–
ATG	–	–	nt	+	+
ATO	+++	+++	–	+++	+
BTA	–	–	+	–	–
BTC	–	–	–	+++	–
BTH	–	–	–	–	–
KRF/KOF	++	+++	–	+++	+
LF	–	–	–	–	–
MK	–	–	+	+++	+
AGC	–	–	–	+	–
TL	–	–	–	–	–
JY	++	++	–	+++	–
BTI	–	–	–	–	nt
HLA allele ^c	DQA1*0103/ DQB1*0603	DQA1*0103/ DQB1*0603	DQA1*0201/ DQB1*0201	DPB1*0201	DRB3*0101

^a Epstein–Barr virus (EBV)-LCL were preincubated with the relevant synthetic peptide for 2 h at 37°C, washed, and cocultured with TCC cells as described in Materials and Methods. EBV-LCL without peptide were used as negative control. AF and JN are the autologous EBV-LCL for TCC AF19,24,28, and 31 and JN26, respectively.

^b Values shown are stimulation indexes (SI), defined as mean c.p.m. of triplicate cultures with peptide-pulsed EBV-LCL divided by mean c.p.m. of triplicate cultures with EBV-LCL without peptide. +++ (SI > 20) ++ (SI 10–20) + (SI 3–10) and – (SI < 3). Results are representative for two experiments. ^c The HLA restriction element for the TCC was determined by analysing the reactivity patterns of the TCC with the typed EBV-LCL (shown in Table 2) and inhibition studies (Table 1).

HLA-DQB1*0301, but did not recognize the peptide presented by APC that expressed either molecule. This was confirmed using peripheral blood mononuclear cells typed for the expression of HLA-DQB1*0201 and HLA-DQB1*0301. Moreover, the DQA1*0102,0501, DQB1*0201, 0602-expressing EBV-LCL BTC could not act as APC for JN26 either.

Interestingly, however, the HLA-DQA1*0201 and *0302 molecules share distinct epitopes that are not shared by DQBA1*0102 or *0501 [25], and the results therefore suggest that the recognition of Bet v 1 by JN26 is restricted by an HLA-DQB1*0201/DQA1*0201 molecule that cross-reacts with a HLA-DQB1*0201/DQA1*0302 molecule.

Finally, using HLA-typed EBV-LCL (Table 3) as well as PBMC (data not shown), AF31 was shown to recognize Bet v 1 presented by HLA-DRB3*0101-expressing APC.

TCRAV and TCRBV usage of Bet v 1-specific TCC

RT-PCR analysis showed a striking heterogeneity of AV and BV gene usage by the Bet v 1-specific TCCs (Table 4).

The TCRs of the TCCs AF28 and AF31 specific for the same epitope (BP21) both used the BV6 gene segment. The corresponding AV genes, AV20 and AV22, are not related to each other. The characteristics of all CDR3 regions matched those reported by Moss *et al.* [24]. There was a predominance of charged and polar residues present in the loops. Hydrophobic amino acid residues were concentrated towards the V region end of the loops.

Comparison of the CDR3 region sequences of TCCs recognizing BP21

CDR3αs. The three CDR3α regions with a length varying between nine and 10 residues, all contained a negatively charged aa, and the N-terminal a.a. was hydrophobic (Table 4a). In addition, two of the CDR3α regions showed a concentration of OH-group bearing amino acid residues (AF28: SSY; JN26: SXXTY).

CDR3βs. Two of the three CDR3α-regions shared the amino acid residues 'VSS' at the same position (AF28 and

Table 4. Nucleotide and deduced amino acid sequences of TCRA and TCRB CDR3 regions of independent TCCs specific for (a) Bet v 1 21–33 and (b) Bet v 1 37–45

T-cell clone	TCRA gene segment	CDR3 α	TCRB gene segment	CDR3 β
BP21: AFILDGDNLPKV				
AF28 V20S1J12	GTGGTGAGGGATAGCAGCTATAAATTGATC VVRDSSYKLI		V6S3J2S3C2	TTTAGCGGGGTCTAGCACAGATACGAGTAT FSGVSSDTQY
AF31 V22S1J45	CTGGGAGGAGGTGCTGACGGACTCACC LGGGADGLT		V6S3J2S1C2	CTCTAGCGGTAAGCTCCTACAATGAGCAGTTT LLAVSSYNEQF
JN26 V21S1J40	GCAAGCGAGGGAACCTACAAATACATC ASEGTYKYI		V2S1J1S2C1	CAGGGAACCTAACTATGGGTACACC QGTNYGYT
BP37: AISSVENIE				
AF19 V6S1J31	ATGAGCCCTGCGTTGACGGTCTCTTTATGCCAGACTCATG MSPCV DGRLYARLM		V13S3J2S1C2	GGGACTAGCGGCATCCTACAATGAGCAGTTT GTSASYNEQF
AF24 V14S1J45	TATAGGAGCGGACTATATTCAGGAGGAGGTGCTGACGGACTCACC YRGLYSGGGADGLT		V24S1J1S1C1	ACCCGGCGGACACTGAAGCTTTC TRRDTEAF

CDR3 region length was defined according to Rock *et al.* [7]. TRCAV and TCRBV segments were assigned according to the standardized T cell receptor (TCR) nomenclature. The underlined CDR3 aa are encoded by the J segments. The complete TCRV sequences are available at the EMBL nucleotide Sequence Database under accession numbers Y15198–Y15207.

AF 31). In both cases, they were followed by OH-group bearing residues (T resp. Y) resulting in a three-a.a.-long stretch of OH-group bearing amino acid residues. The TCR of TCC JN26 also showed a high predominance of OH-group bearing a.a. (four of eight a.a. composing the CDR3 β -region). The second important feature of the CDR3-regions was the rather long hydrophobic stretch at the N-terminus comprising four a.a. displayed in two of three TCR beta chains (AF31: LLAV and AF28: FSGV, which can be called an 'essential hydrophobic stretch' because the polar characteristics of the serine will be compensated by the sterically much more dominant hydrophobic phenylalanine).

Comparison of the CDR3 region sequences of TCCs recognizing BP31

CDR3 α s The CDR3 α regions of the two clones specific for this epitope were 14 or 15 residues in length (Table 4b). The amino acid residues LY were shared between the CDR3 α s of AF19 and AF24. In the case of AF19, the L was directly preceded by the positively charged a.a. arginine (R).

CDR3 β s AF 19 showed a concentration of four OH-group bearing a.a. separated by hydrophobic a.a. (TSASY). AF 24 showed a high usage of both positively and negatively charged a.a. with accompanying OH-group bearing threonines (TRRDTE, i.e. six of eight a.a. of the CDR3).

Discussion

In the present study, we describe the cognate MHCII-peptide–TCR interaction of allergen-specific T cells and furthermore document the presence of a promiscuous and an allele-specific T-cell epitope within the same region of Bet v 1, the major birch pollen allergen. In a previous epitope mapping study using recombinant Bet v 1 fragments, it was demonstrated that the majority of T-cell clones from Danish patients allergic to birch pollen allergens reacted with peptides corresponding with amino acid positions 1–80 or 21–60, but not with 42–121, 60–100, 81–159, and 101–140 [23]. This led us to conclude that the TCCs reacted with a fragment containing Bet v 1 a.a. 21–41. However, some of the TCCs did not react with a panel of overlapping peptides, whereas three reacted with 19–33. This prompted us to test these Bet v 1-specific TCCs for reactivity with two Bet v 1-derived peptides containing residues 21–41 or 31–51. The latter peptide was chosen to rule out any involvement of the flanking region. To our surprise, two of the clones reacted with this peptide.

The minimal T-cell epitopes of all five clones were determined with a panel of shorter peptides. The epitope peptides BP21 and BP37 displayed different physico-chemical properties which made it plausible to expect

Table 5. Characteristics of Bet v 1-specific TCC^a

TCC	Donor	Epitope	HLA restriction	TCRAV	TCRBV
AF19	AF	37–45	DQA1*0103/DQB1*0603	V6S1J31	V13S3J2S1C2
AF24	AF	37–45	DQA1*0103/DQB1*0603	V14S1J45	V24S1J1S1C1
AF28	AF	21–33	DPB1*0201	V20S1J12	V6S3J2S3C2
AF31	AF	21–33	DRB3*0101	V22S1J45	V6S3J2S1C2
JN26	JN	21–33	DQA1*0201/DQB1*0201	V21S1J40	V2S1J1S2C1

^a The characteristics of the Bet v 1-specific TCC as shown in Fig. 1 and Tables 1–4 are summarized. HLA, human leucocyte antigen.

different characteristics in the contact regions of the CDR3 loops. BP21 showed a highly charged and polar core region flanked on each side by a cluster of several hydrophobic residues. A proline separated an additional positive charge (K) at the C-terminal end. All three CDR3 α loops of TCRs specific for BP21 contained a negatively charged residue in a position that can be expected to be highly exposed and therefore able to interact with the positive charge at the C-terminus of BP21. Two of the three CDR3 β loops (AF28, AF31) specific for BP21 contained a cluster of hydrophobic amino acids at their N-terminal portion which makes the interaction with the hydrophobic areas of BP21 very likely. All three CDR3 β -loops contained a high concentration of OH-group bearing residues (AF28, AF31) or in addition to that a polar residue (JN26). These areas could interact with the highly charged and polar middle portion of BP21.

In contrast, BP37 was characterized by pairs or single residues carrying polar side chains or a negative charge separated by single hydrophobic residues. Both CDR3 α possessed in their centre portion a hydrophobic leucine followed by a polar tyrosine. The LY pair was encoded by randomly added nucleotides. The presence of the hydrophobic leucine residue in this position is rather unusual [24] and may be essential to interact with a respective area in BP37. The CDR3 β loops contained a high number of OH-group bearing residues (AF19) or a high concentration of charged residues (AF24), suggesting that these loops could interact with the charged and polar portions of BP37 via hydrogen bonds. This hypothesis is supported by the work of May *et al.* [26] where superimposed hydropathy plots showed that electrostatic properties rather than rigid sequence motifs determine T-cell specificity.

Diversity as well as restricted gene usage of TCR repertoires against a known antigen or in the context of a disease has been the subject of previous publications. In a study of clonal dominance in a TCR repertoire against a HLA-DR3-restricted hsp65 peptide, it was reported that this specific MHC class II-peptide complex was able to select for TCR V genes and amino acids in the CDR3 region [27]. On the other hand, conservations within the TCR usage were

more generally found in human class-I-restricted responses [28,29], probably a result of the very strict length requirements for MHC class I peptides. For MHC class II peptides, however, the length of the peptide is more flexible [30]. This might lead to different conformations of the MHC-peptide complex and an increase in the availability of TCR contact residues, resulting in a less restricted TCR repertoire. Another reason for diversity could be that immunodominant epitopes might generate a more diverse TCR repertoire than subdominant epitopes [31].

MHC class II restriction patterns of allergen-specific human T cells appear to be very diverse, as is T-cell epitope recognition. HLA-DR, HLA-DRB3, HLA-DP, and HLA-DQ restriction has been demonstrated for several allergens [15]. As HLA restriction of Bet v 1-specific T-cell clones has not been addressed in detail previously, the TCCs were tested in inhibition experiments with neutralizing HLA-specific MoAb, and in presentation experiments with tissue typed APCs. Interestingly, all three BP37 reactive TCCs were restricted by HLA-DQA1*0301/B1*0603. On the contrary, the TCCs reactive with BP21 were either HLA-DPB1*0201, HLA-DQA1*0201/B1*0201 or HLA-DRB3*0101-restricted.

These data clearly show, that the region 21–33 of Bet v 1 constitutes a promiscuous T-cell epitope that can be presented to specific T cells by various HLA-class II antigens. These findings (summarized in Table 5) may have implications for vaccine development as such an epitope may be presented to and recognized by T cells of the majority of the population. Specific immunotherapy has been shown to result in decreased proliferation and an increased IFN- γ /IL-4 production ratio of allergen-specific CD4⁺ T cells [32–34]. In addition, it has been recently shown that the infiltration of allergen-specific T cells and eosinophilic granulocytes in the nasal mucosa is decreased after specific immunotherapy, and an increase was found in the number of IL-12 producing cells [35]. These findings strongly suggest that T cells may be the principal target cells of specific immunotherapy, either directly, or via the induction of TH1 cell differentiation cytokines such as IL-12.

Even though T cells from the majority of the population

may react with P21–33, a few observations argue against the use of promiscuous T-cell epitopes of Bet v 1 for SIT. Bet v 1-specific CD4⁺ T cells have previously been shown to recognize an array of T-cell epitopes, and several immunodominant areas were shown to be present on the molecule, including P21–33 [15]. In addition, the identification of up to 35 different isoallergenic sequences for Bet v 1, some of which have amino acid substitutions in positions 21–33, raises the question which isoform sequence is the most relevant to derive synthetic peptides for immunotherapy. This is illustrated by the observation that an amino acid substitution in a naturally occurring isoform of Bet v 1 at position 25 (D to G) abrogated reactivity of all 21–33 reactive TCCs, irrespective of their HLA restriction (J. N. Larsen and R. J. J. van Neerven, unpublished observations). In contrast, a substitution at position 30 (F-V) in another isoform had no effect. Interestingly, the effect of this single a.a. substitution on the recognition of TCCs restricted by three different HLA-class II molecules may indicate that the TCR of these 21–33 reactive TCC may have a conserved CDR3 motif. Alternatively, the residue in position 25 may interact with a framework HLA determinant shared by HLA-DR, HLA-DQ, and HLA-DP molecules.

In conclusion, in the present study, we demonstrate the existence of a highly promiscuous T-cell epitope in Bet v 1, which is likely to be recognized by TCC of a large number of birch pollen-allergic individuals. However, the biological diversity of birch pollen allergens, together with the notion that several important T-cell epitopes have been identified on the Bet v 1 molecule, indicate that a peptide derived from this epitope may not be suitable for immunotherapy itself. Finally, in view of the diversity of TCR usage of TCCs that recognize this epitope, our results do not support the development TCR-directed immunotherapy for birch pollen allergy.

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