Original Article

In Vitro and In Vivo Biological Activities of Old and Fresh *Cupressus arizonica* Pollen

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Abstract. *Background:* Respiratory allergy to the pollen of Cupressaceae is becoming more and more common every year in the Mediterranean area.
Objective: The purpose of this study was to see whether the allergenic potency of *Cupressus arizonica* pollen diminished after a 6-year period (1994-2000).
Materials and Methods: Among the Cupressaceae, we selected the pollen of *C arizonica*. The mode of sampling in 1994 and in 2000 was the same and the pollen was collected on the same tree and stored at room temperature. To compare its biological and allergenic activities data was collected with the following methods: cytohistology of Alexander, 2,3,5-triphenyltetrazolium chloride enzyme staining, skin testing, nasal provocation test, radioallergosorbent test (RAST), RAST inhibition, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and immunoblotting to detect protein content. Thirty-eight patients with respiratory allergy to Cupressaceae were selected.
Results: We found no decrease in the allergenic potency of the pollen, but did find that viability and germinating power had disappeared completely after 30 to 40 days. Moreover, the amount of protein in the old pollen was half the amount found in the fresh one. Skin prick testing showed identical results with the old and the fresh pollens.
Conclusions: The allergenic in vivo and in vitro activity of cypress pollen is retained for years after its collection. This activity seems to be independent of the viability of pollen grains and of the total protein content. This may explain the presence of clinical symptoms in patients out of the pollen season.

Key words: *Cupressus arizonica*. Allergenicity. Pollen viability

Resumen. *Antecedentes:* La alergia respiratoria al polen de cupresáceas está cobrando cada año más importancia en el área mediterránea.
Objetivo: El objetivo de este estudio fue determinar si existe una disminución de la actividad alergénica del polen de *Cupressus arizonica* tras un período de 6 años (1994-2000).
Materiales y métodos: De entre las cupresáceas se seleccionó el polen de *C arizonica*. La técnica de muestreo en 1994 y en 2000 fue la misma y el polen se obtuvo de la misma planta. El polen se conservó a temperatura ambiente. Para comparar las actividades biológicas y alergénicas se utilizaron los métodos siguientes: método citohistológico de Alexander, método enzimático de sal de tetrazolio, prueba de punción cutánea, prueba de provocación nasal, prueba radioalergoadsorción (RAST), inhibición de RAST, electroforesis en gel de poliacrilamida con dodecil-sulfato de sodio e inmunotransferencia y contenido proteico. Se seleccionaron 38 pacientes con alergia respiratoria a las cupresáceas.
Resultados: Según estos criterios no se halló disminución alguna de la actividad alergénica del polen, pero se observó eliminación de su viabilidad y su poder de germinación, que desaparecieron por completo al cabo de 30-40 días. Además, el contenido de proteínas en el polen viejo fue la mitad del contenido hallado en el polen reciente. La prueba de punción cutánea mostró resultados idénticos con el polen viejo y el reciente.
Conclusiones: La actividad alergénica *in vivo* e *in vitro* del polen de ciprés se mantiene durante años tras su obtención. Esta actividad parece ser independiente de la viabilidad de los granos de polen, así como del contenido proteico total. Esto puede explicar la presencia de síntomas clínicos en pacientes incluso fuera de la estación polínica.

Palabras clave: *Cupressus arizonica*. Alergenicidad. Viabilidad del polen

Introduction

Respiratory allergy to the pollens of the Cupressaceae family (mostly *Cupressus sempervirens* but also *Cupressus arizonica* and *Juniperus oxycedrus*) was a mere curiosity 40 years ago [1] but has gradually increased in prevalence from being a minor pollinosis starting in 1975 [2] to being a growing problem, such that 30% of our patients afflicted with pollinosis are allergic to this pollen [3-6]. That is one of the reasons why it seems important to investigate the gradual changes in the allergenic potency and viability (germinating power) of pollen conserved after a few years of storage at room temperature. It is also easy to see the importance of knowing whether pollens left in the environment still keep their allergenic potency, as this would explain the persistence of symptoms out of the pollen reason.

Even though there have been several immunological studies on cypress pollens resulting in the characterization and cloning of the major allergens, at present there is little information on the duration of the stability and allergenic activity of these pollens over time. We decided to study the pollen of one member of the Cupressaceae family, specifically, that of *C arizonica* because it is easy to obtain an extract. As far as we know, no study has been devoted to this topic.

Material and Methods

Briefly, pollen samples from the same plant of *C arizonica* were collected in 1994 and 2000. Viability and morphologic characteristics were assessed by staining methods at each collection. Diagnostic extracts were prepared with the two samples of pollen and used for skin prick tests in a cypress-allergic population and for radioallergosorbent test (RAST) inhibition assays using the sera from the same patients.

Pollen Sampling and Preservation

The pollen was stored in 1994 and 2000 at the Department of Vegetable Biology, Perugia University, Central Italy, from the same plant of *C arizonica*, and with the same procedure. The 1994 pollen was kept at room temperature in paper envelopes at Perugia University where it was collected. The collected pollen was immediately examined by cytochemical and enzymatic methods (see below). Viability of the pollen grains over time was repeatedly assessed at 24-hour intervals on the days after collection, until the percentage of viable grains fell below 2%. This was done only by the enzymatic staining, since the reliability of the histocytochemical method of Alexander [7] for whether verifying pollen viability is poor.

Extracts of *C arizonica* Pollen

The samples of *C arizonica* pollen from 1994 and 2000 were defatted before undergoing 5% (wt/vol) aqueous extraction in 0.125 mol/L ammonium bicarbonate for 4 hours at 4°C under stirring. The suspension was centrifuged at 20 000*g* for 1 hour at 4°C, supernatants were extensively dialyzed against distilled water and treated with ammonium sulfate. To achieve protein precipitation, ammonium sulfate was slowly added to obtain an 80% saturated solution. After 4 hours of stirring at 4°C, precipitated proteins were recovered by centrifugation at 20 000*g* for 1 hour at 4°C, dissolved in one fifth of the initial volume with water, dialyzed extensively against water to eliminate the residual salt and lastly against 0.05 mol/L ammonium bicarbonate. Protein content of extracts was determined according to Bradford [8] using the commercial BioRad Protein Assay Dye Reagent (Bio Rad, Milan, Italy) and BSA as reference standard.

Alexander Histocytochemical Method

The Alexander staining method [7] allows differentiating aborted and nonaborted grains; thus it is commonly used to evaluate if a grain of pollen is differentiated or not. On the other hand it does not allow proper evaluation of pollen viability, since the mixture can also stain nonviable grains. Briefly, the Alexander stain is prepared with 10 mL of absolute alcohol, 10 mg of malachite green, 50 mL of distilled water, 25 mL of glycerol, 5 g of phenol, 5 mg of chlorine hydrate, 50 mg of acid fuchsin, 5 mg of acridine orange, and 1 to 4 mL of acetic acid. The percentage of the aborted pollen usually increases with the age of the plant. Grain size and wall thickness are used to evaluate the grade of maturation.

Enzymatic TTC method

The salt 2,3,5-triphenyltetrazolium chloride (TTC) stains active enzymes and therefore can estimate the viability of pollens, fungi, plant ovaria, and leaves [10]. The staining mixture was prepared according to Comtois and Schemenauer [11], by mixing 1 mL of 10% TTC solution and 9 mL 60% sucrose solution.

A small amount of pollen was stained with a few drops of the solution on a glass slide and the sample was examined under an optical microscope after 24 hours. Four colors were graded, as follows: colorless, not viable; light pink, low viability; light red, viable; intense red, viable close to degeneration.

Patients

Patients with ascertained sensitization and respiratory allergy to cypress were selected. The presence of asthma or rhinitis was diagnosed on a clinical basis, whereas sensitization to *C arizonica* was assessed by means of a skin prick test with a commercial extract (Stallergenes, Saronno, Italy) and capsulated hydrophilic carrier polymer

Table. Patient Characteristics and Skin Prick Test Results*

Patient Number	Age	Gender	Symptoms	Histamine Wheal Diameter, mm	Pollen 1994 Wheal Diameter, mm	Pollen 2000 Wheal Diameter, mm	Other Sensitizations
1	36	M	A	8	6	9	DPF, Par
2	36	M	AR	9	7	8	DPF
3	32	F	C	10	8	9	–
4	37	M	AR	8	9	10	Par
5	37	F	AR	9	10	9	DPF, GR
6	38	F	AR	8	8	9	DPF
7	42	M	AR	8	9	8	–
8	33	M	A	10	9	9	DPF, Par
9	38	F	AR	8	10	10	DPF, Par
10	41	M	AR	9	6	8	Par, GR
11	33	M	AR	10	7	10	BE
12	34	F	AR	9	9	9	DPF
13	35	F	C	8	8	9	–
14	40	M	AR	9	8	7	DPF
15	39	M	A	8	8	8	Par
16	33	F	AR	8	10	10	DPF, Par
17	35	M	AR	8	6	9	DPF
18	35	M	C	9	8	9	–
19	40	F	AR	7	8	8	DPF
20	39	M	AR	9	8	10	Par
21	41	M	AR	8	7	9	–
22	38	F	C	9	9	9	–
23	42	M	AR	8	8	9	OE, GR
24	33	M	AR	7	7	8	BE
25	38	F	AR	8	9	8	DPF
26	37	F	AR	9	10	10	DPF, Cat
27	44	M	A	9	8	9	Par
28	30	F	AR	10	8	7	DPF, Par
29	35	M	AR	8	8	9	Par
30	38	M	AR	8	9	10	GR, OE
31	35	M	AR	9	7	11	Par
32	40	F	A	8	8	9	DPF, Par
33	35	F	AR	8	9	10	DPF
34	41	F	AR	9	9	8	DPF
35	33	F	AR	8	7	9	BE
36	38	F	AR	10	9	11	Par
37	37	F	AR	8	7	9	DPF
38	37	M	A	8	7	9	DPF
	37 ± 11.5	20 M, 18 F		8.58 ± 1.93	8.21 ± 1.93	9.01 ± 2.1	6 mono, 32 poly

* C indicates conjunctivitis; AR, allergenic rhinitis; A, asthma; BE, betula; DPF, *Dermatophagoides farinae* (mites); GR, grasses; Par, *Parietaria judaica*; OE, *Olea europea*; mono, monosensitized.
Data in the last row are mean ± SD for age and wheals (histamine, pollens).

(CAP) RAST assay (Pharmacia, Uppsala, Sweden). A mean wheal diameter of 5 mm or more and a CAP-RAST class of 4 or higher were required. Skin tests were also carried out in all subjects in triplicate using the extracts of the 1994 and 2000 pollens, by the same operator according to current guidelines. Positive (histamine 0.1%) and negative (saline buffer) controls were always included. Since it was difficult to find monosensitized subjects, the etiological role of cypress in polysensitized patients was confirmed by specific nasal challenges. An extract of *C arizonica* in water solution, standardized in RAST units per milliliter (RU/mL), was prepared at different increasing concentrations (250, 500, 1000, 2000, and 4000 RU/mL). The response to nasal challenge was measured by inspiratory peak flow meter (Youlten apparatus, Ditta Lanzoni, Bologna, Italy) reading [12] and by clinical scores. Each symptom (itching, sneezing, rhinorrhea, and congestion) was graded from 0 (absent) to 3 (severe). The threshold concentration was defined as that causing a 20% fall in the peak inspiratory nasal flow or eliciting a symptom score of 7 or greater.

All the patients gave their informed consent.

SDS-PAGE and Immunoblotting

We carried out sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of old and fresh

C arizonica pollen extracts in a 10% polyacrylamide precast Nupage Bis-Tris gel according to manufacturer's instructions (Novex, Prodotti Gianni, Milan, Italy) at 180 mA for 1 hour. Resolved proteins were stained with Coomassie Brilliant Blue and transferred onto a nitrocellulose membrane according to Towbin [13]. The membrane was saturated in Tris buffered saline containing 5% defatted dry milk before incubation with a positive or negative pool of human sera diluted 1:2 in saturating buffer. Bound specific IgE were detected by 1:2000 peroxidase-conjugated goat anti-human IgE serum (KpL, Celbio, Milan, Italy) in saturating buffer, using an ECL Blotting Kit (Amersham, Milan, Italy) as the substrate.

RAST Inhibition Analysis

Once equalized the concentration of both extracts at 30 µg/mL, RAST inhibition experiments to compare their allergenic activity were performed according to Ceska [14]. Two-fold dilutions (20 µL) of each sample (old and fresh pollen extracts) in 1% PBS-BSA were incubated for 3 hours in tubes with 30 µL of a pool of sera from the cypress-allergic patients. Subsequently a *C arizonica*-coated bead (Sferikit, Lofarma, Milan Italy) was added to each tube and incubated overnight. Bound specific IgE were detected with goat ^{125}I-labeled anti-human IgE (KpL, Celbio, Milan, Italy) diluted in PBS-1% BSA in order to obtain 30 000 cpm/50 µL). After rinsing, residual radioactivity on each bead was measured by a gamma scintillation counter and data were expressed as percentage of inhibition with respect to a maximum binding obtained without inhibitor.

Results

Results are shown as means ± SD.

Pollen

The Alexander test for differentiation revealed no appreciable difference between the pollens collected in 1994 and the fresh ones. The percentages of nonaborted grains were almost identical (94.1% ± 0.83% for the fresh pollens and 94.7% ± 0.86% for the old pollens).

On the other hand, enzymatic staining with TTC showed remarkable differences between the samples of different ages. At collection of the fresh pollen, only 59.6% ± 3.21% of grains were viable. After 20 days the percentage of viable pollen decreased to 38.4% ±2.96, and after 40 days the percentage was 1.8%. Thus, the maximum period of viability of the pollen samples from 2000 was about 30 to 40 days, which is far superior to the pollen of the other gymnosperms. Obviously, no viable pollen was detected in the 1994 sample. Further tests carried out in 2001 on the 2000 pollen samples confirmed the time limit of this biological activity. If the allergenic activity of the *C arizonica* pollen persists over time, this cannot be attributed to its viability.

Skin Prick Tests

Thirty-eight patients fulfilled the inclusion criteria: 20 of them were male and the mean age was 37 ± 11.52 years. All subjects had a positive nasal challenge with the cypress extracts, but only 6 were monosensitized. All of them suffered from allergic rhinitis and 12 also had intermittent to moderate asthma. The results of the skin tests performed in triplicate are listed in the Table. There was no difference between the two pollen samples in elicited skin reactivity (*t* test and Friedman's analysis of variance, $P = .5$).

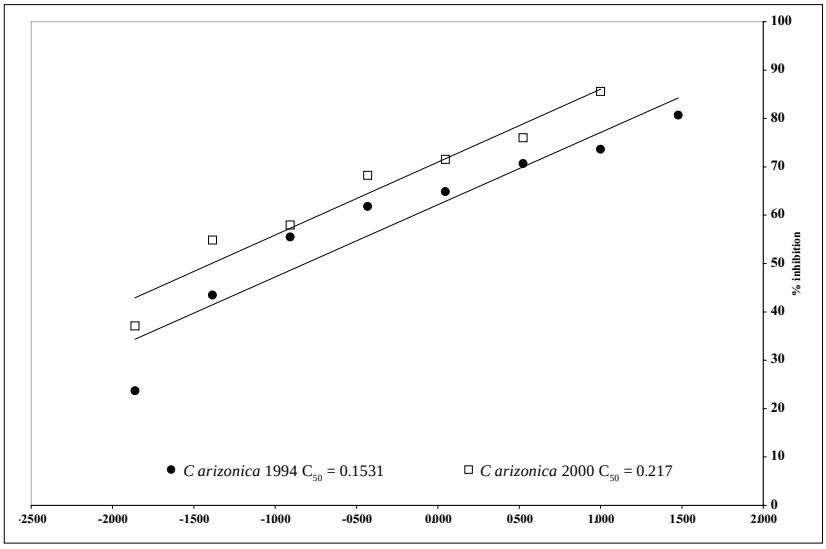

Figure 1. Comparison of allergenic potency between old and fresh *Cupressus arizonica* pollen extract. The point of comparison is the volume of extract necessary to obtain 50% of inhibition (C_{50}).

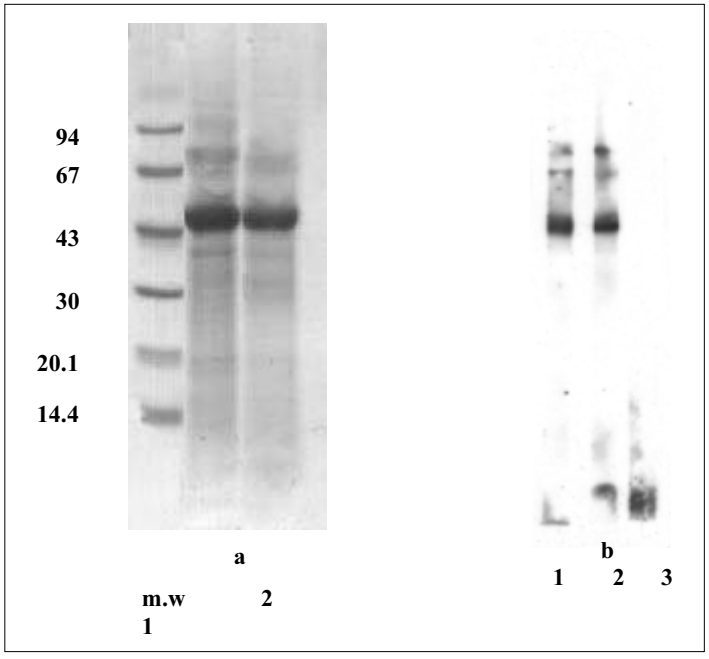

Figure 2. a) sodium docecyl sulfate polyacrylamide gel electrophoresis profile; lane 1: old (1994) *Cupressus arizonica* pollen extract; lane 2: new (2000) *C arizonica* pollen extract. b) immunoblotting profile; lane 1: old (1994) *C arizonica* pollen extract against IgE positive serum; lane 2: new (2000) *C arizonica* pollen extract against IgE positive serum. The negative serum (data not shown) showed no evidence on any band.

Immunological Assays

Protein content of old and fresh *C arizonica* extracts were respectively 0.360 and 0.650 mg/mL. The SDS-PAGE immunoblotting experiments showed a similar profile for both extracts. In particular, the major allergen of *C arizonica* (band at about 45 KDa) was well represented and recognized by the specific IgE in human sera from cypress-pollen allergic patients (Figure 1). To compare the allergenic potency of old and new *C arizonica* extracts we also performed RAST inhibition experiments. As indicated in Figure 2, the values for the volume of extract necessary to obtain a 50% inhibition of the response of the two extracts were almost equal, confirming that the IgE binding capacity of the two extracts is comparable.

Discussion

Considerable work has been carried out on cypress pollen to identify the major allergens of *C arizonica* (Cup a 1 [15] and Cup a 3 [16]) and of *C sempervirens* (Cup s 1 [17] and Cup s 3 [18]). However, since it is likely that other allergens will be found, we thought that it would be preferable for this comparative study to use a complete raw extract containing all the possible allergens. A literature search yielded no comparisons of the allergenic potency of old and fresh pollens after a few years of preservation at room temperature. We wondered if there are factors influencing the antigenicity of pollens: location of the point of collection on the tree (north, south), storage, collection period (season), pollution and tree diseases. The pollens were collected from the same parts of a disease-free tree (no insects or fungi).

A main finding is that there was no change in allergenic potency, that is, the ability of *C arizonica* pollen collected under identical conditions in 1994 and 2000 to bind specific IgE antibodies. This finding is relevant because small amounts of pollens can persist in unused corners of houses or buildings for a long period of time, possibly explaining the persistence of symptoms outside the blooming season. It is also known that pollen, especially of conifers, is transported by the wind for long distances, for instance from the states of Oklahoma to Texas in the United States of America [19]. If we accept the findings of the relatively perennial nature of the allergenic activity of Cupressaceae pollens, we have to face the fact that, in areas with strong and dominant winds such as the mistral in the Provence region of France, pollen can be transported to areas devoid of conifers and persist there for a long time.

The second interesting finding is that the amount of proteins measured by the Bradford technique in the old pollen was half that of the fresh one. However this observation is insufficient to confirm that carbohydrates, which are abundant in the Cupressaceae pollens, play a part. They may, however, participate as epitopes, in some cases, in the overall allergenicity of the pollen, as already demonstrated, though it is known that carbohydrates are very often linked to proteins [20].

The third relevant observation is that in contrast with the persistence of allergenic potency, viability and germinating power have almost completely disappeared. So the two characteristics of Cupressaceae pollens, allergenic potency and germinating power, are independent.

In summary, after a 6-year period of storage at room temperature, *C arizonica* pollen conserved its allergenic potency but lost its germinating power and half of its

protein content. It is likely that these findings, knowing the cross reactivity between all the members of the Cupressaceae and Taxodiaceae families, might be extended to other tree pollen families. The demonstration of the persistence of allergenic activity that is independent of viability may also have some practical implications, as it can explain the presence of symptoms out of the pollen season in subjects clinically sensitized to cypress.

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