

Crossreactivity and T-cell epitope specificity of Bet v 1-specific T cells suggest the involvement of multiple isoallergens in sensitization to birch pollen

S. H. SPARHOLT, J. N. LARSEN, H. IPSEN, C. SCHOU and
R. J. J. VAN NEERVEN

ALK ABELLÓ, Hørsholm, Denmark

Summary

Background Allergen-specific T lymphocytes play an important role in the pathophysiology of atopic disease. Detailed studies of their epitope-specificity and crossreactivity are required for the development of novel approaches for specific immunotherapy.

Objectives The aim of the study was to characterize the fine specificity of Bet v 1-specific T cells from allergic donors.

Methods Polyclonal T-cell lines (TCL) and T-cell clones (TCC), specific for Bet v 1, the major birch (*Betula verrucosa*) pollen allergen, were isolated from the peripheral blood of three birch-allergic patients. Their epitope-specificity was studied using overlapping synthetic peptides, and crossreactivity with other tree pollen allergens of the *Fagales* order was evaluated. In addition, the Bet v 1-specific TCC were studied for their phenotype and cytokine production.

Results All isolated Bet v 1-specific TCC (19/21 CD4⁺, 2/21 CD8⁺) reacted with affinity purified Bet v 1, but showed different reactivities with recombinant Bet v 1 (rBet v 1), and with group 1 allergens from other *Fagales* species. Epitope mapping of rBet v 1-reactive TCC with synthetic peptides of Bet v 1 showed the presence of four T-cell epitopes. Polyclonal T-cell lines reacted with 13 different peptides, and displayed even broader crossreactivity with group 1 pollen allergens from other *Fagales* members.

Conclusion This study demonstrates that apart from T-cell epitopes of rBet v 1, many other crossreactive or Bet v 1 isoallergen-specific epitopes exist. This indicates that isoallergenic variation plays an important role in the induction of Bet v 1-specific and crossreactive T-cell responsiveness to allergens.

Keywords: T lymphocyte, Bet v 1, cytokine, T-cell epitope, crossreactivity, isoforms, IgE

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Introduction

It is well established that the type I allergic immune response is regulated by CD4⁺ TH₂ cells that produce large amounts of IL-4 and IL-5, cytokines that, respectively, induce IgE synthesis and eosinophilia [1–3]. The presence of allergen specific TH₂ cells has been demonstrated in

involved tissues such as atopic dermatitis lesional skin [4,5], in atopic dermatitis skin after allergen challenge [6], in nasal mucosa of rhinitis patients [7,8], and in the lungs of asthma patients [8,9]. Their presence in affected tissues strongly indicates that they play an important role in the pathophysiology of atopic disease.

The increasing knowledge of the DNA and amino acid sequences of major allergens has made it possible to investigate the epitope specificities of such allergen-specific T lymphocytes. Several groups have demonstrated that house dust mite *Dermatophagoides pteronyssinus* major

Correspondence: Dr R.J.J. van Neerven, ALK ABELLÓ group, Bøge Alle 10–12, DK-2970, Hørsholm, Denmark.

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allergens Der p 1 and Der p 2 contain multiple T-cell epitopes along the entire sequence of the allergen molecule [10–13]. This has also been shown to be the case for bee venom phospholipase A2 [14], Lol p 1 [15], Fel d 1 [16], and the major birch pollen allergen, Bet v 1 [17]. However, from one of the T-cell clones (TCC) in the latter study it was not possible to determine the epitope specificity, as it did not respond to a recombinant form of Bet v 1, which may be explained by the fact that natural Bet v 1 represents a mixture of isoforms [18,19].

During recent years it has been documented that group 1 allergens from tree pollen of the *Fagales* order consist of series of isoallergens with single or multiple amino acid substitutions [18,20,21]. In a previous paper we have shown that Car b 1, the major allergen from hornbeam, consists of many different isoallergens, of which several have been cloned and sequenced [20]. Some of the isoallergens of Car b 1 had a sequence identity of up to 75% with a sequence of Bet v 1 [20,22]. In addition to Car b 1, several isoallergens of other group 1 tree pollen allergens, namely Cor a 1 [21] and Bet v 1 [18, and J.N. Larsen, manuscript in preparation] have been cloned and sequenced.

Recent findings showed that serum IgE from different birch allergic patients exhibits distinct isoform reactivity patterns in 2D-gel electrophoretic systems, suggesting that amino acid substitutions in these isoallergens alter IgE epitopes [19]. On the other hand, IgE crossreactivity between different members of the *Fagales*, as well as immunochemical similarities have also been described, which reflects their high sequence homology [23].

Isoform specificity and T cell crossreactivity of *Fagales* group 1 allergen-specific TCC have only been addressed in a few studies [24–26]. The aim of the present study was to study the T-cell epitope specificity and the crossreactivity patterns of Bet v 1-specific T cells in detail, in order to evaluate the significance of isoallergenic variation of Bet v 1 and group 1 tree pollen allergens of other members of the *Fagales* for selective activation of allergen-specific T lymphocytes.

Materials and methods

Patients and IgE reactivity

Patients JN (HLA-DR7,8; DQw2,w3), AF (HLA-DR4,13; DQw1,w3), and MK (HLA-DR4,17; DQw2,w3) are clinically allergic to birch pollen. Type I allergy was further demonstrated by positive skin-prick test (Soluprick SQ, ALK, Denmark) and by the presence of birch-(Bet v) pollen specific IgE (Magic Lite SQ specific IgE, ALK, Denmark) [27–29]. Sera were further characterized for the presence of hornbeam- (*Car b*), hazel- (*Cor a*), alder- (*Aln*

g) and oak- (*Que a*) specific IgE (Magic Lite SQ specific IgE, ALK, Denmark).

Antigens

Preparation of group 1 enriched protein fractions of five tree pollens Group 1 enriched protein fractions (denoted as Bet v G75, *Aln g* G75, *Que a* G75, *Cor a* G75 and *Car b* G75) of the five *Fagales* species were prepared from aqueous extracts as described by Ipsen and Hansen [23,30]. In brief, size exclusion chromatography of 150 mg (dry weight) of the pollen extracts (*Bet v*, *Aln g*, *Que a*, *Cor a* and *Car b*) was performed on a G-75 superfine (Pharmacia, Uppsala, Sweden) 90 cm × 2 cm² (K15/100 Pharmacia, Uppsala, Sweden) column, equilibrated with 0.125 M NH₄HCO₃, pH 8.3. The fraction containing the major allergens (assayed by fused rocket immunoelectrophoresis and fused rocket radio immunoelectrophoresis) were pooled and lyophilized. Typically 15–30 mg of dry weight material was obtained.

Affinity purification of native Bet v 1 Natural Bet v 1 was purified by immunosorbent affinity chromatography. 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 description. Six milligram of 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 5 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.

Recombinant Bet v 1 rBet v 1 was produced by a fed-batch fermentation procedure as earlier described [31]. In brief, *Escherichia coli* K-12, strain JM105 [32], harboring a recombinant plasmid composed of the gene encoding Bet v 1 inserted into the non-fusion expression plasmid pKK223–3 [33] was grown to high cell density in a fermenter. rBet v 1 was purified from a soluble cytoplasmic lysate by affinity chromatography using solid phase monoclonal anti-Bet v 1 antibody as described above.

Overlapping synthetic peptides of Bet v 1 A panel of 17 peptides were synthesised according to the aa sequence of Bet v 1 using a solid phase technique (BT7400 Peptide synthesiser, Cambridge Research Biochemicals). The peptides were 15 aa long overlapping by five or six. Several of the peptides inducing proliferative responses on the clones were resynthesised using solid phase techniques on an

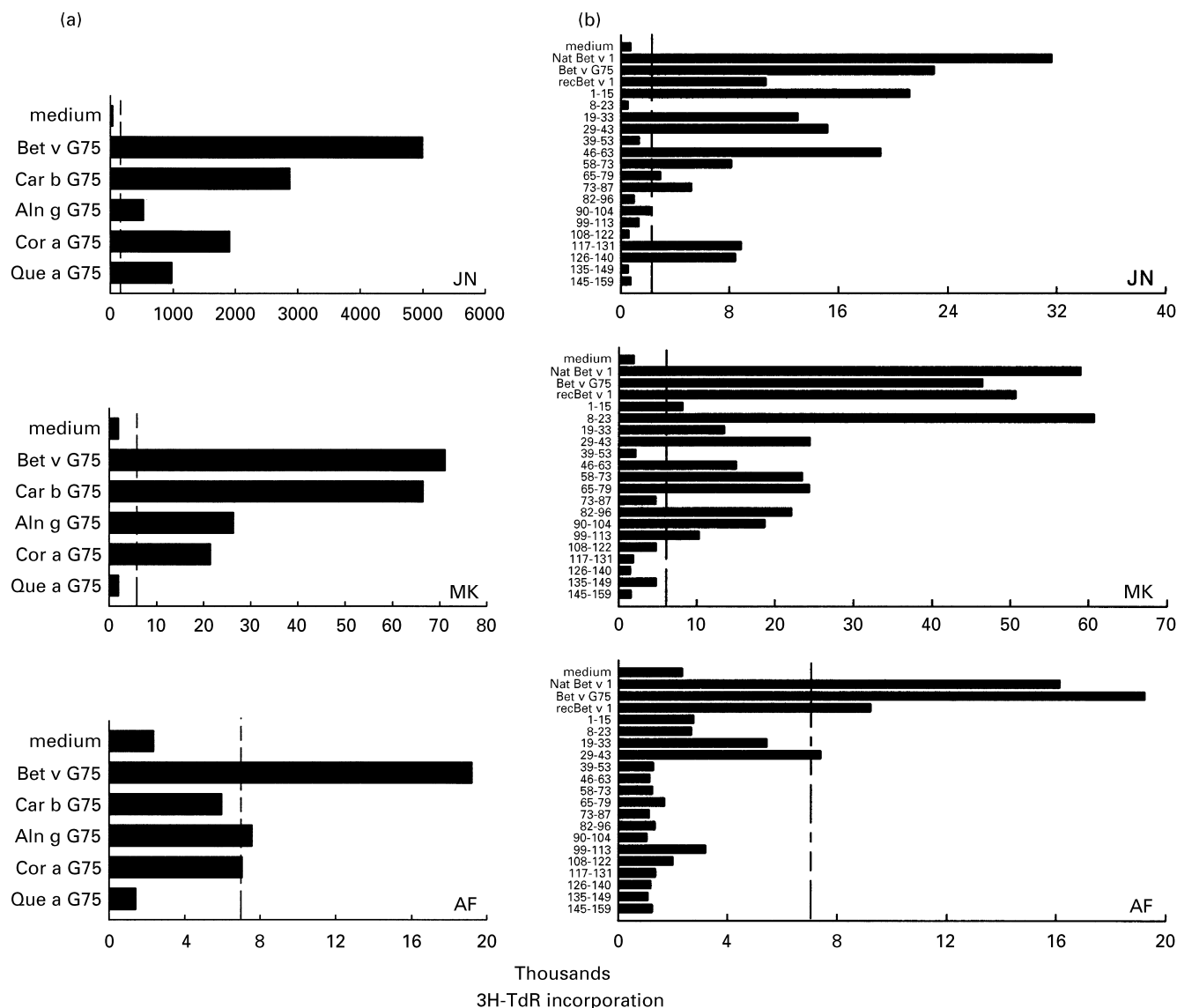


Fig. 1. Bet v 1-specific T-cell lines of three allergic donors ($2\text{--}4 \times 10^4/\text{well}$) were tested for reactivity with purified Bet v 1, rBet v 1, and group 1 enriched fractions of five members of the (a) *Fagales* order or (b) a panel of overlapping synthetic peptides of Bet v 1 in the presence of irradiated, autologous EBV-transformed B cells ($10^4/\text{well}$). Values shown are mean cpm of triplicate cultures as determined by ^3H -TdR incorporation. The dotted line represents a stimulation index (SI) of 3, above which results are considered positive.

Applied Biosystems (Foster City, CA, USA) peptide synthesizer. The peptides were determined by high performance liquid chromatography to be more than 90% pure.

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% CO_2 .

EBV transformed B cell lines of the three donors 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 the T cells.

Generation of Bet v 1 reactive T-cell lines and clones

Peripheral blood mononuclear cells (PBMC) were isolated

from freshly drawn heparinized blood by gradient centrifugation on Lymphoprep (Nycomed, Norway). PBMC ($10^6/\text{mL}$) were stimulated with $10\text{ }\mu\text{g/mL}$ Bet v 1 in complete medium. Bet v 1 reactive T-cell lines were established by adding IL-2 on days 5, 6 and 7 (10%, 5%, and 5% v/v, respectively) (Lymphocult-T, Biotest Folex, Germany). This was followed by an antigen-specific restimulation on day 12 of T cells ($2 \times 10^5/\text{mL}$) with $10\text{ }\mu\text{g/mL}$ Bet v 1 in the presence of 5000 rad-irradiated autologous EBV transformed B-cells ($10^5/\text{mL}$), 3000 rad-irradiated allogeneic PBMC ($10^6/\text{mL}$) and $0.5\text{ }\mu\text{g/mL}$ PHA-P (Sigma, St Louis, MO, USA) in complete medium. Interleukin-2 was added on days 3, 4, and 5 (10, 5, 5% v/v, respectively). Cultures were split and expanded in complete medium every 3 days.

Bet v 1 reactive T-cell clones were generated from such short-term lines by limiting dilution and plated at 0.3 or 1 cells/well in Terasaki plates in the presence of Bet v 1, irradiated autologous EBV, allogeneic PBMC, 10% IL-2, and $0.5\text{ }\mu\text{g/mL}$ PHA. After 7–14 days growing clones were expanded in 96 well plates and afterwards in 24 well plates. Fresh feeder cells, antigen, and PHA were added every 10 days.

T-cell line and TCC responses

Cloned T cells were washed twice in RPMI 1640 medium to remove excess PHA and IL-2. Antigen specific proliferation was assessed by stimulation of the T cells, $2\text{--}4 \times 10^4$ cells/well, by antigen using 5000 rad-irradiated autologous EBV-transformed B-cells as APC (10^4 cells/well). The cells were cultured for 72 h, followed by a 16 h pulse in the presence of tritiated methyl thymidine ($^3\text{H-TdR}$) (NEN DuPont, Wilmington, DE, USA), $0.5\text{ }\mu\text{Ci/well}$. Proliferation

was expressed as mean cpm of $^3\text{H-TdR}$ incorporation of triplicate cultures.

Analysis of phenotype of T-cell clones

Phenotype analysis of the TCC was performed using anti-CD2, CD4, CD8 and CD19 coated Dynabeads (Dynal, Oslo, Norway), adapted from reference [34]. Viable T cells were obtained from TCC cultures by gradient centrifugation on lymphoprep. The cells were washed twice in RPMI-1640 medium and resuspended in complete medium at 4×10^5 cells/mL. Anti CD2, anti-CD4, anti CD8 and anti CD19 coated Dynabeads were prepared according to the manufacturer's recommendations. The TCC cells, $250\text{ }\mu\text{L/sample}$, were incubated for 10 min at room temperature with excess amounts (7×10^6 beads/sample) of each of the four Dynabeads. Non-bound cells were magnetically separated from the Dynabead-cell complex and the phenotype of the cells was established by conventional microscopy. This method was compared with regular FACS analysis with comparable results.

Production of supernatants for cytokine measurements TCC ($2\text{--}4 \times 10^4/\text{well}$) were activated for 24 h in the presence or absence of G75 purified Bet v 1 ($10\text{ }\mu\text{g/mL}$) in the presence of autologous, irradiated EBV transformed B cells (10^4 cells/mL) as described above. Supernatants were collected for cytokine measurements after a 24-h culture period.

IL-4 and IFN γ ELISA assays

The presence of IL-4 and IFN γ in the TCC supernatants was

Table 1. Characterization of Fagales-specific IgE in patient's sera

Patient Species	JN		MK		AF	
	Standardized units (SU)	Allergy class (AC)	Standardized units (SU)	Allergy class (AC)	Standardized units (SU)	Allergy class (AC)
<i>Betula verrucosa</i> (white birch)	341	5	221	4	19	2
<i>Carpinus betulus</i> (hornbeam)	4	2	0	0	0	0
<i>Corylus avellana</i> (hazel)	25	3	2	1	0	0
<i>Alnus glutinosa</i> (alder)	62	3	2	1	1	0
<i>Quercus alba</i> (white oak)	2	1	0	0	0	0

The table shows the reactivity of specific serum IgE of all three patients to pollen extracts of *Betula verrucosa* and related species of the *Fagales* order, as determined by the MagicLite system.

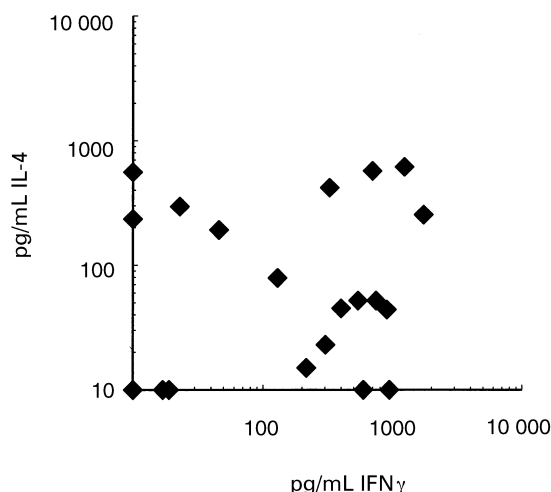


Fig. 2 21 Bet v 1-specific TCC ($2-4 \times 10^4$ /well) were specifically activated with Bet v 1 in the presence of irradiated, autologous EBV-transformed B cells (10^4 /well). After a 24 h culture period, supernatants were collected and IFN- γ and IL-4 concentrations were determined by ELISA.

measured using the Intertest-4 (IL-4) and the Intertest- (IFN γ) ELISA according to the manufacturer's description (Genzyme corporation, Cambridge, MA, USA).

Results

Reactivity of patients serum IgE

Measurement of specific serum IgE of two out of three patients showed reactivity to birch pollen extract as well as to related species of the *Fagales* order, whereas the third patient only had a very weak crossreactivity with only one of the four other *Fagales* species, namely *Alnus glutinosa* (Table 1).

Crossreactivity and epitope-specificity of polyclonal Bet v 1-specific T-cell lines To evaluate whether T cells displayed a similar crossreactivity pattern as serum IgE of the patients, reactivity of polyclonal Bet v 1-specific T-cell lines to group 1 enriched fractions of related species of the *Fagales* was analysed. As is shown in Fig. 1 a, T-cell lines of all three patients display crossreactivity with three or all four other *Fagales* representatives, showing that T cell crossreactivity is even broader than IgE crossreactivity in this system. To further characterize the epitope specificity of the Bet v 1-specific T cells, we chemically synthesized overlapping peptides covering the entire sequence of Bet v 1. The T-cell lines were tested with all peptides in a standard proliferation assay. This analysis of the T-cell lines revealed the presence of 13 distinct T-cell epitopes (Fig. 1b). Donor JN reacted with nine peptides (1–15, 19–33, 29–43, 46–63,

Table 2. Reactivity of Bet v 1-specific TCC with recombinant Bet v 1 and overlapping synthetic peptides

TCC	nat Bet v 1	rec Bet v 1	Epitope [#]
JN08	<u>6.9</u>	<u>6.0</u>	126–140
JN09	<u>94.1</u>	1.0	– [□]
JN10	<u>23.8</u>	<u>24.0</u>	1–15
JN11	<u>8.4</u>	<u>12.3</u>	19–33
HN16	<u>52.9</u>	<u>14.3</u>	1–15
JN26	<u>143.6</u>	<u>185.3</u>	19–33
JN27	<u>34.8</u>	0.7	–
JN29	<u>434.6</u>	1.5	–
JN31	<u>147.4</u>	1.2	–
JN34	<u>5.6</u>	<u>3.7</u>	19–33
JN36	<u>73.1</u>	0.3	–
JN37	<u>45.4</u>	0.9	–
AF03	<u>18.8</u>	<u>17.4</u>	No [§]
AF19	<u>23.4</u>	<u>17.4</u>	No
AF20	<u>54.2</u>	<u>27.6</u>	19–33
AF23	<u>7.2</u>	<u>13.8</u>	99–113
AF24	<u>158.7</u>	<u>154.4</u>	No
AF27	<u>20.1</u>	1.5	–
AF28	<u>27.9</u>	<u>26.5</u>	19–33
AF31	<u>5.6</u>	<u>5.4</u>	19–33
AF34	<u>24.1</u>	<u>14.3</u>	99–113

The table shows the reactivity of 21 TCC to purified native and recombinant Bet v 1 ($10 \mu\text{g/mL}$). Results are shown as specific incorporation relative to the medium values (stimulation index, SI). SI values above 3 are underlined and are considered positive.

[#] TCC reactive with recombinant Bet v 1 were tested with a panel of overlapping synthetic peptides to identify T-cell epitopes. Values shown are amino acid sequences of identified T-cell epitopes. [□] Epitope could not be determined because TCC does not react with recombinant Bet v 1. [§] None of the synthetic peptides was recognized.

58–73, 65–79, 73–87, 117–131, 126–140), donor MK with 10 peptides (1–15, 8–23, 19–33, 29–43, 46–63, 58, 73, 65–79, 82–96, 90–104, 99–113), whereas donor AF reacted with only one peptide (29–43).

Generation and characterization of Bet v 1-specific T-cell clones

Twenty-one T-cell clones reactive to natural Bet v 1 were obtained from donors JN and AF from peripheral blood drawn during birch pollen season. Phenotype analysis showed that 19/21 were CD4⁺, whereas only two clones were CD8⁺ (data not shown). Supernatants from Bet v 1-activated TCC were collected to measure the production of IL-4 and IFN γ . Figure 2 shows that most TCC simultaneously produce IL-4 and IFN γ whereas only a few TCC exclusively produce IL-4 or IFN γ . This is in accordance with findings of Ebner *et al.* who also demonstrated that Bet v 1-specific T

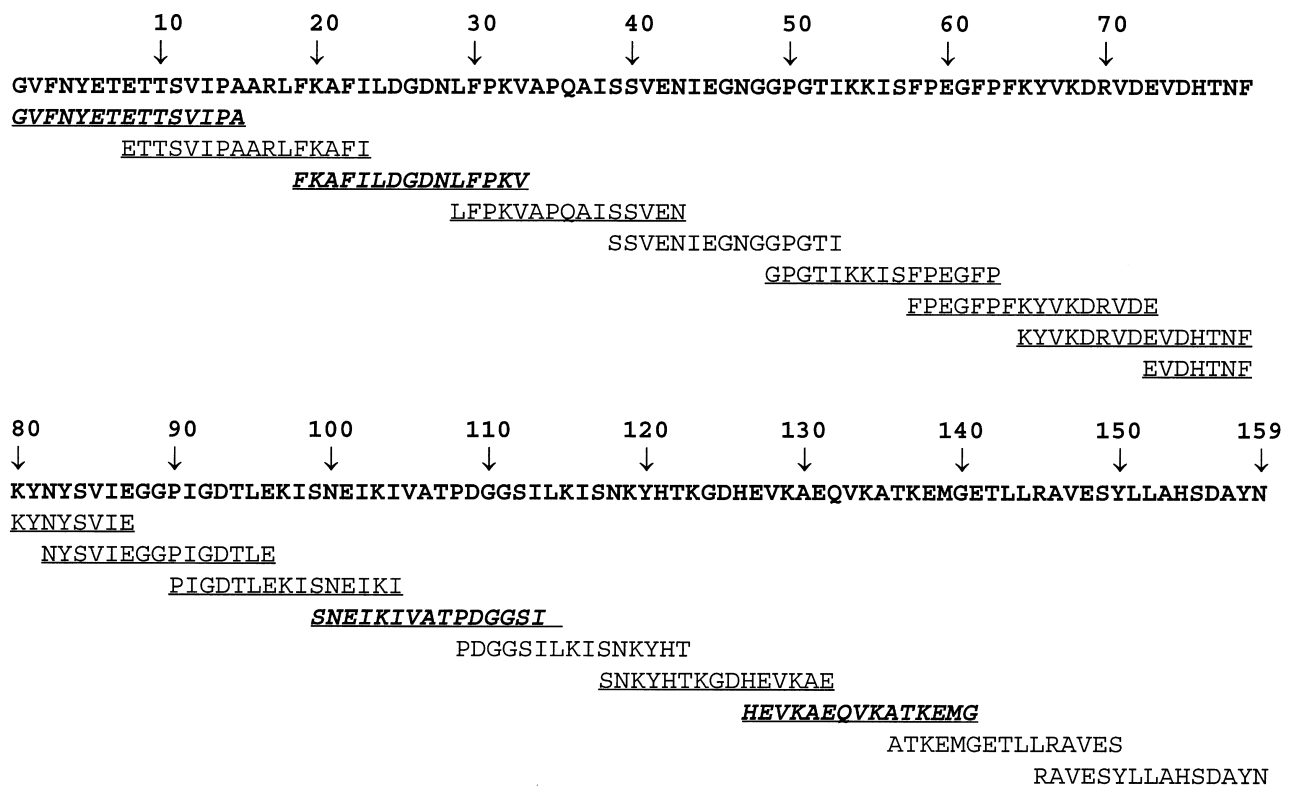


Fig. 3. The figure shows the repertoire of overlapping synthetic peptides covering the amino acid sequence of Bet v 1 used for mapping of T-cell epitopes. Underlined sequences represent peptides that are only recognized by TCL, whereas underlined, italic boldface sequences are recognized by TCL and TCC.

cells from allergic individuals produce both IL-4 and IFN γ [17].

Reactivity of Bet v 1-specific TCC with recombinant Bet v 1 Group 1 allergens from the *Fagales* order isolated from tree pollen extracts are known to consist of many different isoallergens with single or multiple amino acid substitutions. To investigate whether isoallergenic variation is of importance *in vivo*, we tested the responses of these TCC towards a recombinant form of Bet v 1 representing only one of the isoforms. Table 2 shows that 14 out of the 21 TCC reactive to native Bet v 1 did respond to this recombinant form of Bet v 1, whereas seven TCC did not, suggesting that they react with additional Bet v 1 isoforms present in the native Bet v 1.

Epitope mapping of Bet v 1-specific T-cell clones

The T-cell clones that reacted with rBet v 1 were tested for reactivity with the synthetic peptides of Bet v 1. As shown in Table 2, four distinct T-cell epitopes could be identified, namely 1–15 (JN10, JN16), 19–33 (JN11, JN26, JN34, AF20, AF28 and AF31), 99–113 (AF23 and AF34), and 126–140 (JN08). All clones showed strong reactivity with

only a single peptide, whereas a weak crossreactivity was observed with the TCC AF20, AF28, JN11 and JN26, that all reacted with peptide 19–33. Those TCC also reacted weakly with peptide 29–43, suggesting that the most important amino acids of the epitope may reside in the overlap area. The remaining three TCC (AF03, AF19 and AF24) did not react with any of the peptides. Figure 3 summarizes the T-cell epitopes identified in Bet v 1 on the polyclonal and clonal level.

Crossreactivity of Bet v 1 -specific TCC with group 1 allergens from other *Fagales* species Because 7/21 of the TCC did not react with the recombinant Bet v 1 isoform tested, crossreactivity with other group 1 *Fagales* allergens was studied to evaluate their fine specificity. Table 3 shows the crossreactivity of the Bet v 1 reactive TCC to group 1 enriched fractions of related species of the *Fagales* order, Car b (hornbeam), Aln g (alder), Cor a (hazel), and Que a (oak) as well as the reactivity to Bet v 1. The TCC were highly reactive with Bet v 1. Ten out of 21 TCC exhibited crossreactivity with different patterns. These clones could be further subdivided into four groups as three of the clones crossreacted only to Car b 1 (JN31, JN37, AF20), four TCC crossreacted with both Car b 1 and Cor a 1, one TCC reacted

Table 3. Crossreactivity of Bet v 1-specific TCC with Fagales group 1 allergens

TCC	Bet v G75	Car b G75	Aln g G75	Cor a G75	Que a G75
JN08	<u>20.4</u>	1.0	1.2	1.4	1.0
JN09	<u>82.2</u>	0.8	1.0	1.2	1.0
JN10	<u>26.7</u>	1.4	2.0	<u>20.1</u>	1.7
JN11	<u>8.3</u>	0.6	0.6	<u>0.5</u>	0.4
JN16	<u>105.0</u>	0.9	0.4	0.8	1.0
JN26	<u>189.0</u>	1.0	0.7	0.8	0.7
JN27	<u>110.2</u>	<u>58.4</u>	0.7	<u>62.1</u>	0.9
JN29	<u>587.6</u>	<u>259.8</u>	2.0	<u>140.0</u>	2.6
JN31	<u>118.4</u>	<u>8.2</u>	1.0	<u>0.9</u>	1.6
JN34	<u>8.3</u>	0.9	0.6	0.7	0.8
JN36	<u>202.2</u>	<u>89.7</u>	1.4	<u>13.4</u>	1.3
JN37	<u>27.2</u>	<u>7.1</u>	1.8	1.0	0.7
AF03	<u>9.0</u>	0.8	2.3	0.7	0.9
AF19	<u>32.2</u>	1.6	<u>25.1</u>	0.5	0.5
AF20	<u>35.8</u>	<u>3.8</u>	2.8	2.4	1.9
AF23	nt [§]	nt	nt	nt	nt
AF24	<u>144.9</u>	<u>15.7</u>	<u>144.8</u>	2.4	1.0
AF27	<u>14.7</u>	1.6	0.6	1.2	0.8
AF28	<u>18.9</u>	1.0	1.9	0.7	0.1
AF31	2.6	0.8	1.3	0.9	1.9
AF34	<u>3.4</u>	<u>8.1</u>	<u>12.5</u>	2.3	0.8

The table shows the crossreactivity of nineteen Bet v 1-specific TCC with major allergen enriched fractions of related species of the *Fagales* order. Results are shown as specific 3H-TdR incorporation relative to the medium values (stimulation index, SI). SI values above 3 are underlined and considered positive. [§] Not tested.

only with Aln g (AF19), and two reacted with both Car b and Aln g (AF24, AF34). The remaining TCC responded exclusively to Bet v 1. These results suggest, that apart from the rBet v 1 reactive TCC we have identified at least four distinct crossreactivity patterns, which may represent as many T-cell epitope-specificities of additional isoallergens in the Bet v 1 reactive T-cell repertoire of these donors.

Discussion

In this study we have shown that the T-cell response of allergic patients to Bet v 1 is very diverse, and that both Bet v 1 isoallergen-specific, as well as T cells that crossreact with group 1 allergens of other trees belonging to the *Fagales* order can be identified. These data suggest that multiple isoallergenic forms of Bet v 1 are involved in the sensitization to birch pollen.

Most of the Bet v 1-specific TCC described here produced IL-4 and IFN γ simultaneously, whereas a few produced only IL-4 or IFN γ upon Bet v 1-specific stimulation. This is in accordance with findings by Ebner *et al.* [17]. However, in a follow-up study, they could show that TCC from non-allergic controls had a more TH₁-like cytokine production pattern, confirming that allergen-specific T cells from aller-

gic patients have a higher IL-4/IFN γ production ratio than do non-allergic individuals [35]. We are currently establishing Bet v 1-specific TCC from a non-allergic individual to investigate this in our culture system.

Traditionally T-cell epitopes on antigens have been mapped by the use of overlapping peptides. Previous studies on T-cell responses to the house dust mite allergens, Der p 1 and 2 [10–13], bee venom phospholipase A2 [14], rye grass major allergen Lol p 1 [15], the major cat allergen Fel d 1 [16], and to Bet v 1 [17] all suggest that multiple T-cell epitopes are scattered all along the molecules.

By using synthetic peptides based on the sequence of one recombinant isoform of Bet v 1 [22], we could demonstrate the presence of multiple T-cell epitopes of Bet v 1 throughout the molecule. Even though we only studied a limited number of patients, it appears that peptide 19–33 may be a relatively important epitope in the Danish population. Other studies in Austria have previously shown that 77–92 is the most frequently recognized epitope [17, 24].

Many of the Bet v 1-specific TCC in the present study, however, did not react with rBet v 1, suggesting that they may be specific to a different isoallergen of Bet v 1 that is present in purified natural Bet v 1. This is in contrast with earlier studies with Bet v 1-specific TCC, where almost all

native Bet v 1-specific T cells reacted with rBet v 1 [17, 24, 25]. These differences in the recognition of rBet v 1 and epitope-specificity may reflect a difference in genetic background of the patients involved in these studies, but may also be the result of a different exposure to *Fagales* pollen isoallergens.

As a consequence, T-cell epitope mapping by the application of overlapping peptides may not be sufficient to perform a thorough evaluation of epitope-specificity of Bet v 1 reactive T lymphocytes. For this reason we decided to study the crossreactivity of the TCC with a panel of group 1 allergens of other members of the *Fagales* order.

Several of these *Fagales* major allergens have been cloned and sequenced and of others N-terminal aa sequences are available. These data document a high degree of sequence homology between species [20,22,23]. Furthermore, previous studies have demonstrated crossreactivity with other *Fagales* members on the IgE level [23]. This is in accordance with the IgE reactivity of the patients' sera and as expected, the T-cell response of these donors. Natural Bet v 1 -specific T-cell lines showed strong proliferative response to Bet v 1 as well as crossreactivity to group 1 enriched fractions of Car b, Cor a, Aln g, and Que a. Investigation of the TCC revealed several groups of reactivity, suggesting the clones to have different epitope, and possibly isoallergen, specificities (Table 2). These data confirm and extend the previously described crossreactivity patterns of Bet v 1-specific TCC to Cor a 1 and Aln g 1 [25].

From the crossreactivity pattern of the TCC (Table 2) it can be seen that among the TCC that did not react to recombinant there are at least four distinct epitope specificities involved, probably representing as many Bet v 1 isoallergens. This is in accordance with the diversity seen in the IgE binding repertoire to the different isoforms in a 2D-gelelectrophoresis system [19].

Only one recent study to date has directly addressed the occurrence of T-cell reactivity with different isogenic forms of allergens [24]. In this study, isoform-specificity of natural Bet v 1-specific TCC was studied, showing that some amino acid substitutions are permissive, whilst other substitutions abolish T-cell reactivity. These data are in line with the finding in the present study that indicate that Bet v 1-specific TCC that do not react with a rBet v 1 isoform do crossreact with other *Fagales* group 1 allergens. In addition, the correlation between *in vitro* IgE binding and *in vivo* skin reactivity of several high or low IgE binding recombinant Bet v 1 isoforms was demonstrated. T-cell reactivity to low IgE binding isoforms was similar to T-cell reactivity to high IgE binding isoforms. It was suggested that such non-IgE binding Bet v 1 isoforms that have retained their T-cell activating capacities may be used for specific immunotherapy.

We have earlier shown that Car b 1 consists of many different isoallergens of which some exhibit up to 75%

sequence identity with the rBet v 1 isoform. It may be that the crossreactivity that is found between group 1 allergens of the related species is accomplished through sequence homology of only a minor fraction of isoforms of the different species. This is supported by the observation that one of the TCC that does not recognize rBet v 1 reacts with three out of 10 recombinant Car b 1 isoforms tested (R.J.J. van Neerven, unpublished results), and that Schenk *et al.* similarly described a Bet v 1-specific TCC that did not react with rBet v 1 but displayed crossreactivity with two out of four recombinant Cor a 1 isoforms [26].

It is clear that isoallergenic variation is not limited to tree pollen allergens, but can also be found in the mite allergens Der p 1 and Der p 3 [36,37], in the ragweed allergen Amb a 1 [38], and in group 1 and 5 allergens from temperate grasses [39,40]. These observations suggest that multiple isoallergens play an important role in the sensitization to environmental allergens. As a result, if recombinant allergens or peptides are considered in the development of new treatment strategies for type 1 allergic disorders, the significance of isoallergenic variation should be evaluated.

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