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Analysis of IgE binding proteins of *Celtis tala* pollen

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Celtis tala is a tree, widely distributed in Argentina. Its significance as a source of allergic sensitisation is still unclear. Our aim was to partially characterise the IgE binding proteins of *C. tala* pollen. Skin prick tests using *C. tala* pollen extract were carried out in 25 patients with respiratory allergy. *Celtis tala* extract elicited positive skin reactions in five patients. Sensitised patients showed increased specific IgE levels to *C. tala* by immunodot. Immunoblotting assay demonstrated IgE binding to proteins of 50 kDa and 55 kDa. *Celtis tala* pollen extract is allergenically potent requiring approximately 50 ng of self-protein for 50% inhibition of IgE binding in enzyme-linked immunosorbent assay (ELISA) inhibition, whereas no cross-inhibition was detected with other types of pollen widely distributed in Argentina (*Helianthus annuus* and *Prosopis* spp.). Thus, these results taken together indicate that proteins of 50 kDa and 55 kDa may be considered as specific allergens of *C. tala* pollen.

Keywords: allergen; *Celtis tala*; pollen; seasonal rhinitis; serum IgE

Introduction

Allergic diseases of the respiratory system caused by pollinosis are quite frequent and have a great clinical impact in industrialised countries (Ibarrola et al., 2004; ISAAC, 1998), being in part responsible for rises in the morbidity rate, lack of productivity and increased costs for the health care system (Weiss & Sullivan, 2001). However, among the 250,000 well-described pollen-producing plant species, less than 100 are significant in terms of inducing pollen allergy (D'Amato et al., 1998). Pollens from wind-pollinated plants are the most potent allergen sources, whereas pollens from insect-pollinated plants less frequently elicit allergic symptoms (Durham, Gould, & Hamid, 1997). The characterisation of allergens from plant pollens is a necessary task to improve the diagnosis and treatment of this increased clinical disorder and can help to explain relationships among biologic function, protein structure, and allergenic activity (Aalberse, 2000; Puc, 2003).

Celtis tala (Gill. ex Planchon), a member of the *Ulmaceae* family, is an autochthonous tree in South America and widely distributed in Argentina (Nitiu, 2003). As with all the species belonging to the *Celtis* family, *C. tala* is a typically anemophilous tree (Green & Dettmann, 2004). The pollen of *C. tala* has been found to be one of the most important causes of pollinosis and allergic sensitisation in the north and central areas of Argentina

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during the pollination period between August and December (Nitiu, 2003; Nitiu, Mallo, & Romero, 2003; Teijeiro et al., 2002; Weber, 2005).

Córdoba city (31°19'S and 64°13'W) is located in the Córdoba province in the central region of Argentina. Pollen season in Córdoba can be divided in two periods. The first one goes from August to November with a predominance of arboreal pollen and the second one goes from October to April with a predominance of grass pollen grains (Teijeiro et al., 2002). Airborne pollen in the central part of Argentina is mainly represented by *Platanus*, *Fraxinus*, *Cupressaceae*, *Poaceae*, *Urticaceae*, *Cyperaceae*, *Myrtaceae*, *Celtis*, and *Casuarina* *Morus* (Nitiu, 2006). It has been demonstrated that the *Celtis* airborne pollen content represented between 1998 and 2000, an average of 2.62% compared to arboreal pollen, reaching a maximum of 10% in October (Nitiu, 2003). The average pollen count was 15–30 grains/m³, with a maximum airborne pollen concentration of 275 grains/m³ (Nitiu, 2003). In other reports, *Celtis* pollen is considered to be one of the important pollen types which exceed 1% of the total relative concentration of pollen present in the atmosphere, together with other species such as *Fraxinus*, *Poaceae*, *Cupressus*, *Ambrosia*, *Urticaceae*, *Artemisia*, *Myrtaceae*, *Casuarina*, *Asteraceae*, and *Cyperaceae* (Nitiu et al., 2003). Considering the most allergenic species according to skin prick test sensitivity, August–November is a very risky period in Córdoba for patients affected with seasonal symptoms caused by pollen trees. In the case of grasses and weeds the period extends from October to April (Teijeiro et al., 2002).

Despite *C. tala* being described as a potential allergenic (Nitiu et al., 2003; Teijeiro et al., 2002; Weber, 2005), its allergic components have still not been properly investigated, which affect the accurate diagnosis and safe immunotherapy of *C. tala* allergy. The present study was therefore aimed at identifying IgE binding of *C. tala* pollen by immunoblot using hypersensitive sera from allergic patients to *C. tala* pollen. In addition, the specific allergens of *C. tala* with predominant pollen species were delineated by inhibition assays.

Materials and methods

Patients

Twenty-five patients were recruited from the Service of Allergy and Immunology of Oulton Institute for Diagnosis and Treatment, Córdoba, Argentina. From August to December of 2003 (spring and summer season), these patients presented allergic symptoms of asthma and/or rhinitis. The criteria for asthma were symptoms suggestive of it, including self-reported asthma, dyspnea with wheezing or cough with wheezing, and at least one positive bronchodilation test (Viinainen et al., 2005). The definition of rhinitis includes a heterogeneous group of one or more of the following nasal symptoms: sneezing, itching rhinorrhea, or nasal congestion (Phipatanakul, 2005). The detailed history including age, sex, duration of illness, family history, onset of symptoms, seasonality, etc. of each patient was recorded and clinical investigations were carried out with informed consent. The Human Ethics Committee of the Institute approved the study protocol.

Skin testing

Skin prick tests were carried out on the forearm surface of patients with *C. tala* pollen extract reconstituted in phosphate buffer saline (1:500 w/v in PBS: Na₂HPO₄ 10 mM, KH₂PO₄ 3 mM, NaCl 120 mM, pH 7.5). The patients were also skin prick tested with a

routine battery of aeroallergens of our area (Ceti Laboratories, Córdoba, Argentina). The battery included grass pollen, tree pollen, weed pollen, house dust mite and animal dander. For skin prick tests, each drop was pricked using a separate sterile lancet, and the reaction was read after 15 min. Histamine chloride 10 mg/ml was used as a positive control and the diluent as a negative control (Greer Laboratories, Lenoir, NC). Positive reaction was defined if the wheal and erythema size were greater than 3 mm and 5 mm after subtraction of the diameter of the negative control. The same examiner carried out each skin prick test.

Sera were collected for immunobiochemical assays from patients with positive skin prick tests to *C. tala* pollen extract. Sera were also collected from healthy volunteers and pooled for use as a negative control. The total concentration of serum IgE was determined using a commercial enzyme immunoassay.

Pollen protein extracts

Polleniferous materials were collected from *C. tala* (Tala) and four other predominant pollen species namely, *Eucalyptus* sp. (Eucalyptus), *Prosopis* spp. (Algarrobo), *Prosopis caldenia* (Caldén), and *Helianthus annuus* (Sunflower), which were obtained in fields from the Province of Córdoba (Argentina) during peak flowering season. Collection and processing of pollen material was done carefully by trained pollen collectors.

The protein extraction was carried out by the method of Park, Ko, Kim, Jeoung, and Hong (1999). Briefly, 200 mg of pollen was defatted with diethyl ether and then extracted in 100 mL of carbonate buffer (125 mM NH_4HCO_3 , 15 mM NaN_3 , pH 7.5) for 24 h at 4°C with constant stirring. The supernatant was separated by centrifugation at $27,000 \times g$ for 30 min at 4°C, filtered through 0.22 μm membrane under sterile conditions and lyophilised. Protein concentration was determined according to the method of Bradford (1976).

Specific IgE detection

Immunodot assay

To detect the specific IgE reactivity to different types of pollens we performed an immunodot assay. After standardisation analysis, the optimal condition for immunodot assay was as follows: 4 μg of total protein, extracted from each type of pollen, was loaded on nitrocellulose membranes. After washing the unbound antigen with tris-buffered saline (TBS) (250 mM Tris, 750 mM NaCl; pH 7.3), free sites were blocked with 5% defatted dried milk in TBS for 1 h at 37°C. This was followed by three washing with TBS-T (TBS with 0.05% Tween 20) and overnight incubation with individual patients' sera (1:5 v/v TBS-T) at 4°C. The membrane was washed five times with TBS-T, incubated with peroxidase-conjugated goat anti-human IgE (Sigma Co., St. Louis) (1:500 v/v in TBS-T) for 2 h at room temperature and revealed by chemoluminescence reaction (ECL Reagent, NEN Life Science Products). To express the relative amount of serum specific IgE against the different types of pollen extract the intensity of immunodots was quantified by densitometric analysis using the image software (UVP Life Science Software, Vision Works® LS Image Acquisition and Analysis) and relative signals were expressed in density units (DU) as follows: (-): 0–300 DU; 1+: 301–1350 DU; 2+: 1351–2400 DU; 3+: 2401–3450 DU; and 4+: 3451–4500 DU.

Immunoblot assay

To recognise allergenic proteins, SDS-PAGE was carried out by the method of Laemmli (1970) under reducing conditions using 12% polyacrylamide gels. One hundred microgram protein of pollen extracts was boiled for 5 min at 100°C in two-fold concentration of sample buffer containing 50 mM dithiotritol. The proteins were electrophoresed at a constant voltage of 120 V and bands were detected by staining with 0.1% Coomassie brilliant blue R-250 or transferred to nitrocellulose membrane in 25 mM Tris pH 8.3 containing 192 mM glycine and 20% methanol (Towbin, Staehelin, & Gordon, 1979). Standard proteins of known molecular weight (Sigma Co., St. Louis) were also electrophoresed on the same gel along with sample proteins. Non-specific sites were blocked with 5% defatted dried milk in TBS for 1 h at 37°C. The membrane was washed with TBS and incubated overnight at 4°C with pooled or individual patients' sera (1:5 v/v in TBS-T) positive to *C. tala* extract. After washing five times with TBS-T and once with TBS, peroxidase-conjugated goat anti-human IgE (1:500 v/v in TBS-T) was added to the blotted membrane and incubated for 2 h at room temperature. Finally, the blots were revealed with chemoluminescence reaction as is indicated above.

Pollen extracts of four predominant pollen species were also transferred onto nitrocellulose membrane and incubated with pooled patient sera positive to *C. tala* pollen.

Specific IgE ELISA inhibition

To evaluate the specificity of IgE binding to *C. tala* pollen extract, ELISA inhibition tests were carried out. IgE binding was inhibited by pre-incubating the hypersensitive pooled patients' sera (1:10 v/v in TBS-T) with various amounts (2–2000 ng) of *C. tala* pollen and other different pollen extracts (*H. annuus* and *Prosopis* spp.) for 16 h at 4°C. The pooled patients' serum was prepared from five individual patients' sera which were skin prick test positive to *C. tala* extract. The pre-incubated mixture was then added to microtitre plate coated with *C. tala* pollen protein (1 µg/well) and ELISA was performed. The pooled serum without an inhibitor was used as a positive control. Percentage inhibition was calculated as under:

$$\left[1 - \frac{\text{absorbance with competitor}}{\text{absorbance of control}} \right] \times 100$$

Statistical treatment of data

The differences between study results were estimated using the *t*-test for unpaired data sets and regression analysis (Pearson correlations). Differences were considered significant when the *p*-value was ≤ 0.05 .

Results*Specific IgE estimation**Skin prick test and immunodot assays*

From 25 patients admitted to this study, only five patients showed a strong positive reactivity to *C. tala* pollen extract by skin prick test. The mean ($\pm$ standard deviation) values of the wheal and erythema size in these five patients were 6.24 (± 2.99) mm and

Table 1. Main clinical characteristics of sensitised patients' group.

Patient N°	Age	Sex ^a	Diagnose ^b	Season	Total IgE (UI/ml) ^c
1	57	M	AR	Spring	239
2	56	M	AR	Spring	358
3	29	F	AR + A	Spring	410
4	53	F	AR	Spring/Summer	597
5	45	M	AR	Spring/Summer	164

^aM, male; F, female; ^bAR, rhinitis; A, asthma; ^cupper limit of normal reference value: 90 UI/ml.

15.28 (± 6.86) mm, respectively, which were significantly different to the mean values of non-sensitised patients ($p < 0.01$). The main clinical characteristics of these five sensitised patients are presented in Table 1, showing that these patients had increased serum level of total IgE in respect to normal control. In order to evaluate the presence of specific IgE against *C. tala* in individual patients' sera an immunodot assay was used. Table 2 shows the results of immunodot assays demonstrating that the five patients studied presented specific IgE against *C. tala* pollen. The totality of these *C. tala* sensitised patients showed distinct levels of relative signal of specific IgE reactivity against *C. tala* pollen. Then, from these *C. tala*-sensitised patients, we analysed specific IgE reactivity of the individual patients' sera against protein extracts from other types of predominant pollen species using immunodot assays. The totality of these *C. tala* sensitised patients showed distinct levels of relative signal of specific IgE reactivity against *Prosopis* spp., *H. annuus*, *Eucalyptus* spp., and *P. caldenia* pollen extracts (Table 2).

Immunoblot assays

By immunoblot analysis, we determined the IgE binding components of *C. tala* pollen from sensitised patients. Individual patients' sera presented IgE reactivity against two protein bands of approximately 50–55 kDa (Figure 1A). Interestingly, these proteins were identified in all of the sensitised patients studied, indicating that 50–55 kDa proteins can be considered the major allergenic components of *C. tala* pollen. In addition, we evaluated the IgE binding components of these five patients with immunodot IgE positive reactivity

Table 2. Specific IgE levels against *C. tala* and other predominant plant pollens of individual patient sera with positive skin prick test to *C. tala* pollen.

Patient N°	Immunodot ^a				
	<i>C. tala</i>	<i>Prosopis</i> spp.	<i>H. annuus</i>	<i>Eucalyptus</i> spp.	<i>P. caldenia</i>
1	1+	2+	4+	—	1+
2	1+	—	—	1+	—
3	2+	1+	—	—	—
4	3+	1+	1+	—	—
5	4+	1+	—	—	—

^aDU, density units. Negative relative signal (—): between 0–300 DU, Positive relative signal: 1+: between 301–1350 DU, 2+: between 1351–2400 DU, 3+: between 2401–3450 DU and 4+: between 3451–4500 DU.

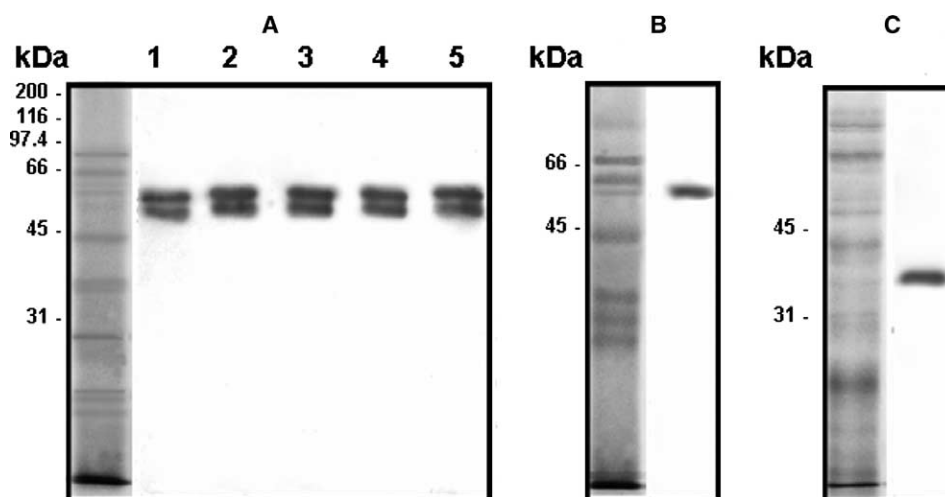


Figure 1. (A) IgE immunoblot of *C. tala* pollen extract using sensitised individual patients' sera. The numbers indicate the *C. tala* sensitised patients showed in Tables 1 and 2. (B) IgE immunoblot of *H. annuus* pollen extract using pooled sera of patients 1 and 4 of Table 2. (C) IgE immunoblot of *Prosopis* spp. pollen extract using pooled sera of patients 1, 3, 4, and 5 of Table 1. In each panel at the left line are showed the SDS-PAGE profile of *C. tala*, *H. annuus*, and *Prosopis* spp. pollen extract, respectively. MW: Molecular weight markers.

against other types of predominant pollens (Table 2). When the immunoblot assay was performed with electroblotted protein extracts of *H. annuus* pollen, the pooled serum from sensitised *C. tala* pollen patients with positive relative signal for this pollen (patients 1 and 4 in Table 2) only detected an IgE binding component of 55 kDa (Figure 1B). On the other hand, when the immunoblot assay was performed with electroblotted proteins extracted from *Prosopis* spp. pollen, the pooled patients' serum with positive relative signal for this pollen (patients 1, 3, 4, and 5 in Table 2) detected an IgE binding component of 38 kDa (Figure 1C). Immunoblotting of *P. caldenia* and *Eucalyptus* spp. pollen extracts with the pooled patients' serum with positive relative signal for each type of pollen (patients 1 and 2 in Table 2, respectively), none IgE binding component was detected (data not shown). As negative control, pooled normal human serum did not show IgE binding components to any electroblotted protein extracts of *C. tala* or other type of predominant pollen species studied (data not shown). Thus, these results indicate that sensitised patients to *C. tala* pollen showed serum IgE reactivity against two proteins of 50 kDa and 55 kDa.

ELISA inhibition

Cross reactivity of *C. tala* allergens was further investigated by means of ELISA inhibition assay with pooled patients' sera (Table 2) using *H. annuus* and *Prosopis* spp. pollen extracts as inhibitors. *C. tala* extract required approximately 50 ng of self-protein for 50% inhibition, whereas *H. annuus* and *Prosopis* spp. extracts did not show significant IgE binding inhibition even at 2000 ng of extract (Figure 2). Thus, these results taken together suggest that proteins of 50 kDa and 55 kDa can be considered as specific allergens of *C. tala* pollen.

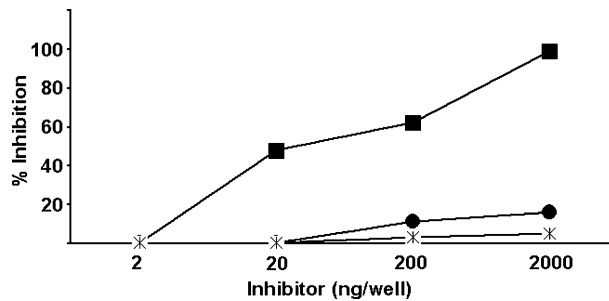


Figure 2. IgE ELISA inhibition of *C. tala* pollen extract. *C. tala* extract (1 µg/well) was coated and incubated with a mixture containing pooled patients' sera (1:10 v/v in TBS-T) and 2, 20, 200, and 2000 ng of extract as inhibitors (■, *C. tala*; ●, *Prosopis* spp.; *, *H. annuus*). The bound IgE was determined using anti-human IgE-HRP. The values represent the mean of three independent experiments.

Discussion

The pollination period of *C. tala* in our geographic area is from September to December. Although a few previous reports have reported that *C. tala* pollen is an important allergen in people living in north and central areas of Argentina (Nitiu, 2003; Nitiu et al., 2003; Teijeiro et al., 2002; Weber, 2005), neither its sensitisation rate nor its allergenic component has been properly studied. This work was designated to detect allergenic components of *C. tala* pollen. In this sense, using skin prick tests and immunodot assays we identified patients sensitised to *C. tala* pollen. Out of 25 patients with allergic sensitisation to different types of pollen, five patients showed positive skin prick tests and specific IgE reactivity to *C. tala* pollen extracts. These patients also showed some IgE reactivity against other types of pollens (as determined by skin prick tests and immunodot assays), i.e. *H. annuus*, *Prosopis* spp., *Eucalyptus* spp. and *P. caldenia*, which are also widely distributed in Argentina (Asturias et al., 1998; Nitiu et al., 2003; Teijeiro et al., 2002). Then, by immunoblot assays we determined that these five sensitised patients presented IgE reactivity against two protein bands of approximately 50 and 55 kDa, respectively (Figure 1A). On the other hand, in other types of pollen, pooled patients' sera only showed IgE reactivity against a single protein of 55 kDa for *H. annuus* and 38 kDa for *Prosopis* spp. pollen extracts (Figures 1B and C). By ELISA inhibition tests we demonstrated that 50 ng of self-protein was required for 50% inhibition of IgE binding, whereas the IgE binding to *C. tala* pollen extracts was not inhibited by *H. annuus* and *Prosopis* spp. pollen extracts (Figure 2). Therefore, we have identified for the first time allergenic components of *C. tala* pollen as two proteins of 50 kDa and 55 kDa, which did not present IgE reactivity against other plant pollen extracts that are widely distributed in the same area of *C. tala*. Although further studies are required, including the increase of the number of sensitised patients, this partial characterisation could be promising for the development of diagnostic tests and the formulation of immunotherapy vaccines for patients allergic to *C. tala* pollen.

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