

T-Cell Receptor-Mediated Cross-Allergenicity

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Key Words

Allergy · Allergen · T-Cell receptor · T-Lymphocyte · Cross-reactivity

Abstract

Background: Profilins are conserved and ubiquitous plant and animal proteins. We wanted to discover whether the T-cell response to conserved epitopes on birch and grass profilins could account for cross-allergenicity in subjects allergic to these two pollens. **Methods:** Thirty-one patients allergic to grass and birch were recruited for the study. Grass and birch reactive T lymphocytes were studied by measuring proliferation to birch and grass allergen, respectively, followed by V β T-cell receptor family-specific polymerase chain reaction and heteroduplex analysis. T-cell clones were derived from patients with cross-proliferating T cells. **Results:** In 25 of 31 subjects the T-cell response to grass was quite distinct from that to birch. In contrast, in 6 of 31 individuals grass T cells cross-proliferated to birch and this was reproduced in 4 patients by birch profilin. CD4 Th2 cell clones were derived which promiscuously recognized homologously conserved regions on birch and grass profilins. **Conclusion:** We conclude that a functionally relevant T-cell response to conserved regions of panallergens underlie cross-allergenicity in a subset of allergic patients. These results suggest that a reciprocal modulation of the response to one sensitizing allergen can occur following

natural exposure to or immunotherapy with another allergen. These results have relevance in the management of patients with multiple allergies.

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Introduction

Profilins are important panallergens, i.e., allergen components highly conserved in heterogeneous allergen sources [1, 2]. IgE to the birch profilin, Bet v 2, displays a wide spectrum of cross-reactivity because they recognize heterologous profilins from several inhalant and food allergens [3–6]. This phenomenon likely represents the consequence of extreme conservation, among plants and even animals, of ubiquitous proteins that presumably perform vital functions [7]. The present observation can be extended to panallergens in general, including pathogen-related and lipid-transfer proteins [7].

Studies on IgE-based cross-reactivity have demonstrated in great detail the cross-recognition of different allergens via the panallergen components [8–11]. The diagnostic implications of B-cell receptor (IgE)-dependent cross-reactivity among panallergens have been extensively considered [12–17]. In contrast, few studies are available on the T-cell receptor-dependent cross-reactivity to allergens, and these are limited to homologous allergen components within a given allergen source [18–22].

But far more important clinical implications can be envisaged from a cross-reactive T-cell response to allergen mediated by conserved molecules than from mere (IgE-dependent) cross-reactivity. In fact, it is well established that allergen-specific T cells are the major players in the pathogenesis of allergic diseases. Thus, cross-recognition of epitopes of heterologous panallergens by CD4 Th2 lymphocytes could potentially affect the natural history of the disease, depending on natural or immunotherapy-dependent exposure to allergens that, in a T-lymphocyte perspective, were not sensitizing for a given patient.

Here, we investigated the T-cell receptor cross-reactivity via recognition of conserved epitopes of different profilins in a subset of patients allergic to grass and birch. We found that single T-cell clones could recognize phylogenetically conserved epitopes from the grass (Phl p 12) and birch (Bet v 2) profilin molecules.

Characterization of humoral and cellular reactivity to single allergen components in pollen-allergic patients has implications in the design of patient-tailored immunotherapy as well as in the elucidation of the mechanisms of sensitization to multiple allergens.

Patients and Methods

Patients recruited to this study all gave a history of seasonal allergic rhinoconjunctivitis of at least 2 years duration and positive skin-prick test to the following allergen extracts: grass extract, including *Phleum pratense* and tree pollen extract, including *Betula verrucosa* (Alk-Abellò S.p.A., Milan, Italy). The size of the immediate-phase cutaneous responses was assessed 15 min later. The skin prick test was performed according to the recommendations of the European Academy of Allergy and Clinical Immunology [23] and positive results were expressed accordingly (scores ranging from 1 to 4). Median values of skin test scores for grass and birch extracts were 3.37 and 3.16 (interquartile ranges 3–4 and 3.25–4), respectively. Sensitization to these allergens was confirmed by the determination of specific IgE on sera. Allergen-specific IgE assay was performed using Pharmacia's CAP system. The results are reported as fluorescence units and as a percent of the IgE included standard and expressed in KU/l (Pharmacia, Uppsala, Sweden). Median values of grass- and birch-specific serum IgE were 21.24 and 8.18 KU/l (interquartile ranges 12.93–5.47 and 4.56–9.34), respectively. Thirty-one adult and pediatric patients (median value of age 18.8 years, interquartile ranges 14.75–24) were recruited for the study. The total IgE median value was 239.16 KU/l (interquartile ranges 87.25–293.5). They had all received tetanus toxoid vaccination in the last 5 years. Twelve of these patients suffered from mild asthma. None of the patients had received any steroidal therapy (local or systemic) in the last 3 months. Antihistamine drugs were taken on an 'as needed' basis. Informed consent was obtained from adult patients or from parents of pediatric patients.

Reagents

Complete medium used for cell cultures was prepared as follows: RPMI 1640 (PBI, Milan, Italy) was supplemented with gentamicin 5 µg/ml, glutamine 2 mM (PBI) and 5% normal human serum (Danish Red Cross Laboratories, Utrecht, The Netherlands). Allergens were lyophilized commercial preparations of *Phleum pratense* and *Betula verrucosa* from Alk-Abellò (Lainate, Milan, Italy) whose total protein concentrations were calculated using a commercial kit (Biorad Laboratories, Segrate, Italy). Major allergen content in T-cell cultures (Bet v 1 and group V allergens, respectively) was 10 µg/ml in both cases. Recombinant Bet v 2 was purchased from Biomay (Vienna, Austria) and used in cell culture at 10 µg/ml final concentration. Recombinant IL-2 was from Roche (Nutley, N.J., USA). Peptides were synthesized by the solid-phase F-moc method using an Applied Biosystems model 433A peptide synthesizer (Foster City, Calif., USA) [24]. Peptides were purified by semi-preparative reverse phase-high pressure liquid chromatography (RP-HPLC). Linear gradients of acetonitrile in water/0.1% trifluoroacetic acid were used to elute bound peptides. The purity of the peptides (>95%) was confirmed by analytical RP-HPLC, and their mass determined by matrix-assisted laser desorption/ionization time-of-flight analysis with a Voyager-RP Biospectrometry Workstation (PE Biosystems, Inc.). Observed experimental values were in agreement with the theoretical calculated ones. The limulus amoebocyte gelification test (PBI) was used to exclude the presence of bacterial endotoxins in antigens.

T-Cell Epitope Prediction

Allergen sequences were loaded into the HLA-DR ligand prediction software (TEPITOPE) to predict promiscuous HLA-DR ligands. TEPITOPE is based on 25 virtual matrices [25–27] that cover a significant part of human HLA class-II peptide-binding specificity. We selected the HLA-DR alleles most frequent in the Caucasian population, i.e., DRB1*0101 (DR1), DRB1*0301 (DR3), DRB1*0401 (DR4), DRB1*0701 (DR7), DRB1*0801 (DR8), DRB1*1101 (DR5), and DRB1*1501 (DR2), and set the TEPITOPE prediction threshold at 5%. To identify DR ligands within Bet v 2 and Phl p 12 sequences, we selected peptides that were predicted to bind to at least three different allotypes. The human Fcε peptide RKGFRLLNPHPKPNP (Fcε_{240–254}) was included as control. The Phl p 12, Bet v 2 and human Fcε sequences are available from GeneBank/EMBL/DDBJ under the following accession numbers: X77583, M65179 and X06948, respectively.

Direct and Cross-Proliferation Assays

Cell preparations containing T lymphocytes were obtained from each patient starting from peripheral blood mononuclear cells (PBMC) isolated by standard gradient separation (Ficoll-Hypaque, Pharmacia, Uppsala, Sweden) from heparinized blood drawn by venipuncture in the forearm. Cells were cultured with allergen for 5 days; recombinant IL-2 (10 IU/ml) was added for 2–3 days. Cells were then starved from IL-2 for 24 h, and subsequently harvested and stimulated with the same protocol used for the primary stimulation, in the presence of 3,000 rad irradiated autologous PBMC as a source of antigen-presenting cells. The starving procedure allowed reduction of the background proliferation (i.e., in the absence of allergen) in the secondary stimulation, as assessed in parallel 48-hour proliferation assays (not shown). After the second round of stimulation a further 24-hour IL-2 starvation was applied and, subsequently, the direct and the cross-proliferation assays were per-

formed. These were standard 48-hour proliferation assays in which 50,000 cells were plated in 96-well flat-bottom plates (Costar, PBI) in the presence of 100,000 autologous 3,000-rad-irradiated PBMC as a source of antigen-presenting cells. Either the same allergen (homologous stimulation) used to derive the T-cell lines was used or the other one (heterologous stimulation), respectively. Titrated thymidine (^3H -TdR, Dupont, Cologno Milan, Italy) was added (1 μCi /well) in the last 6–18 h of culture. Cells were harvested with a FilterMate Universal Harvester (Perkin Elmer Life Sciences, Boston, Mass., USA) and incorporated thymidine, expressed as counts per minute (cpm), following scintillation counting was used as a measure of allergen-specific cell proliferation (TopCount NXT Microplate Scintillation and Luminescence Counter, Perkin-Elmer Life Sciences). Controls were included (T cells without antigen in the presence of irradiated autologous PBMC). The stimulation index (SI) for each antigen was calculated as the ratio between the mean ^3H -TdR incorporation in test wells versus control wells (containing medium only). In previous experiments in mono-sensitized subjects, allergic to either grass or birch pollens, cpm readings in cultures containing the irrelevant heterologous allergen did not significantly differ from those observed with medium only (not shown). A T-cell line was defined as cross-proliferating when proliferation to the heterologous allergen was above the mean plus 3 SD of the cpm value obtained in control microcultures. All assays were done in quadruplicate and mean values were used for calculation.

Generation of Bet v 2-Specific T-Cell Clones and Characterization of Their Cytokine Profiles

In order to derive Bet v 2-specific T-cell clones from grass-stimulated T cells, Bet v 2 (5 $\mu\text{g}/\text{ml}$) was used in the second round of stimulation in the presence of autologous irradiated PBMC. Cells were cultured for 5 days, then IL-2 (20 IU/ml) was added for 2–3 days and a 48-hour ^3H TdR proliferation assay was performed (following 24-hour IL-2 starvation) with the grass extract and the recombinant Bet v 2, respectively. In 2 patients (patients 26 and 29) where proliferation to Bet v 2 was equal to or higher than proliferation to grass (not shown), T lymphocytes from these cell lines were cloned as described [28]. The specificity of T-cell clones for grass, birch, recombinant Bet v 2 and peptides (6 μM) was determined in 48-hour proliferation assays performed using T cells from the clone and 3,000-rad-irradiated autologous PBMC as antigen-presenting cells in a 1/2 ratio. Supernatants were taken after 24 h of culture from antigen-stimulated clones and frozen at -80°C for analysis of IL-4, IL-5 and interferon- γ . A sandwich ELISA assay was used with proper combination of capture/detection monoclonal antibodies specific to each cytokine (BD Biosciences, Heidelberg, Germany), according to the manufacturer's instructions. The detection range of each assay was from 16 to 2,000 pg/ml. The HLA typing of the 2 patients was as follows: patient 26, DRB1*0301/*1101, DR52, and patient 29, DRB1*02/*07, DR53. The HLA-DR restriction analysis of T-cell clones was performed using autologous PBMC as antigen-presenting cells and blocking antibodies: anti-DR (L243, 1/2 diluted culture supernatant), anti-DQ (SVLP3) or anti-DP (B7.21) both as 1/1,000 diluted ascites.

V β Family-Specific PCR

V β family-specific PCR analysis of grass and birch T-cell lines was performed as described [28, 29]. Briefly, total RNA was extracted using the procedure described by Chomczynski and Sacchi

[30]. One microgram was used for first-strand cDNA synthesis using an oligo (dT) primer and MMLV reverse transcriptase (BRL, Gaithersburg, Md., USA). Polymerase chain reaction (PCR) was then performed using a panel of V β -specific oligonucleotides, paired with a C β primer (5'-TGCTGACCCCACT-GTCGACCTCCTTCCCAT-3') [31].

Heteroduplex Analysis

Ten of the 30- μl PCRs were used to directly visualize the PCR product on a 1% agarose gel. The remaining 20 μl of PCR-amplified products were subsequently run on a 12% native polyacrylamide gel electrophoresis (PAGE) as previously described [28, 29, 32]. Briefly, 20 μl of each amplified fragment was heated at 94°C for 5 min to denature DNA, re-annealed for 1 h at 50°C , and kept at 4°C until gel loading. This temperature is permissive for re-annealing between DNA strains with minor mismatches (heteroduplexes) as well as between homologous strains (homoduplexes). Gene fragments differing for single-point mutations in the N region are detected with this assay [32]. V β -amplified fragments derived from grass and birch T-cell lines were run in parallel lanes. Gels were run at 10 mA overnight at 4°C , stained with ethidium bromide and photographed.

After electrophoresis, DNA was transferred from the acrylamide gel to nylon membranes (Hybond plus, Amersham), as described [29].

Statistical Analysis

The two-tailed Student's *t* test for paired data was used to compare the proliferation values of T-cell lines to allergens with the proliferation values to medium only. The two-tailed probability of the χ^2 distribution was used to compare the number of subjects with T cells expressing one or more common V β family gene in different patients subgroups. The analysis was done with standard statistical software (GraphPad Software Inc., San Diego, Calif., USA). Values of $p < 0.05$ were considered statistically significant.

Results

Proliferation Assays with T Cells from Individuals Sensitized to Grass plus Birch Allergens Show Distinct Patterns of Direct and Cross-Proliferation

Cumulative data on the reactivity of freshly isolated PBMC to grass, birch and tetanus toxoid are shown in table 1. Proliferation of grass-stimulated T-cell lines to grass was highly significant as compared to control microcultures (medium only) as expected (fig. 1a; $p < 0.0001$). Proliferation of grass-stimulated T-cell lines to an irrelevant antigen (tetanus toxoid) did not differ from proliferation to medium only, thus confirming the specificity of the T-cell lines. In contrast, proliferation of grass T-cell lines to birch extract was significantly higher than control (fig. 1a; $p < 0.05$). According to the criteria for cross-proliferation defined in the Patients and Methods section, 6 individuals (patients 7, 26, 28, 29, 30 and 31) were selected whose grass T-cell lines proliferated to birch at val-

Fig. 1. a Cumulative results of cross-proliferation assays. Following 2 cycles of in vitro stimulation and a 24-hour IL-2 starvation period, proliferation of grass T-cell lines was evaluated upon challenge with the antigen indicated on the x-axis presented by autologous antigen-presenting cells. Proliferation was measured as ^3H -TdR incorporation and expressed as counts per minute (cpm), indicated on the y-axis. Values of p refer to paired t test results of the indicated groups of data versus the respective control (no antigen). n.s. = Not significant. **b** Proliferation of grass T-cell lines from 'cross-reactor' patients to birch and to Bet v 2. Proliferation was measured as ^3H -TdR incorporation and expressed as counts per minute (cpm; on the y-axis). Patients who scored positively in the cross-proliferation assay depicted in figure 1a were analyzed with this assay (individual patients are indicated on the x-axis). Antigens used for proliferation are shown in the legend.

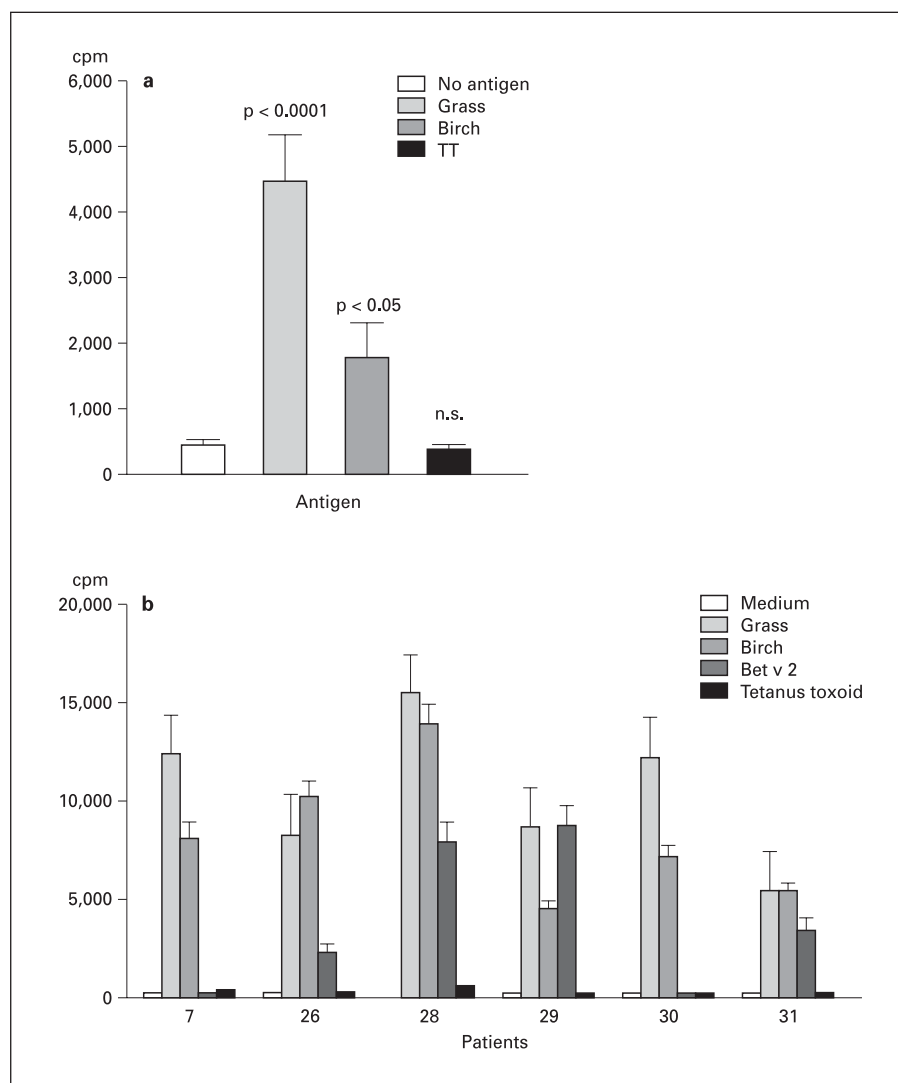


Table 1. Cumulative data on reactivity of PBMC to grass, birch and tetanus toxoid

	No antigen	Grass	Birch	Tetanus
cpm	456 ± 226	4,567 ± 1,232	5,655 ± 1,654	6,776 ± 2,433

Results refer to 5-day proliferation assays with freshly isolated PBMC in the presence of the indicated antigens. Values (mean ± SD) indicate proliferation measured as ^3H -TdR incorporation and expressed as counts per minute (cpm).

ues above the mean plus 3 SD of control microcultures. Blood was taken from these subjects at a second visit, and grass T-cell lines were derived again.

Bet v2 Panallergen Accounts for Cross-Proliferation of Grass T-Cell Lines to Birch in Single Patients

We wanted to know if a known panallergen could be responsible for the cross-reactivity of grass-stimulated T cells in the 6 individuals identified above. We found that in 4 of them proliferation to Bet v 2 was above the cutoff value (mean plus 3 SD of the proliferation values in control microcultures), while no significant proliferation to an irrelevant antigen (tetanus toxoid) was observed (fig. 1b).

TcR Analysis of Grass and Birch T-Cell Lines Confirms Cross-Reactivity in 6 of 31 Patients

Vβ gene-specific PCR analysis of birch and grass T-cell lines from each patient was performed in parallel. In individuals in whom no T-cell cross-reactivity was observed with the proliferation assay, the Vβ gene usage was different in most cases (common Vβ gene usage for at least 1 gene family in 5 of 25 cases). A representative result (patient 3) is shown in figure 2a. In contrast, in the 6 individuals who displayed cross-proliferation at the T-cell level, a widely overlapping Vβ family gene usage was observed at PCR analysis (common Vβ gene usage for at least 1 gene family in 6 of 6 cases; $p < 0.05$). Individual results of PCR analysis are listed in table 2.

When amplified fragments belonging to the same Vβ family were obtained from the compared birch and grass T-cell lines in a given patient, heteroduplex analysis of PCR products could be performed. We found that a pattern characterized by distinct TcR composition was prevalent in patients whose allergen-specific T-cell lines were not cross-proliferating (fig. 2b). In contrast, similarity in the clonal composition of grass and birch T-cell lines was indicated by co-migrating 'twin' bands in patients who

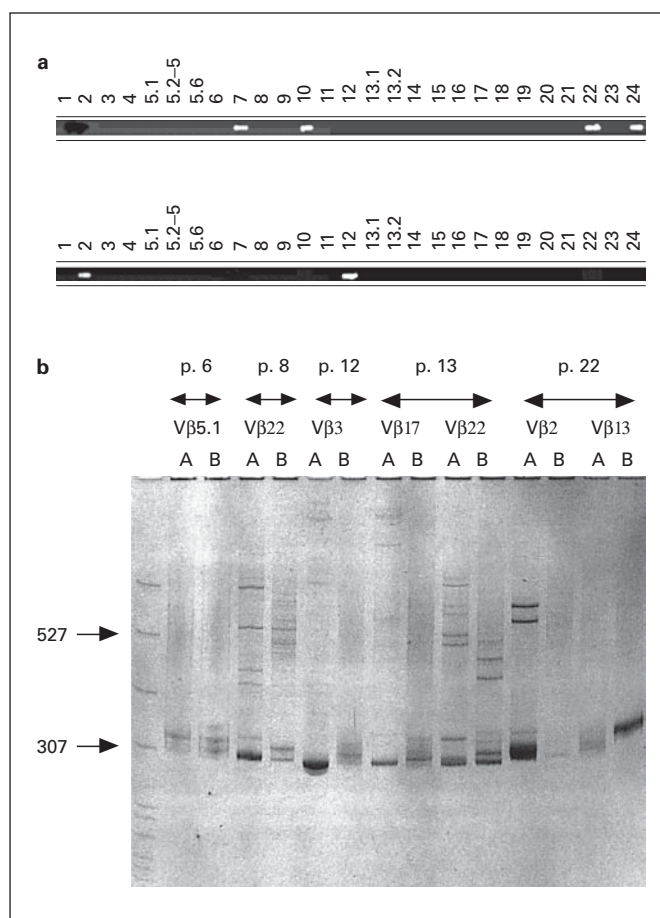


Fig. 2. **a** Family-specific Vβ TcR analysis of grass (top) and birch (bottom) T-cell lines from patient 3. Bands correspond to ethidium bromide-stained fragments, run on agarose gel. **b, c** Heteroduplex analysis of Vβ family fragments from patients who were negative ('non-cross-reactor', **b**) and positive ('cross-reactor', **c**) in the cross-proliferation assay. Fragments belonging to the same gene family and obtained from grass (A) and birch (B) T-cell lines were run in parallel. Patients' (p) identification number is indicated on the top of each lane. Arrows indicate the fragment length at that point, in base pairs, according to a molecular weight marker, which was run in parallel. Bands correspond to Cβ-hybridized fragments following transfer from polyacrylamide gels.

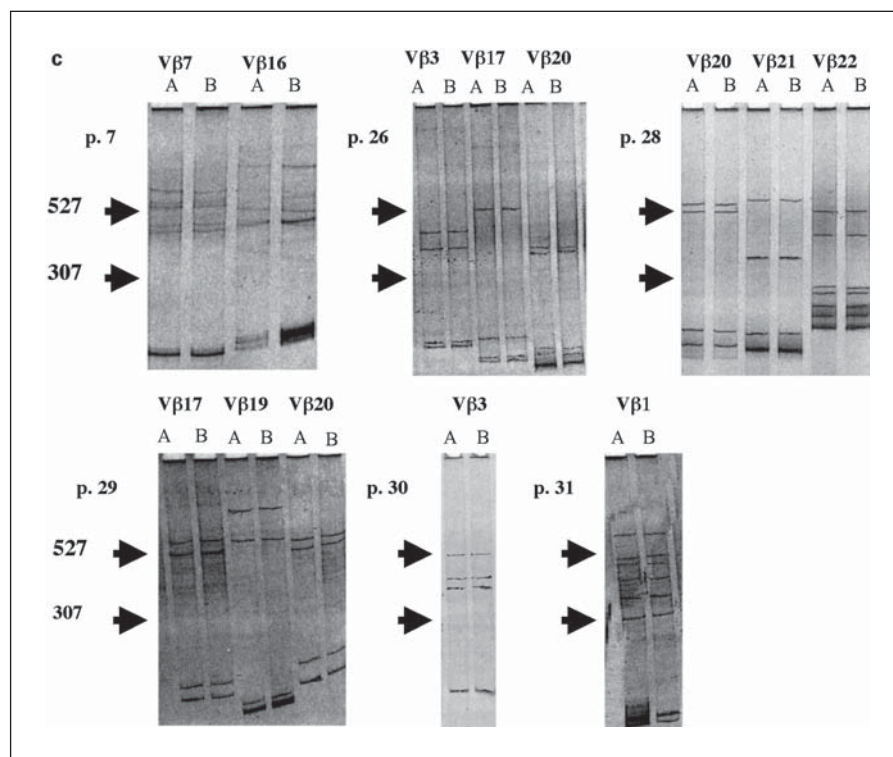


Table 2. V β gene usage of T-cell lines (TCL) from individual patients

Patient No.	Grass TCL	Birch TCL
1	6, 10, 18	4, 5.1, 7
2	5.1, 6	2, 3, 11
3	7, 10, 22, 24	2, 12
4	5.1, 10	17, 22
5	6, 12, 16	5.2, 7.5
6	<u>5.1</u> , 17	<u>5.1</u> , 12
7	<i>1</i> , 7, <i>16</i>	2, 7, <i>16</i>
8	7, <u>20</u>	13.1, <u>22</u>
9	5, 13.1	3, 7
10	3, 8, 11	2, 6, 9, 19
11	2, 16	1, 5.1, 17
12	6, 3, 13	2, 3, 11
13	2, <u>17</u> , <u>22</u>	5.1, <u>17</u> , <u>22</u>
14	6, 22	3, 12, 21
15	6, 24	7, 16
16	13, 16, 22	1, 6, 10
17	1, 5.1	6, 7, 18
18	5.1, 7	2, 7, 11
19	2, 12, 22	5.1, 7
20	1, 13.1	5.2, 9
21	5.2, 12	7, 9, 11
22	<u>2</u> , <u>13</u> , 22	<u>2</u> , <u>13</u> , 22
23	3, 6, 19	1, 9, 11
24	3, 6, 22	5.1, 7, 13.1
25	5.1, 13.1	1, 8, 19
26	<i>1</i> , <u>3</u> , <u>17</u> , <u>20</u>	2, <u>3</u> , <u>17</u> , <u>20</u>
27	3, 6, 17	5.1, 19
28	<i>1</i> , <u>20</u> , <u>21</u> , <u>22</u>	3, <u>20</u> , <u>21</u> , <u>22</u>
29	2, <u>17</u> , <u>19</u> , <u>20</u>	6, <u>17</u> , <u>19</u> , <u>20</u>
30	<u>3</u> , <i>11</i> , 19	<u>3</u> , 6, <i>13.1</i>
31	<u>1</u> , 17, 22	<u>1</u> , 16, 24

Patients displaying cross-reactivity in the proliferation assay are displayed in italics. Common V β gene usage is underlined.

had scored positive in the cross-proliferation assay (fig. 2c). In single cases, clonal identity could be further confirmed by direct sequencing of the T-cell receptor hyper-variable region of the V β genes from gel-eluted twin bands (table 3).

In silico Analysis of Sequences from Birch and Grass Profilins: Alignment and MHC Class-II T-Cell Epitope Prediction

By using the T-COFFEE Version_1.41 software for multiple alignment (34), a region of prevalent homology was identified on the profilin sequences, including those from other pollen allergens (shaded area in table 4). In close proximity to this same region most anchor residues identified by the TEPITOPE algorithm were clustered (table 4, underlined residues). Peptides which were used for in vitro studies were the following: Phl p12₂₇₋₄₁ and its homologous Bet v 2₂₉₋₄₃; Phl p 12₂₂₋₃₆ and its homologous Bet v 2₂₄₋₃₈; Phl p 12₃₈₋₅₂ and its homologous Bet v 2₄₀₋₅₄, and Phl p 12₆₃₋₇₇ and its homologous Bet v 2₆₅₋₇₉.

T-Cell Clones from 2 Patients with Cellular Cross-Reactivity to Grass and Birch Recognize Corresponding Epitopes from Grass Phl p12 and from Birch Bet v 2

Bet v 2-specific T-cell clones were derived from patients 26 and 29 through a screening procedure based on the sequential recognition of the grass extract (used for preliminary expansion of T cells) and of the Bet v 2 recombinant molecule (used for further expansion and selection after cloning). A panel of 27 CD4 T-cell clones, proliferating when in vitro challenged with both the grass extract and Bet v 2, in the presence of autologous antigen-presenting cells, were established from these patients.

Table 3. T-cell receptor N-region sequences of comigrating homoduplex bands obtained from PCR of grass and birch T-cell lines, respectively

Patient No., V β	TCL	V	N-D-N	J	
29, V β 17	Grass	GCC AGT AGT	ATA GCC TAC CTT CGA	CCC CAG CAT	J β 1.5
	Birch	GCC AGT AGT	ATA GCC TAC CTT CGA	CCC CAG CAT	
	a.a.	A S S	I A Y L R	P Q H	
3, V β 3	Grass	GCC AGC AGT	TTA GGG GGG CTC GGG	GAA AAA CTG	J β 1.4
	Birch	GCC AGC AGT	TTA GGG GGG CTC GGG	GAA AAA CTG	
	a.a.	A S S	L G G L G	E K L	

Nucleotide sequences and corresponding amino acids (a.a., indicated according to one-letter codes) are shown. The V β , D β and J β gene families are indicated. TCL = T-cell line.

Table 4. Alignment of profilins from pollen allergens: anchor residues for MHC class-II binding

		-----10-----20-----30-----
Phl p 12 (X77583)	MSWQTYVDEHLMCEI-EGHH---	<u>L</u> ASAAI <u>I</u> GHDGT
Bet v 2 (M65179)	MSWQTYVDEHLMCDI-DGQASN-	SLASAI <u>V</u> GHDGS
Cyn d 12 (Y08390)	MSWQAYVDDHLMCEI-EGHH---	LTSAAI <u>I</u> GHDGT
Ole e 2 (Y12425)	MSWQAYVDDHLMCDI-EGHEDHRLTAAAI <u>V</u> GHDGS	
Par j 3 (Y15208)	MSWQAYVDDHLMCDVGDGNT---	PASAAI <u>I</u> GHDGS
Hev b (Y15042)	MSWQTYVDERLMCEI-EGNH---	LTAAAI <u>I</u> GQDGS
	****:***:***: :*: :*:***:***:	
		----40-----50-----60-----70
Phl p 12	VWAQSADEFQFKPEEITGIMKDFDEPGHLAPT <u>G</u> MFVA	
Bet v 2	VWAQSSSFQFKPQEITGIMKDFEEPGHLAPT <u>G</u> LHLG	
Cyn d 12	VWAQSAAFPQFKPEEMANIMKDFDEPGFLAPT <u>G</u> LFLG	
Ole e 2	VWAQSATFPQFKPEEMNGIMTDFNEPGHLAPT <u>G</u> LHLG	
Par j 3	VWAQSANFPQLKPEEVTGIMNDFNEAGFLAPT <u>G</u> LFLG	
Hev b 8	VWAQSSNFPQFKSEEITAIMSDFDEPGTLAPT <u>G</u> LHLG	
	*****: ** :*:*: :*:**:*.* *****:..	
		-----80-----90-----100-----
Phl p 12	GAKY <u>M</u> VIQGEPRVIRGKKGAGGITIKKTGQALVVGI	
Bet v 2	GIKY <u>M</u> VIQGEAGAVIRGKKGSGGITIKKTGQALVFGI	
Cyn d 12	PTKY <u>M</u> VIQGEPGAIVIRGKKGSGGVTVKKTGQALVIGI	
Ole e 2	GTKY <u>M</u> VIQGEAGAVIRGKKGSGGITIKKTGQALVFGI	
Par j 3	GTKY <u>M</u> VIQGESGAVIRGKKGSGGATLKKTGQAIVIGI	
Hev b 8	GTKY <u>M</u> VIQGEAGAVIRGKKGPGGVTVRKTNQALIIGI	
	*****.* *****.** *:***:***:..**	
		-110-----120-----130
Phl p 12	YDEPMTPGQCNMVVERLGDY <u>L</u> VEQGM	
Bet v 2	YEEPVTTPGQCNMVVERLGDY <u>L</u> IDQGL	
Cyn d 12	YDEPMTPGQCNMVIEKLGDY <u>L</u> IEQGM	
Ole e 2	YEEPVTTPGQCNMVVERLGDY <u>L</u> VEQGM	
Par j 3	YDEPMTPGQCNLVVERLGDY <u>L</u> LEQGL	
Hev b 8	YDEPMTPGQCNMIVERLGDY <u>L</u> LEQGM	
	.:*****:~*:*****:~*~*	

Alignment of profilin sequences from pollen allergens. The accession number according to the GeneBank/EMBL/DBJ database is shown in the first block of alignments. Allergen nomenclature is according to IUIS/WHO Allergen Nomenclature Subcommittee [Bull World Health Organ 1994;72:797–806]. Alignment was performed with the T-COFFEE Version 1.41 software. The symbols, * and :, indicate identity and homology, respectively. The higher homology sequences, cumulatively considered, are shown with a shaded background. Anchor residues for most common MHC class-II alleles, as predicted by the TEPITOPE algorithm, are underlined (see Patients and Methods for details).

Most of these could not be further characterized due to poor in vitro cell growth following multiple re-stimulations. Three clones were further studied, which recognized Bet v 2 peptides designed on the basis of TEPITOPE prediction. In particular, from patient 26 a T-cell

clone was identified (TCC G.R.-A), which recognized peptide Bet v 2_{65–79}. From patient 29 two T-cell clones were identified which recognized 2 distinct predicted Bet v 2 peptides; namely, TCC G.O.-A recognized peptide Bet v 2_{40–54} and TCC G.O.-B recognized peptide

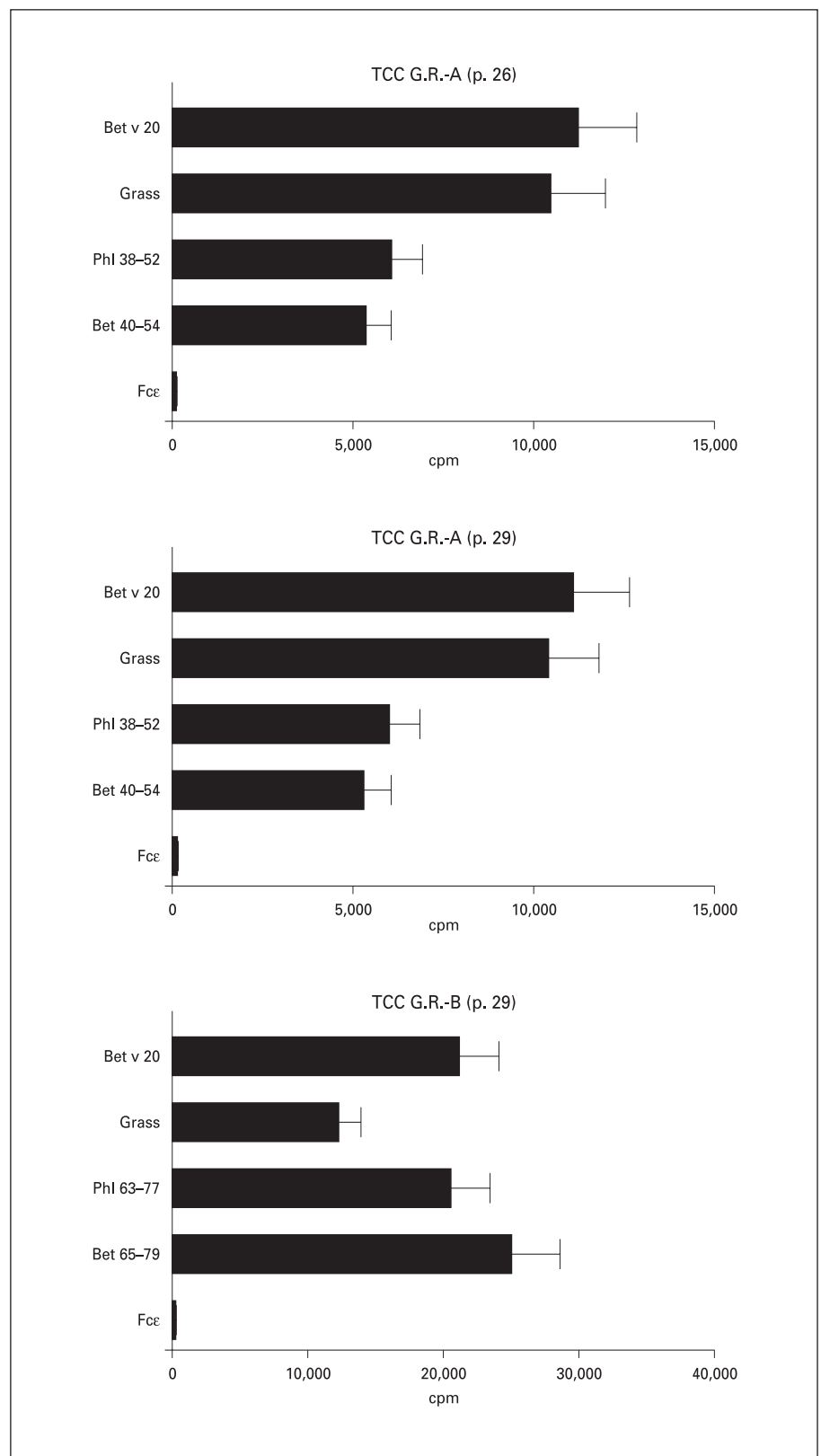


Fig. 3. Epitope mapping of three Bet v 2-specific T-cell clones established from grass T-cell lines. Proliferation was measured as ^3H -TdR incorporation and expressed as counts per minute (cpm; on the x-axis). Patients (P) identification is shown for each clone. Antigens and peptides used for stimulation are shown on the y-axis.

Bet v 2₆₅₋₇₉ (fig. 3). The two cell clones from patient 29 expressed different TcR V β (not shown). The three T-cell clones specific to predicted Bet v 2 epitopes were CD4 positive and had lymphokine profiles compatible with a Th2 phenotype (not shown). Clone TCC G.R.-A also significantly proliferated to peptide Phl p 12₆₃₋₇₇, which corresponded to the sequence of the grass profilin homologous to Bet v 2₆₅₋₇₉. Similarly, clones TCC G.O.-A and TCC G.O.-B recognized peptides Phl p 12₃₈₋₅₂ and Phl p 12₆₃₋₇₇ (fig. 3), respectively, which are homologous to the corresponding Bet v 2 peptides. No proliferation to control peptides was observed with any clone. All clones were DR restricted, since proliferation to the allergen of each clone was inhibited by a blocking monoclonal antibody specific to DR (L2342), but not DP (B7.21) or DQ (SVLP3) (not shown).

Discussion

In this work we evaluated T-cell receptor-mediated cross-reactivity to allergens in a cohort of patients sensitized to both birch and grass.

By using a cross-proliferation assay with purified allergen extracts, we found that 6 of 31 individuals displayed a significant T-lymphocyte-dependent cross-reactivity (i.e., grass T-cell lines proliferated to birch). We found that recombinant Bet v 2, the birch profilin, could reproduce this cross-reactivity in 4 of 6 patients. In the 2 remaining patients (patients 7 and 30) the clonal size of the profilin-specific T cells might have been below the limit of detection of the assay. Alternatively, in these patients' cross-reactivity might have been induced by different panallergens (e.g., Bet v 4, a calcium-binding protein highly homologous to grass Phl p 7). Unfortunately, we could not cross-check whether birch T-cell lines, which were similarly cross-reactive upon stimulation with grass (not shown) could respond Phl p 12 (the Bet v 2 homologous), since it was not available.

In order to confirm that cross-reactivity was T-cell receptor-dependent, we then used V β family-specific PCR. We found that the grass and birch T-cell lines of the 6 patients with cross-reactive T lymphocytes more often shared V β family genes as compared to 'non-cross-reactive' patients ($p < 0.05$). Furthermore, when a higher resolution approach was used (heteroduplex analysis), a distinct clonal composition was found in T-cell receptor 'non-cross-reactive' patients, while the expansion of identical clones was prevalent in the 'cross-reactive' patients. Notably, the expansion of identical clones was observed

also in patients 7 and 30, whose T-cell cross-proliferation could not be accounted for by Bet v 2, further suggesting that in these cases other panallergens could explain T-cell receptor-mediated cross-reactivity.

We then derived CD4 T-cell clones from 'cross-reactive' patients and epitope-mapped them by using an *in silico* prediction with a validated algorithm [27] on the basis of Bet v 2 and Phl p 12 sequences. We found T-cell clones that recognized with similar efficiency one of the predicted Bet v 2 epitopes and its corresponding Phl p 12 counterpart. The fact that these epitopes mapped to the most conserved part of the profilin molecule support the hypothesis that also at the T-cell level it is indeed the structural homology that is making profilins the most important panallergens. To our knowledge, this is the first time that T-cell cross-reactivity of allergen-specific T lymphocytes via a panallergen epitope is demonstrated at the single T-cell level.

In principle, a B-cell receptor cross-reactivity, based only on the recognition by IgE antibodies of common conformational epitopes on conserved molecular motifs, could *per se* explain all clinical and biological observations on cross-allergenicity. In this scenario, there would be a single repertoire of T cells specific, for example, for a given profilin that would obviously support production of profilin-specific IgE. In contrast, we show here that in a significant proportion of patients IgE cross-reactivity can result from the T-cell help provided to B cells by CD4 Th2-lymphocytes activated by a different panallergen. Notably, the 4 patients with Bet v 2 cross-reactive T lymphocytes had high titers of serum IgE binding to Bet v 2 and Phl p 12 (not shown). In this perspective, IgE cross-reactivity is not to be considered the pure epiphenomenon of the conservation of structural motifs among panallergens. In fact, the T-cell response has by far the prevalent pathogenic role in the generation, maintenance and determination of the clinical severity of the allergic disorders. Thus, natural exposure to, or immunotherapy with allergens containing 'third-party' profilins could directly modulate the clonal size and/or the functional phenotype of T lymphocytes specific to a single ('*bona fide*' sensitizing) profilin. This might significantly influence the clinical outcome of the allergic disease either in a favorable or a negative manner.

On the basis of our results it can be speculated that, in the natural history of allergy, individuals who initially have a single allergic sensitization could become polysensitized through the mechanism of epitope spreading of the allergen-specific immune response. This may be initiated by the cross-recognition of allergenic compo-

nents highly homologous in different allergenic sources, such as panallergens. Therefore profilins, which per se are minor allergens, i.e. they are recognized only by a subset of patients, could have a peculiar relevance by supporting

the generation of multiple sensitizations. Our results stress the relevance of component-resolved diagnosis [12] in the characterization, the follow-up and the management of allergic patients.

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