POLCALCINS FROM DIFFERENT POLLEN SOURCES SHOW DIFFERENT IgE REACTIVITY

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ABSTRACT

Background: Polcalcins are highly cross-reacting pollen panallergens able to sensitize less than 10% of allergic patients. All pollen species are considered able to sensitize patients to this panallergen.

Objective: We aimed to assess the presence of polcalcin in different pollen extracts for allergen immunotherapy

Methods: Sera from polcalcin reactors as well as rPhl p7 and rBet v 4 were checked in an ELISA inhibition assay, using the recombinant polcalcin as 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 a 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 polcalcin and grass pollen immunotherapy is likely to be an option for polcalcin hypersensitive patients.

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

Resumen

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.

El objetivo de este trabajo fue analizar la presencia de esta polcalcina en diferentes extractos de pólenes que se utilizan en inmunoterapia.

El suero de pacientes reactores a polcalcina, así como frente a as rPhl p7 y a rBet v 4 fue analizado mediante ensayo de ELISA inhibición, utilizando polcalcina recombinante como sustrato y extracto de pólenes como inhibidores.

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 two EF-hand calcium-binding proteins present in pollen of all flowering plants, including grasses, trees, and weeds [1-7]. About 40 members of this protein family have been identified so far. Polcalcins are minor allergens able to sensitize less than 10% of the whole pollen allergic patients [8], although this proportion may change among subjects sensitized to specific pollen sources [1, 9-11] . Their high degree of sequence homology is at the basis of their extensive cross-reactivity [12-14], with Phl p 7 (the grass polcalcin) appearing as the strongest IgE-binder [12]. The clinical relevance of sensitization to polcalcins is unclear as no investigations on the role of these aeroallergens by nasal or bronchial challenge tests have been carried out so far; in a recent clinical study, a minority of patients showed respiratory symptoms throughout the flowering period of all pollen species while most had symptoms limited to one specific period, generally springtime [8]. All pollen species are considered able to induce polcalcin sensitization although this is difficult to demonstrate because most reactors show primary hypersensitivity to more than one 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 of all pollen species might theoretically be able to desensitize hypersensitive patients, although this has not been investigated so far. The area of Milan, Italy, shows both Mediterranean and central European climate features, being characterized by the presence of significant levels of most relevant pollens present 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 polcalcin sensitization. We analyzed different pollen extracts for their content in polcalcin and assessed the immune reactivity of the different polcalcins by the use of two recombinant members of this allergen family.

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PATIENTS AND METHODS

Sera from 9 polcalcin-hypersensitive adults (M/F: 3/6) were used for the study. All showed skin reactivity to at least four different pollen sources on SPT with commercial extracts of grass, mugwort, ragweed, pellitory, plantain, birch, plane, olive, and cypress (Allergopharma, Reinbeck, Germany) [8)], and a strong IgE reactivity to Phl p 7 (grass polcalcin) on ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden).

Extracts of grass, birch, ragweed, pellitory, and olive pollen (Allergon, Ängelholm , Sweden) were freshly prepared using defatted pollen, extracted (8 %) in 0.1M phosphate-buffered saline, pH 7.4 (PBS) shaking over-night at 4 °C. After centrifuging, the supernatant was harvested and dialyzed against the same buffer. Protein contents, measured after Bradford [16] were 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 patients' sera reactivity to recombinant grass and birch polcalcins, rPhl p 7 and rBet v 4 (Thermo Fisher Scientific, Uppsala, Sweden), in an ELISA test. Briefly, rPhl p 7 or rBet v 4, both 0.25 µg/100 µl of coating buffer (15 mmol/L Na_2CO_3 ,35 mmol/L $NaHCO_3$, pH 9.6) per well were used in the coating of 96-microtitre plates (Maxisorp Nunc, Roskilde, Denmark). After washings with 0.1 M phosphate-buffered saline, pH 7.4 (PBS), and 0.05% Tween 20 (Sigma, Milan, Italy), wells were saturated with 2% bovine serum albumin (BSA) in PBS (dilution buffer) for 2 hours at room temperature. Subsequently, after further washing, 100 µl of control and patient sera diluted 1:4 in dilution buffer were added to the wells and incubated for 2 hours at room temperature. Wells were then washed and bound specific IgE was detected by adding a peroxidase-conjugated anti-human IgE from goat (diluited 1:3000, Biospacific, Emeryville, CA, USA); the development of the colorimetric reaction was induced by using tetramethyl-benzidine/H_2O_2 as substrate. The enzyme reaction was stopped after 20 minutes by the addition of 1 mol/L HCl, and absorbance values were read at 450 nm by a spectrophotometer. IgE levels were expressed as optical density units (OD x 1000). Based on the mean + 2SD of IgE levels found in normal control values less < 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, the clinical and serological data of 52 consecutive polcalcin-hypersensitive patients seen at the Clinica San Carlo were reviewed. All patients showed skin reactivity to at least 4 seasonal allergen sources out of grass, mugwort, ragweed, pellitory, plantain, birch, plane, olive, and cypress. Polcalcin reactivity was shown by IgE levels to Phl p 7 exceeding 1 kU/L (ImmunoCAP, ThermoFisher) and in 23/23 cases also by strong skin reactivity on SPT with a date palm polcalcin [8]. All these patients underwent the measurement of IgE to markers of primary sensitization to seasonal allergen sources, including 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 by ImmunoCAP. Levels exceeding 0.35 kU/l were considered positive.

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RESULTS

Results of ELISA inhibition experiments using recombinant Phl p 7 and Bet v 4 as substrate 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 a 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 control did not induce any inhibition.

The findings in 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 51/52 of cases (98%), followed by hypersensitivity to ragweed (79%), and birch (62%) pollen. Genuine olive, cypress, ragweed, and pellitory sensitization was detected in 38% or less. Only 6/52 (12%) patients were sensitized to one single pollen source (grass in 5 cases, ragweed in 1 case).

DISCUSSION

This study started with the aim to confirm the presence of polcalcin in freshly prepared extracts of pollen from different sources for a possible use in allergen immunotherapy for allergic patients. To this end, sera from polcalcin reactors and two recombinant polcalcins from different sources (Phl p 7 from grass and rBet v 4 from birch) were used for inhibition experiments. To our surprise, the two recombinant polcalcins showed marked differences in IgE reactivity: while all pollen extracts were able to significantly inhibit IgE reactivity to rBet v 4, with rPhl p 7 this strongly happened only with grass pollen extract. We cannot rule out that the use of a pool of sera rather than individual sera from allergic patients may have somehow influenced the results, as IgE reactivity to polcalcins could vary among patients. In effect, using the serum from a single patient the level of inhibition induced by birch extract increased from 23 to 50% (table 1). Further, as we did not measure polcalcin in pollen extracts used to perform the experiments the results might simply reflect different polcalcin levels present 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 or, alternatively, that results are the due to the use of a polcalcin isoform that does not show the whole repertoire of allergenic epitopes present on the natural counterpart. This would explain why all extracts were able to neutralize most Bet v 4 IgE reactivity, and why all extracts (other than grass) were not able to neutralize Phl p 7 IgE reactivity, which was on the other hand abolished by grass pollen extract. Unfortunately, no polcalcins other than rPhl p 7 and rBet v 4 are currently on the market in order to further support this interpretation. Our findings suggest that grass pollen could be responsible for polcalcin sensitization, at least in this geographic area. In effect, 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 mono-sensitized to ragweed. These observations are in keeping with clinical data from our group that most polcalcin reactors have symptoms mostly in spring (8) and confirm previous findings by Tinghino and co-workers showing that Phl p 7 is the strongest IgE-binding polcalcin [14]. The different content of polcalcin in various pollen sources along with the burden of each

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pollen in this geographic area might explain how patients get sensitized to polcalcin, as has been shown before for profilin [17]. Further, previous studies found that polcalcin sensitization correlates with a higher number of primary pollen sensitizations and with disease duration [18]. The current results seem to support these findings, suggesting again a higher complexity of this type of patients.

Although the demonstration of the polcalcin IgE reactivity was based on the detection of IgE to Phl p 7, we don't believe that our population was characterized by a relevant selection bias, as all these subjects showed multiple skin reactivity to pollen extracts and in 23/23 cases reacted to polcalcin from date palm pollen, which is completely missing in this area. Thus, grass pollen polcalcin seems to contain specific epitope(s) 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 of our results might be that several isoforms of the different natural polcalcins exist, but we were able to investigate only two recombinant proteins; therefore we cannot exclude that the available isoforms do not contain the whole repertoire of allergenic epitopes present on 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 not depend only on the primary sensitization source but also on the content of polcalcin in current grass formulas for immunotherapy, although this aspect should be investigated by checking the specific IgG4 kinetics to Phl p 7 during the immunotherapy course.

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Table 1: ELISA inhibition studies using rPhl p 7 as substrate and fresh pollen extracts as inhibitors.

Serum	rPhl p 7 or rBet v 4 (OD)	% Inhib by Birch extract	% Inhib by Grass extract	% Inhib by Ragweed extract	% Inhib by Olive extract	% Inhib by Parietaria extract	% Inhib by HDM extract
B.C. (Phl p 7)	1932	50	91	17	////	////	5
Pool Phl p 7	2085	23	87	11	33	27	0
Neg. Serum (Phl p 7)	389	////	////	////	////	////	////
Pool Bet v 4	3110	64	87	46	79	71	1

IgE reactivity to rPhl p 7 and rBet v 4 is expressed in optical density units (OD x 1000). B.C. is the serum from an individual patient; Pool: serum pool from all 9 study patients. The degree of inhibition is expressed as % of reduction of IgE reactivity following serum pre-incubation with the different pollen extracts.

////: not done.

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%)