ORIGINAL ARTICLE

Cupressus arizonica Pollen: A New Pollen Involved in the Lipid Transfer Protein Syndrome?

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Abstract

Background: Lipid transfer proteins (LTP) are responsible for systemic manifestations in food allergy. Their relationship with pollinosis is not clear. In our area, many patients allergic to multiple LTP-containing foods present pollinosis due to *Cupressus arizonica*.
Methods: We selected 6 patients with cypress pollinosis and food allergy to peach. Skin prick tests (SPT) were performed for pollens (grass, cypress, wall pellitory, plane tree, and olive tree) and plant foods (hazelnut, kiwifruit, peach peel, maize, wheat, peanut, lettuce, apple, mustard, and melon). In vitro assays included specific immunoglobulin (Ig) E to *C arizonica* and peach LTP (Pru p 3), enzyme allergosorbent test (EAST) inhibition, immunoblotting, immunoblotting-inhibition, and immunocytochemical techniques for the detection of Pru p 3–like LTP in cypress pollen grains.
Results: SPT were positive for *C arizonica*, peach, lettuce, mustard, and hazelnut in all patients. Specific IgE to *C arizonica* and Pru p 3 was positive in all but 1 patient, whose Pru p 3 IgE was negative. Immunoblotting under nonreducing conditions with *C arizonica* extract and patients' sera showed a band at 14-15 kDa that was inhibited by Pru p 3. Pru p 3 partially inhibited the *C arizonica* pollen extract in EAST-inhibition. Pru p 3–like LTP was localized in the cytoplasm and walls of *C arizonica* pollen grains.
Conclusion: A 15-kDa allergen in *C arizonica* pollen was found in a group of patients presenting peach allergy and respiratory symptoms to cypress. In vitro tests and immunocytochemical techniques indicate that this protein is an LTP.

Key words: Cypress pollen. Food allergy. Lipid transfer protein. Pollinosis. Pru p 3.

Resumen

Fundamento: Las proteínas de transferencia de lípidos (LTP) son responsables de manifestaciones sistémicas en la alergia alimentaria. Su relación con la polinosis no está clara. En nuestra región, muchos pacientes alérgicos a múltiples alimentos que contienen LTP presentan polinosis por *Cupressus arizonica*.
Métodos: Se seleccionaron seis pacientes con polinosis por ciprés y alergia al melocotón. Se realizaron pruebas cutáneas (SPT) a pólenes (gramíneas, ciprés, parietaria, plátano de sombra, olivo) y alimentos (avellana, kiwi, piel de melocotón, maíz, trigo, cacahuete, lechuga, manzana, mostaza, melón). El estudio in vitro incluyó IgE específica a *C. arizonica*, LTP del melocotón (Pru p 3), EAST-inhibición, inmunoblotting, inmunoblotting inhibición y técnicas inmunocitoquímicas para la detección de LTP Pru p 3-like en los granos de polen del ciprés.
Resultados: Las SPT fueron positivas para *C. arizonica*, melocotón, lechuga, mostaza y avellana en todos los pacientes. IgE específica a *C. arizonica* y Pru p 3 fueron positivas en todos los pacientes excepto en uno, cuya IgE específica a Pru p 3 fue negativa. El inmunoblotting con extracto de *C. arizonica* y el suero de los pacientes mostró una banda a 14-15 kDa en condiciones no reductoras, que fue inhibida por Pru p 3. Pru p 3 inhibió parcialmente el extracto de *C. arizonica* en el EAST inhibición. Se ha localizado una LTP pru p 3-like en el citoplasma y paredes de los granos de polen de *C. arizonica*.
Conclusión: Se ha detectado un alérgeno de 15 kDa en el polen de C. arizonica en un grupo de pacientes que presentan síntomas respiratorios por ciprés y alergia al melocotón. Los estudios *in vitro* indican que podría tratarse de una LTP.

Palabras clave: Polen de ciprés. Alergia alimentaria. Proteina de transferencia de lípidos. Polinosis. Pru p 3.

Introduction

Patients who are allergic to fruit and vegetables are frequently allergic to pollens. This cross-reactivity is due to the existence of panallergens, proteins that are found throughout the vegetable kingdom [1]. In Central and Northern Europe, members of the pathogenesis-related (PR) 10 protein family associated with the major birch pollen allergen Bet v 1 and plant profilins associated with Bet v 2 cause most of the cross-reactivity detected between fruit and vegetable allergens and pollens [2]. Patients affected by these sensitizations usually show mild symptoms restricted to the oropharyngeal cavity (oral allergy syndrome) and seem to be primarily sensitized to pollen allergens. The Mediterranean area is practically unexposed to birch pollen, and another sensitization profile is usually detected. Nonspecific plant lipid transfer protein (LTP) is well known for its role in severe systemic manifestations after food ingestion, especially in Southern Europe. Multiple sensitization to various LTP-containing foods in LTP-allergic patients is a consequence of the high degree of immunoglobulin (Ig) E cross-reactivity between LTPs, even those that are taxonomically distant [3,4], giving rise to the so-called LTP syndrome [5]. The relationship between this syndrome and pollinosis is not clear, as LTPs are considered true food allergens with the capacity to cause primary sensitization that is not dependent on sensitization to LTP-containing pollen [6]. Even so, cross-reactivity between peach LTP (Pru p 3) and pollen LTP from mugwort (Art v 3) and the plane tree (Pla a 3) has been described [4,7,8]. In our environment, we have observed that many patients with LTP syndrome are sensitized to the pollen of *Platanus acerifolia* and *Cupressus arizonica*. *C arizonica* is a species of cypress that is native to North and Central America and is widespread in Mediterranean countries because of their optimal conditions for growth. It is becoming an increasingly frequent cause of allergic diseases in this area [9]. Therefore, we investigated the possible existence of an LTP in *C arizonica* pollen.

Material and Methods

Patients

Patients had seasonal rhinoconjunctivitis, asthma, or both in the cypress flowering season (usually January to March in the Mediterranean area) and a positive history of immediate-type reactions to peach and other plant foods.

Skin Prick Tests

Skin prick tests (SPT) with commercial extracts (BIAL-Arístegui, Bilbao, Spain) were performed with pollens (grass, cypress, wall pellitory, plane tree, and olive tree) and plant foods (hazelnut, kiwifruit, peach peel, maize, wheat, peanut, lettuce, apple, mustard, and melon). SPT were performed on the volar surface of the forearm using a standard 1-mm–tip lancet following the recommendations of the European Academy of Allergy and Clinical Immunology [10]. Histamine hydrochloride (10 mg/mL) and saline solution were used as positive and negative controls, respectively. The SPT result was considered positive if the larger diameter was greater than 3 mm compared to the negative control.

Enzyme Allergosorbent Test (EAST) and EAST Inhibition

Specific IgE levels were quantified using the enzyme allergosorbent test (EAST) (Hytec-specific IgE EIA, Hycor Biomedical, Kassel, Germany) as described by the manufacturer. *C arizonica* pollen extract and Pru p 3 were coupled to cyanogen bromide–activated paper disks [11] at 1 mg/mL and 100 μg/mL, respectively. For inhibition experiments, sera were preincubated with serial dilutions of Pru p 3 (100, 10, 1, 0.1 μg/mL). These aliquots were then incubated overnight at 4° C with cyanogen bromide–activated disks and, after washing, bound IgE was detected as described by the manufacturer.

Immunoblotting and Immunoblotting Inhibition

Proteins from *C arizonica* pollen extract and the purified Pru p 3 allergen were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions, electroblotted onto a polyvinylidene fluoride membrane, and incubated overnight at 4°C with undiluted sera. For inhibition experiments, sera were preincubated overnight at 4ºC with each inhibitor before exposure to the membranes. Bound IgE was detected by incubation with antihuman IgE-horseradish peroxidase conjugate (Southern Biotech, Birmingham, Alabama, USA; diluted 1/2000), and blots were developed using the ECL Plus Western Blotting Detection System (GE-Healthcare, Uppsala, Sweden).

Transmission Electron Microscopy

Fixation and embedding. Freezing protocol: Freeze substitution was used to achieve the in situ localization of water-soluble proteins and to preserve ultrastructure and antigenicity. *C arizonica* pollen was chemically fixed at 4ºC with a mixture of 4% p-formaldehyde and 0.2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Freeze substitution was performed in an automatic freeze substitution system (AFS, Leica, Vienna, Austria) using 0.5% uranyl acetate in methanol at –90ºC for 72 hours, and samples were warmed to –50ºC at a rate of 5ºC per hour. After several methanol rinses, samples were infiltrated in Lowicryl HM20 for 6 days and polymerized at –50ºC with UV lamps. Ultrathin sections were prepared using a Leica ultramicrotome (Leica UCT, Vienna, Austria).

Immunogold labeling. Sections of anthers were incubated with anti-Pru p 3 polyclonal antibodies (Bial-Aristegui; diluted 1/500) in 5% fetal bovine serum (FBS) in phosphate-buffered saline (PBS) for 30 minutes. After 3 washes with 0.2% FBS in PBS for 20 minutes, sections were incubated for 20 minutes using protein A coupled to 10-nm colloidal gold particles (purchased from Dr. G. Posthuma, Utrecht University, The Netherlands) at a 1:60 dilution in 5% FBS in PBS. Samples then underwent 3 washes with PBS for 10 minutes and 2 washes with distilled water. Sections were also incubated

with pre-immune serum to control the specific labeling of the primary antibody. Other sections were incubated after omitting the primary polyclonal antibody to control the nonspecific binding of the colloidal gold–conjugated antibody. They were observed using a JEOL 1010 transmission electron microscope (JEOL, Tokyo, Japan).

Results

The study sample comprised 6 patients (3 men and 3 women; mean age, 27.6 years), all of whom presented a positive SPT result to *C arizonica*, peach, lettuce, mustard, and hazelnut. Specific IgE to *C arizonica* and Pru p 3 ranged from 0.4 to 13.4 IU/mL and 0.4 to 100 IU/mL, respectively. Patient 6 had negative results to Pru p 3 specific IgE. Demographic data, sensitization profile, clinical data, and specific IgE titers are reported in the Table.

Table. Demographic and Clinical Data

Patient	Age, y/sex	Sensitization		Allergy to Cypress	Symptoms With Plant Foods	Specific IgE, IU/mL	
		Pollen	Plant Food			C arizonica	Pru p 3
1	34/Female	G.C.	H, P, Ap, M, L, Pe	R	P (U, OAS), Ap (OAS), L (D)	0.4	0.5
2	31/Male	G, C, W, Pl	H, P, M, L, Ma, Ap	R	H (OAS), Pe (OAS, An), P (OAS), L (D)	13.4	100
3	23/Female	G, C, Pl, O	H, P, M, L, Ap	R	P (U), Ap (OAS), L (D)	10.8	0.4
4	28/Female	G, C, W, Pl	H, P, M, L, Pe	R, A	P (OAS), H (OAS), L (An)	2.6	1.2
5	18/Male	C, PI	H, P, M, L, Ap	R, A	P (U), Ap (OAS), L (D)	0.6	2.6
6	32/Male	C, PI	H, P, L, M	R	H (OAS), P (OAS), L (An)	2.3	<0.35

Abbreviations: A, asthma; An, anaphylaxis; Ap, apple; C, cypress; D, dyspepsia; G, grass; H, hazelnut; Ig, immunoglobulin; L, lettuce; M, mustard, melon; Ma, maize; O, olive tree; OAS, oral allergy syndrome; P, kiwifruit, peach peel; Pe, wheat, peanut; Pl, plane tree; R, rhinoconjunctivitis; W, wall pellitory; U, urticaria.

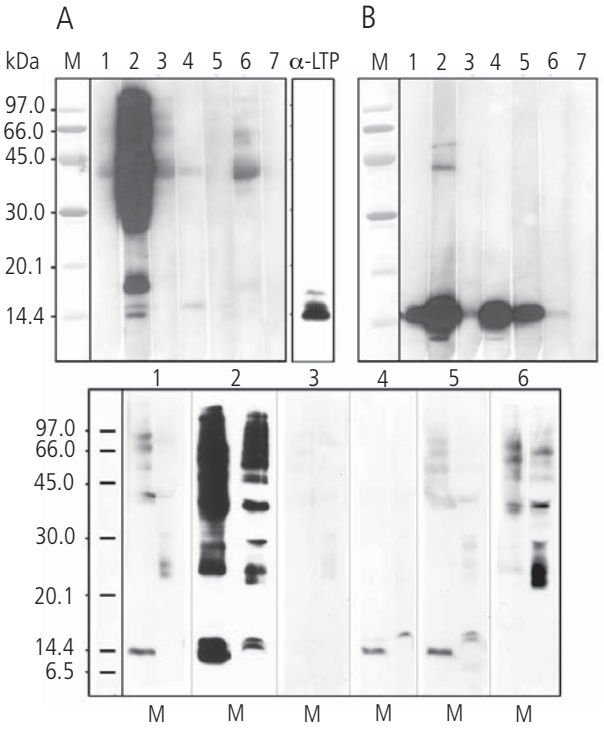

Figure 1. Immunoglobulin E immunoblotting under nonreducing conditions of *Cupressus arizonica* pollen extract (A), purified nPru p 3 (B), lettuce (C), and hazelnut (D) extracts incubated with individual sera (lanes 1 to 6) or rabbit anti-Pru p 3 serum (α-LTP). Lane 7 corresponds to a pool of sera from nonallergic individuals. M indicates molecular weight marker.

The IgE immunoblotting results of Pru p 3 incubated with individual sera agreed with the corresponding specific IgE determinations, except for patient 6, whose specific IgE to Pru p 3 was negative, although it reacted with Pru p 3 in immunoblotting. IgE immunoblotting of *C arizonica* pollen extract under nonreducing conditions revealed a well-defined band at 43 kDa, probably corresponding to Cup a 1 [12], and a double or single band at 14-15 kDa, coinciding with sera from patients with greater reactivity to LTP (Figure 1, patients 2 and 4). A double band of the same apparent molecular mass was detected using rabbit anti–Pru p 3 serum (Figure 1, α-LTP lane). Sera from patients 1, 2, 4, and 5 reacted with proteins of similar molecular mass from lettuce and hazelnut extracts (Figure 1, lanes C and D). Under reducing conditions, these IgE-reacting proteins shifted their apparent molecular mass (<14 kDa) and lost their capacity for IgE binding, as observed with purified Pru p 3 (data not shown). In the light of these findings, the immunoblotting-inhibition experiments were carried out with the sera from patients 2 and 4, showing that IgE binding to the double band (patient 2) or the single band at 15 kDa (patient 4) was completely inhibited by Pru p 3 (Figure 2).

Using immunocytochemical techniques, a Pru p 3–like LTP was localized in the cytoplasm and walls of *C arizonica* pollen grains in mature and hydrated stages (Figure 3).

Finally, EAST inhibition assays were performed with sera from patients 2 and 4 (data not shown). Pru p 3 could not completely inhibit IgE binding to the *C arizonica* pollen extract, probably because the putative cypress LTP is not very

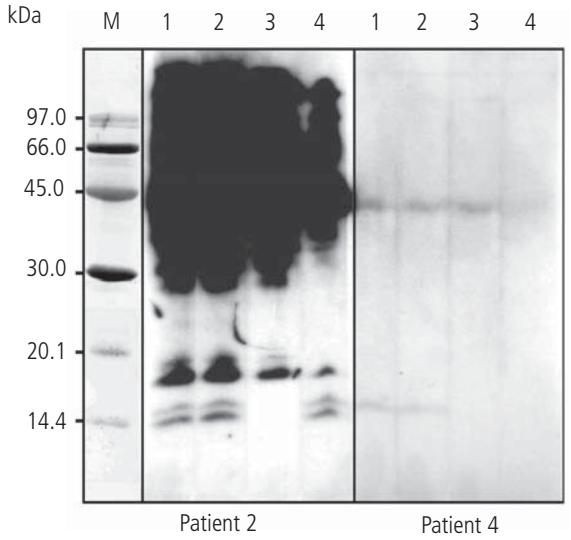

Figure 2. Immunoglobulin E binding of sera from patients 2 and 4 to *Cupressus arizonica* pollen extract run under nonreducing conditions was inhibited by buffer alone (lane 1), bovine serum albumin (100 µg/mL, lane 2), nPru p 3 (100 µg/mL, lane 3), and *C arizonica* pollen extract (2 mg/mL, lane 4).

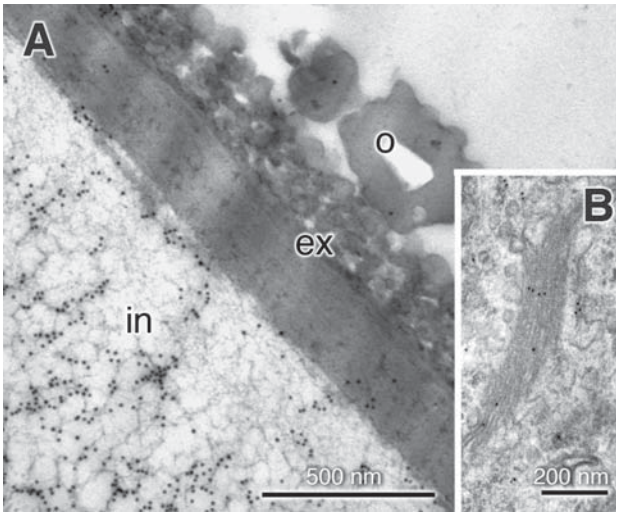

Figure 3. Transmission electron micrograph of *Cupressus arizonica* pollen grains. Immunogold labeling corresponding to Pru p 3. A, Abundant gold particles in the walls, outer layer (ex), inner layer (in), and orbicules (o) of mature pollen grain. B, Detail of hydrated cytoplasm showing the labeled Golgi body.

abundant in the extract (as shown in other pollen extracts [8]) and other predominant proteins could mask it in this kind of assay. Nevertheless, the low inhibition found was specific compared to the control (14% vs 3%).

Discussion

We found a previously undescribed 15-kDa allergen in *C arizonica* pollen in a selected group of patients who not only present respiratory symptoms to cypress, but also food allergy due to sensitization to Pru p 3. This protein seems to be an LTP-like allergen, as shown by immunoblotting inhibition with Pru p 3 and immunocytochemical assays. The group of selected cypress-allergic patients presented sensitization to multiple LTP-containing plant foods, such as peach (Pru p 3), lettuce (Lac s 1), mustard (Sin a 3), hazelnut (Cor a 8), and maize (Zea m 14) (only 1 patient). Five out of 6 patients also presented sensitization to plane tree pollen. It remains to be elucidated whether sensitization to this 15-kDa protein is a result of primary sensitization to cypress pollen or merely an epiphenomenon of the cross-reactivity with plant food LTPs such as Pru p 3. To date, only LTPs of plane tree pollen (Pla a 3) and mugwort pollen (Art v 3) have been shown to cross-react with food LTPs [7-8]. With regard to plane tree pollen, Lauer et al [8] established that plane pollen LTP (Pla a 3) was a major allergen in plane pollen–allergic patients with peach allergy and a minor allergen in patients with no food allergy, in terms of frequency of IgE binding [8]. The case of mugwort is more controversial, as, depending on the study population, either food LTP (Pru p 3) [13] or pollen LTP (Art v 3) [7] has been suggested as a primary sensitizing agent. Pastorello et al [13] found that sensitization to mugwort LTP in a population of peach-allergic patients was a consequence of sensitization to peach LTP, because the concentration of Art v 3 that inhibited the Pru p 3 band in the immunoblotting inhibition was 100-fold higher than the concentration of Pru p 3, whereas the same concentration of both allergens was enough to inhibit the Art v 3 band. In addition, no sensitization to Art v 3 was found in mugwort-sensitized patients with no Pru p 3 IgE [13]. In the study of Lombardero et al [7], patients were recruited based on sensitization to *Artemisia*. They concluded that Art v 3 behaved as the primary sensitizing agent based on the finding of Art v 3 sensitization in mugwort-allergic patients not sensitized to Pru p 3. They also found that Art v 3 could partially inhibit IgE binding to Pru p 3, but that Pru p 3 could not inhibit IgE binding to Art v 3 [7]. In view of this evidence, Zuidmeer and Van Ree [6] suggested the existence of different LTP syndromes, depending on the primary sensitizer and the presence or absence of pollen allergy associated with food allergy.

Detection of this new allergen at 15 kDa in cypress pollen extract related to peach LTP and the EAST inhibition results could support the idea of cross-reactivity. These findings, together with the fact that, in Europe, *C arizonica* is almost exclusive to the Mediterranean area should be taken into account to better understand the LTP syndrome in Southern Europe. Besides, a 35-kDa allergen was recently detected as a major allergen of *C arizonica* in Teheran [14], supporting the variability of the allergenic components of this pollen in different regions. Even so, our patients presented IgE not only against the 15-kDa allergen, but also against a 43-kDa allergen, probably corresponding to the major allergen of *C arizonica*, Cup a 1; therefore, this could account for the respiratory symptoms, as previously proposed for mugwort hypersensitivity [13].

In 1992, Lleonart et al [15] identified a peach-specific low-molecular-mass allergen (around 10 kDa) preferentially located in the peel of the fruit, which was not sequenced. In

1994, Pastorello et al [16] described a low-molecular-mass peach allergen (around 13 kDa) that bound the sera of 90% of peach-allergic patients and cross-reacted with homologous proteins in other Prunoideae fruits (apricot, cherry, and plum) but not with birch or grass pollen allergens. Apple and peach LTPs were not purified until 1999 [17-19]. Since then, LTP-induced allergy has become increasingly important, so much so that, in the last 10 years, more than 35 allergenic LTPs related to fruits, vegetables, and pollens have been described, the most recent in mustard (Sin a 3) [20] and peanut (Ara h 9) [21]. The description of these allergens is helping us to understand patients with multiple sensitizations to various LTP-containing foods, which might also be associated with pollinosis. A cypress LTP could be involved in this cross-reactivity. The purification and sequencing of this allergen will help to confirm our findings and their immunological relationship to peach LTP. More studies are needed to clarify whether *C arizonica* is a relevant pollen in LTP syndrome.

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Conflicts of interest: None.

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