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Article type : Original Article: Experimental Allergy and Immunology

Strong and frequent T-cell responses to the minor allergen Phl p 12 in Spanish patients IgE-sensitized to Profilins

Short title: T-cell responses to Profilin in Spanish patients.

Keywords: Cross-reactivity ,Grass, Phl p12, Profilin, T-cell

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Abstract

Background: Profilins are dominant pan-allergens known to cause cross-sensitization, leading to clinical symptoms such as the pollen food syndrome. This study aims to determine the T-cell response to Phl p 12 in profilin-sensitized patients, by measuring the prevalence, strength and cross-reactivity to clinically relevant profilins.

Methods: The release of Phl p allergens from pollen was determined by mass spectrometry and immunochemistry. T-cell responses, epitope mapping and cross-reactivity to profilins (Phl p 12, Ole e 2, Bet v 2, and Mal d 4) were measured *in-vitro* using PBMCs from 26 Spanish grass allergic donors IgE-sensitized to profilin. Cross-reactivity was addressed *in-vivo* using two different mouse strains (BALB/c and C3H).

Results: Phl p 12 and Phl p 1 are released from pollen simultaneously and in similar amounts. Both T cell response frequency (17/26 donors) and strength were comparable between Phl p 12 and Phl p 1. T-cell cross-reactivity to other profilins correlated with overall sequence homology, and two immunodominant epitope regions of Phl p 12 were identified. Data from mice immunized with Phl p 12 showed that cross-reactivity to Bet v 2 was mediated by conserved epitopes, and further influenced by additional genetic factors, likely to be MHC II.

Conclusion: The strength, prevalence and cross reactivity of T-cell responses towards Phl p 12 is comparable to the major allergen Phl p 1, which supports the hypothesis that T-cells to Phl p 12 can play an important role in development of allergic symptoms, such as those associated with the pollen-food syndrome.

Introduction

IgE sensitization to profilins is strongly associated with the pollen-food syndrome (PFS), also known as oral allergy syndrome (OAS) (1). Allergenic profilins are exclusively derived from plants, they are, highly conserved (thus, immunologically cross-reactive) and classified as minor allergens. First

described as allergenic molecules in 1991 (2) , their presence in all types of plant tissues makes them an archetypical pan-allergen protein family but, until recently, they were believed to induce at most very mild allergic reactions upon exposure, either by inhalation or ingestion.

Sensitization to profilins is probably induced in almost all cases by pollen inhalation, grass pollen being the dominant pollen sensitizer in southern Europe (3), due to its relatively high profilin content compared with other pollen species (4). The role of profilin as a respiratory allergen has recently been demonstrated by using pure profilin in bronchial provocation (4). In this context, profilin-sensitized patients can also develop the PFS. Due to low stability of profilins in acid pH, most of these patients develop only mild food allergy, but patients overexposed to grass pollen can develop potentially severe profilin food allergic reactions (1). These data suggest that profilin plays a relevant clinical role and, due to its pan-allergenic nature, continuous exposure to pollens and vegetable foods could induce a sustained allergic phenotype in sensitized subjects.

Following this hypothesis, profilin induced T-cell reactivity would contribute to this phenotype. T-cell responses to profilin have been reported in a few studies (5-8). Although relatively little is known about the role of minor allergens in overall T-cell reactivity to a sensitizing source, there is increasing evidence that Th2 responses towards proteins not classified as major allergens could contribute substantially to the T-cell response towards whole extract reactivity (9).

Here we examined the specific immune response to Phl p profilin in patients allergic to grass and sensitized to Phl p 12. The release kinetics of allergens (including Phl p 12) from Phl p pollen was compared to the prevalence and strength of T-cell responses towards Phl p 12 and Phl p 1, in profilin IgE sensitized donors recruited in the Madrid area, which is a geographical region where the average profilin sensitization prevalence in pollen allergic patients is 15% (10). Cross-reactivity was addressed by comparing the T-cell responses to different plant profilins and by T-cell epitope mapping using a set of overlapping peptides covering the Phl p 12 sequence. The relationship between T cell epitopes

and cross-reactivity was further studied *in-vivo*, comparing the response towards Phl p12 and Bet v2 in two different mouse strains (BALB/c and C3H).

Materials and Methods

Pollen allergen release kinetics determined by LC-MS/MS

Two g of raw Phl p pollen (ALK) was extracted in 20 ml PBS (pH 7.2), and samples were taken at several time points between 20 seconds and 2 hours after initiation. Pollen grains were removed by filtering through a PD10 PE bed-filter, followed by a 5 μ m (Millex) and a 0.8 μ m (Millex) filters. Samples were stored on ice until analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The protein content was determined using the Pierce BCA assay (Prod #23227, Thermo Fischer Scientific). For LC-MS/MS, a volume corresponding to 40 μ g protein was evaporated, denatured (6 M urea, 0.3 M NH_4HCO_3), reduced (9 mM DTT), alkylated (17 mM Iodoacetamide), and finally digested with 10% (w/w) trypsin at 37 °C overnight. Peptides were separated and analyzed by LC-MS/MS: Reverse phase liquid chromatography (Ultimate 3000 RSLC nano, Thermo Fischer Scientific) with a C18 analytical column (flow rate 300 nl/min, 4 hour gradient from 4-55 % acetonitrile). Eluting peptides were sprayed into an ESI-QTOF mass spectrometer (Maxis, Bruker), spectra acquired in the mass range of 50-2200 m/z at 2 Hz, and MS/MS sequencing at a spectral rate of 4-16 Hz. Data analysis: MS/MS spectra were searched (via MASCOT search engine, Matrix Science) against an in-house allergen database that included the protein sequences of all IUIS Phl p allergens and isoforms thereof. The sum of the relative and semi-quantitative Exponentially Modified Protein Abundance Index (emPAI) scores (11) of all Phl p allergens were set to 100%, and the relative percentage (molar %) of each allergen was calculated.

Pollen allergen release kinetics determined by single radial immunodiffusion

Single radial immunodiffusion (SRID) was performed with polyclonal monospecific rabbit anti-nPhl p 1, 5, or 12 antibodies cast into agarose gel (1% HSA agarose in Tris/Veronal buffer, I=0.02, pH=8.6), and allergens were applied to wells in the gel. After diffusion (approx. 40 hrs), plates were pressed, washed and stained in a 0.5% Coomassie Blue R-250 solution. Quantification was performed relative to an internal standard reference preparation (nPhl p 1, 5, or 12), applied as a dilution series on the same plate.

Donor selection

A prospective study was designed, comprising a detailed clinical history, *in-vivo* tests, and blood drawing for *in-vitro* assays. During a 4-month running period, every patient attending the Allergy Section of La Princesa University Hospital in Madrid, showing profilin sensitization (defined as a positive skin test to a commercially available profilin SPT, (natural purified Pho d 2) (12), confirmed by IgE test) (Table 1) and willing to participate in the protocol, was included. Written informed consent was obtained from all participants, and the study protocol was approved by the Research and Ethic Committee of the Hospital.

In-vitro human T-cell reactivity

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood according to standard procedure and stored in liquid nitrogen. Phl p 12-specific T-cells were expanded *in-vitro* and specific responses measured by Fluorescent based ImmunoSpot (FluoroSPOT). Briefly, frozen PBMCs were thawed, CD8 removed using magnetic CD8 Dynabeads (kat 111.47 D, Dynal), stimulated with recombinant Phl p 12 (10 ng/ml) or natural purified Phl p 1 (10 ng/ml) and expanded for 14 days with addition of IL-2 on days 5, 6, and 7 (Proleucin, Chiron). On day 14, the T-cell lines were tested for specific induced cytokine by stimulation with 2 µg/ml of different profilins,

allergens and controls (in triplicates), at 10^5 cells/well in FluoroSpot plates: IL-5/IFN γ FluoroSPOT (Mabtech FS-0108-10), for 16-20 h at 37°C. Fluorospot were developed and measured according to the manufacturer's protocol. The remaining T-cells were re-stimulated (2×10^5 /ml) with 10 ng/ml of rPhl p 12 or nPhl p 1, in the presence of autologous irradiated PBMC (10^6 /ml) and PHA (0.5 μ g/ml), and cultured for additional 10 days (IL-2 added on days 3, 4, and 5). IL-5/IFN γ response to allergens (or peptides) were measured by FluoroSPOT, as described above, with T-cells (3×10^4 /well) added, and also autologous PBMC (2×10^4 /well, irradiated 3000 Rad) as antigen presenting cells (APC). Criteria for positivity responses were determined relative to cells without stimuli (medium): Data with significant positive responses (student T-test, $p > 0.05$), with at least 20 spot forming cells (SFC) per 10^6 PBMCs above baseline, and with a stimulation index > 2 , were used for comparing the magnitude of response, and all other data were set to zero. Donor coverage was calculated from the number of positive responses relative to the number of valid (PHA positive) T-cell lines. However, only positive responses with $> 5\%$ of maximal response for the individual donor were taken into account in order to eliminate borderline responses.

T-cell responses in mice immunized with natural Phl p 12

Animals (8-10 weeks old, Tactonic, Denmark) were sensitized to nPhl p 12 by means of 2 intraperitoneal injections of 200 μ g of nPhl p 12 emulsified in Al(OH) $_3$ (ALK, Denmark), in a total volume of 250 μ L, on days 0 and 7 of the experimental protocol. Ten days after the second nPhl p 12 sensitization, a single cell suspension of spleen cells was prepared by meshing spleens through a 70 μ m filter. The splenocytes (3×10^6 /ml) were cultured in the presence of overlapping Phl p 12 peptides (4 μ g/ml), nPhl p 12, or nBet v 2 (either 10 μ g/ml (BALB/c mice), or 20 μ g/ml (C3H mice)). On day 5, ^{3} H-Thymidine was added, and 18h later proliferation was measured by scintillation counting. Results are expressed as stimulation

index (SI), calculated out of three replicates. All animal experiments were approved and performed in accordance with Danish and international guidelines and legislations.

Results

Pollen allergen release kinetics

The release of Phl p allergens from raw pollen grains in an aqueous buffer was investigated using both semi-quantitative MS and quantitative single radial immunodiffusion (SRID) assays, in order to compare the kinetics and amounts of released allergens. Using the semi-quantitative MS technique, polypeptide masses from all known IUIS allergens were included in the analysis (i.e., Phl p 1, 2, 4, 5, 6, 7, 11, and 12). Release of allergens was measured at several time points, from 20 seconds to 2 hours after initiation of aqueous extraction performed under non-denaturing conditions and at physiological pH. The relative abundance of the various Phl p allergens in each sample (i.e., at each time point) was calculated as described in the Methods section. All allergens were released from pollens during the investigated period of 2 hours, albeit with variations in both kinetics and amounts (Figure 1A). Phl p 6 was generally the dominating allergen at all time points investigated, and the relative amount of released Phl p 5 was increasing over time. The molar amounts and release kinetics of Phl p 1 and Phl p 12 were comparable between them and clearly lower than Phl p 5 at most times. To further investigate the quantitative aspect of Phl p 12 release relative to major allergens, the release kinetics of Phl p 12, Phl p 1 and Phl p 5 was determined on an absolute scale using SRID assays and purified fractions of the respective allergens as reference material for quantification (Figure 1B). In contrast to the MS analysis, the amounts determined by SRID are absolute and can therefore be compared between time points thereby providing independent confirmation of the MS results..

T-cell responses to Phl p 12 in profilin IgE-sensitized grass-allergic donors

T-cell lines specific to Phl p 12 (Figure 2A) were established from PBMCs of 26 Spanish grass allergic patients sensitized to profilin (Table 1). In parallel, T-cell lines were established against Phl p 1 for most donors, and T-cell responses towards Phl p 12, Phl p 1, Phl p 5, and whole *Phleum* extract, were investigated for all lines generated. In total, we could establish Phl p 12 specific T-cell lines from 58% of the donors, and 83% when T-cell lines were raised against the major allergen Phl p 1. Only 8% and 6% of the Phl p 12 or Phl p 1 T-cell lines, respectively, were reactive when re-stimulated with Phl p 5, which confirmed the specificity of the T-cell assays. The strength of the T-cell responses was assessed by the number of IL-5 producing cells determined by FluoroSpot (Figure 2B). Phl p 12 responses were similar in magnitude to the whole *Phleum* extract and to responses against Phl p 1.

Epitope mapping and T-cell cross-reactivity to clinically relevant profilins

The T-cell cross-reactivity between Phl p 12 and other clinically relevant plant profilins from olive, birch, apple and date palm was determined by re-stimulating T-cell lines raised against rPhl p 12 with purified preparations of these profilin variants. Both the response prevalence (Figure 3A) and strength in particular (Figure 3B) were reduced at lower levels of sequence conservation (relative to Phl p 12), indicating the importance of sequence conservation of specific T-cell epitopes in mediating cross-reactivity. To address this further, T-cell lines specific for Phl p 12 were re-stimulated using a set of overlapping peptides covering the Phl p 12 sequence (Figure 3C). Three immunodominant regions in the Phl p 12 sequence, positions 22-51, 62-81, and 92-121, appear to harbour T-cell epitopes that are recognised by considerably more donors than the rest of the Phl p 12 sequence. These immunodominant T-cell epitopes are located in Ole e 2/Phl p 12 conserved (position 92-121) as well as non-conserved (position 22-51/62-81) regions of Phl p 12, as illustrated in Figure 3D. T-cell responses from individual donors towards these epitopes (not shown) further show that the

majority of cross reactivity to Ole e 2 was found in donors with response to the conserved region (position 92-121).

Influence of MHC-II haplotypes on cross-reactivity of Phl p 12

T-cell activation and cross-reactivity was investigated in strains of inbred mice (BALB/c and C3H) that have different MHC-II haplotypes (H-2D and H-2K, respectively), and they are therefore expected to bind different subsets of peptides. The T-cell response to Phl p 12 was determined using *in-vitro* stimulation of splenocytes isolated from BALB/c and C3H mice immunized with nPhl p 12. Epitopes were determined using the same set of overlapping peptides (Figure 4A), and the cross-reactivity was determined by stimulating T-cell cultures with purified Phl p 12 and Bet v 2 (Figure 4B). Peptide 12 (amino acid position 12-31 in the Phl p 12 sequence) was the only 20-mer causing T-cell activation in BALB/c mice. Sequence conservation between Phl p 12 and Bet v 2 in this region is relatively low (80%), explaining the lack of cross-reactivity to Bet v 2 (Figure 4B). In contrast, Phl p 12 positions 2-22, 12-32, 60-80, and 110-130, elicited significant T-cell activation in C3H mice. Several of these peptides include regions of profilin that are fully conserved between Phl p 12 and Bet v 2, explaining the observed T-cell cross-reactivity to Bet v 2 in Phl p 12 immunized C3H mice.

Discussion

T-cells epitopes involved in allergic responses have been characterized in great detail (13) for most major allergens. In contrast, T cell responses to minor allergens are addressed in far fewer and typically more limited studies (5-7;14). Certain classes of minor allergens from pollens, such as profilins and LTP, are known to cause of food allergy (15) leading to PFS. Understanding what drives the the cellular immunological response to such molecules is therefore of significant clinical significance.

Profilins are ubiquitous proteins, present in all eukaryotic cells and identified as minor allergens in pollen, latex and plant foods. As a class, profilins are categorized as pan-allergens due to their

allergenicity and highly conserved structure leading to highly cross-reactive IgE antibody responses (16). Sensitization to plant food profilins is almost exclusively seen in patients with specific IgE against pollen profilins and can lead to clinical symptoms like pruritus and oedema of the oral mucosa within minutes upon ingestion of profilin containing foods, typically melon, watermelon, tomato, banana, pineapple and orange. The clinical relevance of profilin in PFS has been demonstrated by provocation tests (4;17). Grass pollen profilin is found to be the primary profilin sensitizer in most profilin-allergic patients with PFS (3). However, other pollens such as Mugwort (3), Ragweed (18) or Birch (19) may act as profilin sensitizing pollens, depending on geographical differences in pollen exposure. In areas with higher grass pollen exposure, like Extremadura in the Western part of Spain, profilin allergy is linked to a more severe phenotype (1) with patients suffering from severe profilin mediated food reactions and in general the prevalence of profilin-sensitization and associated PFS is relative high across Spain.

Quantity and speed of allergen release from pollens on the mucosa may influence allergenicity. By mass spectrometry, it was evident that all known allergens were released almost instantly. Phl p 6 was clearly the dominating allergen during the whole time course of extraction (Figure 1A). It constituted approx. 70 % (molar %) of the allergens after 20 seconds and this fraction fell to approx. 45 % after two hours. Despite its high quantity and fast release, sensitization to Phl p 6 in grass allergic populations is less prevalent (44-75 % (20;21)) than to the two major allergens Phl p 1 and Phl p 5 (43-96% and 50-95%), respectively (20)). Further, we found that Phl p 12 was released from pollen in the same timeframe as the other Phl p allergens and in amounts comparable to Phl p 1. Despite these similarities, the difference in IgE sensitization prevalence between the major allergen Phl p 1 and the minor allergen Phl p 12 indicated the amount and temporal release of proteins from phl pollens does not fully predict allergenicity of grass pollen proteins.

To investigate the role of T-cell reactivity towards profilin, we initially sought to determine T-cell responses to Phl p 12 in grass pollen allergic patients living in Denmark. Only a few patients showed weak and inconsistent responses to profilin (data not shown), and it was speculated that it might be

caused by the generally low sensitization to profilin in Scandinavia (around 8%; ALK unpublished data). The present study is therefore based on a cohort of Phl p12-sensitized patients recruited in Madrid, where profilin sensitization is more common, around 15% (10), and often found in association with PFS. In contrast to the Danish cohort, T-cells from the Spanish patients responded vigorously to profilin and were comparable to the major allergen Phl p 1, in terms of both response prevalence and strength. Thus, the T cell response to Phl p12 is influenced by geographical conditions in this case likely to be the level of grass pollen exposure (1). Further, the similar responses to major and minor allergens suggest that their distinction is mainly relevant from a population point of view, and not a meaningful at the patient level. Once sensitized, the immune system responds equally to major or minor allergens, and the difference in allergenicity lies mainly in the initial sensitization of the immune system, starting with sensitization to major allergens like Phl p 1 and Phl p5, followed by molecular spreading to minor allergens like Phl p 12 (22).

Human T-cell responses to profilin have been reported in a few studies. One of them demonstrates T-cell cross-reactivity to defined Phl p 12/Bet v 2 peptide epitopes (7), while others indicate that overall T-cell cross-reactivity between Phl p 12 and Par j 3 is very limited (5;6). The T-cell epitopes identified by Burastero *et al.* (7) show that T-cell cross-reactivity may occur, even for a 15-mer peptide (Phl p 12, 63-77) containing six mismatches between the Phl p 12 and the Bet v 2 epitope. Recently, T-cell epitopes in Amb a 8 (Amb a 8, 41-55 and 25-39) were identified in American patients (14), but cross-reactivity to other profilins was not investigated.

Alignment of the investigated profilin sequences from different plants shows that most potential T cell epitopes had few to several mismatches to the corresponding Phl p 12 epitope, which offers an explanation for the observed correlation between overall sequence homology and T-cell cross reactivity (Figure 3D). The strong reactivity to Ole e 2 found in the current study was thus likely the direct result of the complete sequence identity between Phl p 12 and Ole e 2 in the Phl p 12 92-121 region, even though some cross-reactivity in the Phl p 12 22-51 region cannot be excluded, because only 5 widely dispersed mismatches are found between Phl p 12 and Ole e 2 in this region. In further

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support, T-cell responses of the individual patients (data not shown) showed that cross reactivity to Ole e 2 was found in all donors with response to the corresponding conserved region in Phl p 12 (position 92-121). The correlation between T cell cross-reactivity and epitope conservation was also observed *in-vivo*. Only mice responding to conserved areas were able to recognize both Phl p 12 and Bet v2 (Figure 4) upon immunization with a single component. Furthermore, the difference in epitope recognition patterns between the two strains of mice showed that cross-reactivity to plant profilins was influenced by genetic determinants, most likely the MHC II haplotype of the mouse strain. Translating this observation to humans, it suggests that certain donors might be genetically predisposed to develop cross-reactive T-cell responses to profilin, by a preferential presentation of conserved peptides by their HLA molecules, and thereby being more prone to become multi-sensitized to different species of profilins with potential clinical implications.

In conclusion, we found that Phl p 12 was released from pollen simultaneously and in similar amounts as Phl p 1. In a cohort of 26 IgE-sensitized grass-allergic donors recruited in Madrid, Phl p 12 induced T-cell responses that were comparable in prevalence and strength to the major allergen Phl p 1. Epitope mapping showed that immunodominant areas of Phl p 12 were in some cases conserved, leading to cross-reactivity to other profilins that correlated with sequence homology. T cell responses in mice immunized with Phl p 12 and Bet v2 showed that additional genetic factors, likely to be MHC II, influenced cross-reactivity patterns. Taken together, these results support the hypothesis that T-cells play an important role in the development of allergic symptoms, such as those associated with the pollen-food syndrome.

Acknowledgements

We thank Duarte de Sousa for his work as study nurse.

Conflict of Interest

DB has worked on consultant basis for ALK-Abelló

CB has received speaker's honoraria from ALK-Abelló and Merck.

GL, SB, LHC, IH, KHM, PAW, PSA, PB, MA are employee at ALK- Abelló

Author Contributions

TR, FV and CB were the clinicians responsible for patient recruitment, *in-vivo* SPT tests and patients' sample collection; they also contributed to manuscript writing and approved its final version. SB was responsible for mouse experiments, GL for T-cell experiments and LHC for allergen release kinetic data.

PSA and PAW wrote the manuscript and all authors contributed to this.

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Patient no.	Age (y)/sex	Total IgE (kU/L)	Pollen allergy	Phleum pratense		Plant-food allergy	Profilin	
				SPT*	slgE#		SPT*	slgE#
1	40/M	1350	RC	6	nd	-	6	16.3
2	55/F	81	RC	8	10.7	-	7	0.1
3	32/F	26	RC, BA	10	12	PFS	6	2.5
4	29/F	242	RC, BA	8	9.8	PFS	9	3.5
5	45/M	794	RC	9	23.1	EIA	7	2.8
6	47/F	158	RC, BA	11	29.8	PFS, U	10	1
7	35/M	866	RC	12	86.2	PFS	8	2
8	32/M	533	RC, BA	9	nd	PFS	6	1.4
9	23/M	nd	RC, BA	6	nd	-	7	2.6
10	31/F	170	RC	11	3.9	PFS	9	2.3
11	50/M	15	RC, BA	5	0.8	PFS	5	1.4
12	37/M	166	RC, BA	3	0.4	-	4	0.8
13	44/F	295	RC	4	11.6	PFS	7	0.6
14	48/F	214	RC	10	20.9	PFS	8	0.5
15	41/F	156	RC, BA	12	29.6	PFS	5	0.4
16	24/F	112	RC, BA	5	35.8	PFS	5	3.4
17	25/F	168	-	5	19.4	A	7	1.0
18	53/M	807	RC, BA	9	18.6	-	8	5.0
19	19/M	1349	BA	6	52.1	PFS	8	18.9
20	18/M	299	RC	9	55	PFS	8	6.7
21	39/M	1106	RC	4	27.8	PFS	10	5.3
22	45/F	70	RC	4	4.5	PFS	10	2.1
23	27/F	107	RC, BA	7	22.6	-	6	0.5
24	63/M	1419	RC	7	80	PFS	8	3.7
25	33/M	145	RC, BA	14	19.1	PFS	10	6.3
26	35/M	533	RC	5	7.5	PFS	7	0.4

F, female; M, male; RC, rhinoconjunctivitis; BA, bronchial asthma; nd, not done; PFS, oral allergy syndrome;

EIA, exercise-induced anaphylaxis; U, urticaria; A, anaphylaxis

*SPT to commercial Phleum (ALK, Spain Catalog No. 215) or date palm profilin (ALK, Spain Catalog No. 869), mean wheal diameter in mm

#slgE as determined by ImmunoCAP, in kU/L



