ORIGINAL ARTICLE

IgE Reactivity to Polcalcins Varies According to Pollen Source

Asero R[1], Mistrello G[2], Amato S[2]

[1]Ambulatorio di Allergologia, Clinica San Carlo, Paderno Dugnano (MI), Italy
[2]Lofarma Spa, Milano, Italy

J Investig Allergol Clin Immunol 2016; Vol. 26(6): 362-365
doi: 10.18176/jiaci.0054

Abstract

Background: Polcalcins are highly cross-reactive pollen panallergens. Less than 10% of allergic patients are sensitized to polcalcins. All pollen species are considered able to sensitize patients to this panallergen.
Objective: We aimed to assess the presence of polcalcins in various pollen extracts used in allergen immunotherapy.
Methods: ELISA inhibition experiments were performed with sera from patients sensitized to polcalcin and rPhl p 7 and rBet v 4. Recombinant polcalcin was used as the substrate and freshly prepared pollen extracts as inhibitors.
Results: All pollen extracts induced significant inhibition of IgE reactivity to rBet v 4, whereas only grass pollen extract induced marked inhibition of IgE reactivity to rPhl p 7.
Conclusion: Grass polcalcin probably contains more epitopes than polcalcins from other pollen sources. Grass pollen could be responsible for sensitization to polcalcins, and grass pollen immunotherapy is likely to be an option for polcalcin-hypersensitive patients.

Key words: Polcalcin. Pollen allergy. Panallergens. Sensitization. Cross-reactivity.

Resumen

Antecedentes: Las polcalcinas son panalérgenos de alta reactividad cruzada en pólenes capaces de sensibilizar a un 10% de los pacientes alérgicos. Todas las especies de pólenes se consideran capaces de sensibilizar pacientes mediante este panalérgeno.
Objetivo: El objetivo de este trabajo fue analizar la presencia de esta polcalcina en diferentes extractos de pólenes que se utilizan en inmunoterapia.
Método: El suero de pacientes reactores a polcalcina, así como frente a rPhl p 7 y a rBet v 4 fue analizado mediante ensayo de ELISA inhibición, utilizando polcalcina recombinante como sustrato y extracto de pólenes como inhibidores.
Resultados: En cuanto a los resultados obtenidos, todos los extractos de pólenes indujeron una inhibición significativa de la reactividad de la IgE frente a rBet v 4, mientras que solo el extracto de polen de gramíneas inducía una marcada inhibición de la reactividad de la IgE frente a rPhl p 7.
Conclusión: La polcalcina de gramíneas probablemente contiene más epítopes que las polcalcinas de otras fuentes. El polen de gramíneas podría ser responsable de la sensibilización a la polcalcina y la inmunoterapia con polen de gramíneas es probablemente una opción para los pacientes hipersensibles a polcalcina.

Palabras clave: Polcalcina. Alergia a polen. Panalérgenos. Sensibilización. Reactividad cruzada.

Introduction

Polcalcins are 2 EF–hand calcium-binding proteins present in the pollen of all flowering plants, including grasses, trees, and weeds [1-7]. About 40 members of this protein family have been identified to date. Fewer than 10% of pollen-allergic patients are sensitized to these minor allergens [8], although this percentage may vary among individuals sensitized to specific pollen sources [1,9-11]. The high degree of sequence homology in polcalcins underlies their extensive cross-reactivity [12-14], although Phl p 7 (the grass polcalcin) seems to be the strongest IgE binder [12]. The clinical relevance of sensitization to polcalcins is unclear, as no investigations have been performed on the role of these aeroallergens using nasal or bronchial challenge tests. In a recent clinical study, a minority of patients experienced respiratory symptoms throughout the flowering period of all the pollen species analyzed, although most had symptoms limited to a specific period, generally springtime [8]. All pollen species are considered able to induce polcalcin sensitization; however, it is difficult to demonstrate which allergens a patient is sensitized to, because most reactors show primary hypersensitivity to ≥1 allergen source [15]. Allergen immunotherapy is currently the only treatment able to modify the natural history of allergic disease. In view of the high homology of polcalcins, extracts from all pollen species might theoretically be able to desensitize hypersensitive patients, although this hypothesis has not been investigated to date. The area of Milan, Italy, has features of both the Mediterranean climate and the central European climate and is characterized by the presence of significant levels of the most relevant pollens in Europe, including grass, mugwort, ragweed, pellitory, birch, hazel, plane, cypress, plantain, and Oleaceae (mainly privet and ash). It is therefore an ideal site to investigate the origin of sensitization to polcalcins. We analyzed various pollen extracts to determine their polcalcin content and assessed the immune reactivity of the polcalcins using 2 recombinant members of this allergen family.

Patients and Methods

The study was performed with sera from 9 polcalcin-hypersensitive adults (3 males, 6 females), all of whom experienced cutaneous reactions to at least 4 pollen sources in skin prick tests (SPT) with commercial extracts of grass, mugwort, ragweed, pellitory, plantain, birch, plane, olive, and cypress (Allergopharma) [8] and strong IgE reactivity to Phl p 7 (grass polcalcin) in ImmunoCAP (Thermo Fisher Scientific).

Extracts of grass, birch, ragweed, pellitory, and olive pollen (Allergon) were freshly prepared using defatted pollen, which was extracted (8%) in 0.1 M phosphate-buffered saline (PBS), pH 7.4, after shaking overnight at 4°C. After centrifuging, the supernatant was harvested and dialyzed against the same buffer. Protein content, which was measured following the method of Bradford [16], was 3.3 mg/mL, 0.8 mg/mL, 2.6 mg/mL, 2.3 mg/mL, and 1.0 mg/mL for grass, birch, ragweed, pellitory, and olive pollen extracts, respectively. All extracts were used to inhibit the reactivity of the patients' sera to recombinant grass and birch polcalcins (rPhl p 7, and rBet v 4, Thermo Fisher Scientific) in an ELISA. Briefly, rPhl p 7 or rBet v 4 was used at a concentration of 0.25 µg/100 µL of coating buffer (15 mmol/L Na_2CO_3, 35 mmol/L $NaHCO_3$, pH 9.6) per well to coat 96-well plates (Maxisorp Nunc). After washing with 0.1 M PBS, pH 7.4, and 0.05% Tween 20 (Sigma), the wells were saturated with 2% bovine serum albumin in PBS (dilution buffer) for 2 hours at room temperature. After further washing, 100 µL of control and patient sera diluted 1:4 in PBS was added to the wells and incubated for 2 hours at room temperature. The wells were then washed again, and bound specific IgE was detected by adding peroxidase-conjugated goat antihuman IgE (diluted 1:3000, Biospacific); the colorimetric reaction was developed using tetramethyl-benzidine/H_2O_2 as the substrate. The enzyme reaction was stopped after 20 minutes by the addition of HCl 1 mol/L, and absorbance values were read at 450 nm using a spectrophotometer. IgE levels were expressed as optical density units (OD ×1000). Based on the mean ±2SD obtained in normal controls, values <300 OD were considered negative. In inhibition studies, patients' sera were pre-absorbed overnight at 4°C with 50 µg of pollen or house dust mite extract (negative control) (Lofarma) in a final volume of 200 µL.

In order to detect the origin of polcalcin sensitization in this geographic area, we reviewed clinical and serological data from 52 consecutive polcalcin-hypersensitive patients seen at the Clinica San Carlo, Paderno Dugnano, Italy. All patients experienced cutaneous reactions to at least 4 of the following seasonal allergens: grass, mugwort, ragweed, pellitory, plantain, birch, plane, olive, and cypress. Polcalcin reactivity was demonstrated by levels of IgE to Phl p 7 >1 kU_A/L (ImmunoCAP, Thermo Fisher Scientific) and, in 23 cases, also by strong skin reactivity during SPT with a date palm polcalcin [8]. IgE to markers of primary sensitization to seasonal allergen sources (Phl p 1, Phl p 5, Bet v 1, Art v 1, Amb a 1, Par j 2, Cup a 1, and Ole e 1 [ImmunoCAP]) was determined in all 52 patients. Levels >0.35 kU_A/L were considered positive.

Results

The results of the ELISA inhibition experiments using recombinant Phl p 7 and Bet v 4 as substrates are shown in Table 1. The absorption of the serum pool with all pollen extracts resulted in significant inhibition of IgE reactivity to rBet v 4. In contrast, only grass pollen extract induced marked inhibition of IgE reactivity to rPhl p 7 (87%), whereas the inhibition induced by pre-absorption with all the other extracts did not exceed 33%. A house dust mite extract used as a control did not induce inhibition.

The findings for the 52 polcalcin reactors are shown in Table 2. Component-resolved diagnosis showed that most patients were sensitized to >1 allergen source. Grass pollen hypersensitivity was detected in 51 cases (98%), followed by hypersensitivity to ragweed (79%) and birch pollen (62%). Genuine olive, cypress, ragweed, and pellitory sensitization was detected in ≤38% of patients. Only 6 patients (12%) were sensitized to a single pollen source (grass in 5 cases, ragweed in 1 case).

Table 1. ELISA Inhibition Studies Using rPhl p 7 and rBet v 4 as Substrate and Fresh Pollen Extracts as Inhibitors

Serum	rPhl p 7 or rBet v 4, OD[a]	Inhibition by Birch Extract, %	Inhibition by Grass Extract, %	Inhibition by Ragweed Extract, %	Inhibition by Olive Extract, %	Inhibition by *Parietaria* Extract, %	Inhibition by House Dust Mite Extract, %
BC (Phl p 7)[b]	1932	50	91	17	ND	ND	5
Pool Phl p 7[c]	2085	23	87	11	33	27	0
Negative Serum (Phl p 7)	389	ND	ND	ND	ND	ND	ND
Pool Bet v 4	3110	64	87	46	79	71	1

Abbreviation: ND, not done.
[a]IgE reactivity to rPhl p 7 and rBet v 4 is expressed in optical density units (OD ×1000).
[b]BC is the serum from an individual patient;
[c]Pool: serum pool from all 9 study patients. The degree of inhibition is expressed as the percentage of reduction of IgE reactivity following serum pre-incubation with the different pollen extracts.

Table 2. Sensitization to Individual Pollen Sources in 52 Patients Sensitized to Polcalcin

Pollen source	Polcalcin
Grass	51 (98%)
Birch	32 (62%)
Pellitory	11 (21%)
Olive	20 (38%)
Mugwort	9 (17%)
Ragweed	41 (79%)
Cypress	16 (31%)

Discussion

The aim of the present study was to confirm the presence of polcalcins in freshly prepared extracts of pollen from various sources for possible use in allergen immunotherapy for allergic patients. To this end, inhibition experiments were performed with sera from polcalcin reactors and recombinant polcalcins from 2 sources (Phl p 7 from grass and rBet v 4 from birch). To our surprise, the 2 recombinant polcalcins showed marked differences in IgE reactivity: while all pollen extracts were able to significantly inhibit IgE reactivity to rBet v 4, only grass pollen extract was able to markedly inhibit rPhl p 7. We cannot rule out the possibility that the use of a pool of sera rather than individual sera from allergic patients may have influenced the results, as IgE reactivity to polcalcins can vary between patients. In effect, using serum from a single patient, the level of inhibition induced by birch extract increased from 23% to 50% (Table 1). Furthermore, as we did not measure polcalcin content in the pollen extracts used to perform the experiments, the results might simply reflect differences in levels of polcalcin in the extracts. Nonetheless, since the experiments were carried out using identical concentrations of both sera and rBet v 4 and rPhl p 7, another possible interpretation of our findings is that grass pollen polcalcin contains more epitopes than homologous allergens from all other pollen sources; alternatively, the results could indicate a polcalcin isoform that does not show the whole repertoire of allergenic epitopes of the natural counterpart. This would explain why all of the extracts were able to neutralize most IgE reactivity to rBet v 4 and why all of the extracts except grass were not able to neutralize IgE reactivity to rPhl p 7, which was abolished by grass pollen extract. Unfortunately, this interpretation lacks further support, since no polcalcins other than rPhl p 7 and rBet v 4 are currently on the market. Our findings suggest that grass pollen could be responsible for sensitization to polcalcin, at least in this geographic area. In fact, although most polcalcin reactors were primarily sensitized to several pollen sources (up to 6 in some cases), grass sensitization was detected in all patients except one, who was monosensitized to ragweed. These observations are in keeping with clinical data from our group showing that most polcalcin reactors have symptoms mainly in spring (8) and confirm previous findings by Tinghino et al [14] showing that Phl p 7 is the strongest IgE-binding polcalcin. Variations in polcalcin content between pollens, along with the burden of each pollen in this geographic area, might explain how patients become sensitized to polcalcins, as was previously shown for profilin [17]. Furthermore, previous studies found that sensitization to polcalcins correlates both with a higher number of primary pollen sensitizations and with disease duration [18]. The current results seem to support these findings, again suggesting the high complexity of polcalcin sensitization.

Although demonstration of IgE reactivity to polcalcins was based on the detection of IgE to Phl p 7, we do not believe that the study population was affected by a relevant selection bias, as all patients experienced skin reactions to various pollen extracts, and, in 23 cases, the patients reacted to polcalcin from date palm pollen, which is not present in this area. Thus, grass pollen polcalcin seems to contain specific epitopes that react to IgE from sensitized individuals (at least those living in this geographic area) and that are missing in homologous allergens from other sources. Another possible explanation for our results might be that although we were able to investigate only 2 recombinant proteins, natural polcalcins have several

isoforms; therefore, we cannot rule out the possibility that the available isoforms do not contain the whole repertoire of allergenic epitopes present in their natural counterpart.

On the basis of these observations, we can conclude that grass pollen immunotherapy is probably the treatment of choice in severely polcalcin-sensitized patients living in this geographic area. The choice of a grass extract to treat these patients might depend not only on the primary sensitization source, but also on the content of polcalcin in grass formulas currently used for immunotherapy. However, this aspect should be investigated by checking the kinetics of specific IgG4 to Phl p 7 during immunotherapy.

Acknowledgments

The authors thank Jonas Lidholm (Thermo Fisher Scientific) for the gift of recombinant Phl p 7 and Bet v 4.

Funding

The authors declare that no funding was received for the present study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

1. Engel E, Richter K, Obermeyer G, Briza P, Kungl AJ, Simon B, Auer M, Ebner C, Rheinberger HJ, Breitenbach M, Ferreira F. Immunological and biological properties of Bet v 4, a novel birch allergen with two EF-hand calcium-bindings domains. J Biol Chem. 1997;272:28630-7.
2. Tinghino R, Barletta B, Palumbo S, Afferni C, Iacovacci P, Mari A, Di Felice G, Pini C. Molecular characterization of a cross-reactive Juniperus oxycedrus pollen allergen, Jun o 2, representing a novel calcium-binding allergen. J Allergy Clin Immunol. 1998;101:772-7.
3. Weber RW. Patterns of pollen cross-allergenicity. J Allergy Clin Immunol. 2003;112:229-39.
4. Ferreira F, Hawranek T, Gruber P, Wopfner N, Mari A. Allergic cross-reactivity: from gene to the clinic. Allergy. 2004;59:243-67.
5. Mari A, Wallner M, Ferreira F. Fagales pollen sensitization in a birch-free area: a respiratory cohort survey using fagales pollen extracts and birch recombinant allergens (rBet v 1, rBet v 2, rBet v 4). Clin Exp Allergy. 2003;33:1419-28.
6. Wopfner N, Gruber P, Wallner M, Briza P, Ebner C, Mari A, Richter K, Vogel L, Ferreira F. Molecular and immunological characterization of novel weed pollen pan-allergens. Allergy. 2008;63:872-81.
7. Wopfner N, Dissertori O, Ferreira F, Lackner P. Calcium-binding proteins and their role in allergic diseases. Immunol Allergy Clin N Am. 2007;27:29-44.
8. Asero R, Jimeno L, Barber D. Preliminary results of a skin prick test–based study of the prevalence and clinical impact of hypersensitivity to pollen panallergens (Polcalcin and Profilin). J Investig Allergol Clin Immunol. 2010;20:35-8.
9. Compès E, Hernandez E, Quirce S, palomares O, Rodriguez R, Cuesta J, Sastre J, Villalba M. Hypersensitivity to black locust (Robinia pseudoacacia) pollen: "allergy mirages". Ann Allergy Asthma Immunol. 2006;96:58-92.
10. Pico de Coana Y, Parody N, Fuertes MA, Carnes J, Roncarolo D, Ariano R, Sastre J, Mistrello G, Alonso C. Molecular cloning and characterization of Cup a 4, a new allergen from Cupressus arizonica. Biochem Biophys Res Commun. 2010;401:451-7.
11. Nouri HR, Sankian M, Vahedi F, Afsharzadeh D, Rouzbeh L, Moghadam M, Varasteh A. Diagnosis of Chenopodium album allergy with a cocktail of recombinant allergens as a tool for component-resolved diagnosis. Mol Biol Rep. 2012;39:3169-78.
12. Valenta R, Hayek B, Seiberler S, Bugajska-Schretter A, Niederberger V, Twardosz A, Natter S, Vangelista L, Pastore A, Spitzauer S, Kraft D. Calcium-binding allergens: from plant to man. Int Arch Allergy Immunol. 1998;117:160-6.
13. Mari A. Multiple pollen sensitization: a molecular approach to the diagnosis. Int Arch Allergy Immunol. 2001;125:57-65.
14. Tinghino R, Twardosz A, Barletta B, Puggioni EM, Iacovacci P, Butteroni C, Afferni C, Mari A, Hayek B, Di Felice G, Focke M, Westritschnig K, Valenta R, Pini C. Molecular, structural, and immunologic relationships between different families of recombinant calcium-binding pollen allergens. J Allergy Clin Immunol. 2002;109:314-20.
15. Villalta D, Asero R. Analysis of the allergenic profile of patients hypersensitive to pollen pan-allergens living in two distinct areas of Northern Italy. Eur Ann Allergy Clin Immunol. 2011;43:54-7.
16. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem. 1976;72:248-54.
17. Ruiz-Garcia m, Garcia del Potro M, Fernandez-Nieto M, Barber D, Jimeno-Nogales L, Sastre J, Profilin: a relevant aeroallergen? J Allergy Clin Immunol. 2011;128:416-8.
18. Barber D, de la Torre F, Lombardero M, Antépara I, Colas C, Dávila I, Tabar AI, Vidal C, Villalba M, Salcedo G, Rodríguez R. Component-resolved diagnosis of pollen allergy based on skin testing with profilin, polcalcin and lipid transfer protein pan-allergens. Clin Exp Allergy. 2009;39:1764-73.

Manuscript received December 10, 2015; accepted for publication January 22, 2016.

Riccardo Asero

Ambulatorio di Allergologia, Clinica San Carlo
Via Ospedale 21
20037 Paderno Dugnano (MI), Italia
E-mail r.asero@libero.it