

Allergenic Relevance of *Cupressus arizonica* Pollen Extract and Biological Characterization of the Allergoid

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Key Words

Cypress allergy · *Cupressus arizonica* pollen · Cup a 1 allergen · Potassium cyanate · Allergoid

Abstract

Background: Cupressaceae (cypress) pollens can cause pollinosis in winter. However, the lack of specific commercial extracts combined with the early pollination period of cypress trees make a precise diagnosis difficult. The need for a reliable and effective cypress extract for diagnostic and therapeutic purposes is increasingly felt. **Methods:** Mixed or single *Cupressus arizonica*, *lusitanica* and *sempervirens* pollen extracts precipitated with ammonium sulfate (PPT) were compared by direct RAST, RAST inhibition and SDS-PAGE techniques. The major allergen of *C. arizonica* (Cup a 1), purified by anion exchange chromatography, was checked by immunoblotting experiments before chemical modification, in parallel with a *C. arizonica* extract, with potassium cyanate (KCNO) to obtain a monomeric allergoid. The allergoid extract was characterized for its biological, chemical-physical and immunological features by RAST inhibition, SDS-PAGE and ELISA assays. **Results:** Direct RAST, RAST inhibition, and SDS-PAGE data indicated that the

PPT *C. arizonica* pollen extract showed the most allergenic potential, and it can be considered representative of the *Cupressus* spp. Immunoblotting data confirmed Cup a 1 as a major allergen. RAST inhibition and ELISA showed that modified PPT *C. arizonica* extract had less IgE reactivity than the native, non-modified extract, while preserving the immunogenic capacity typical for an allergoid. Finally, the SDS-PAGE profile of Cup a 1 allergoid was similar to native Cup a 1 allergen, suggesting the modified *C. arizonica* extract shows the characteristics of a monomeric allergoid. **Conclusions:** The PPT *C. arizonica* pollen extract shows good in vitro diagnostic potential and its chemically modified form offers the features of a monomeric allergoid. It might therefore lend itself to the development of a product to be administered by the sublingual or oromucosal route for immunotherapy of individuals with cypress pollinosis.

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Introduction

The elegant shape of the beautiful *Cupressus* spp. (cypress) tree marks the landscape of certain parts of Italy, and Southern Europe in general, where the climatic condi-

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tions, optimal for their growth, have led to large-scale plantation. This, however, has had a negative impact on the surrounding environment on account of the spread of large amounts of pollen.

Inhalation of pollen from cypress trees (the most widespread being *Cupressus arizonica*, *C. lusitanica* and *C. sempervirens*) is becoming an increasing cause of allergic diseases, particularly in the Mediterranean area [1–5]. In Japan, the major source of pollens are the Japanese cypress (*Chamaecyparis obtusa*) and the Japanese cedar (*Cryptomeria japonica*), a member of the closely related family of Taxodiaceae [6–8]. According to recent estimates, cypress pollen allergy can reach more than 20% of all the cases of pollinosis in some areas [9, 10].

The early pollination period of cypress trees (November to February) makes a precise diagnosis difficult because specific allergic symptoms can be confused with seasonal illnesses such as the flu or common colds. In addition, the fact that commercial diagnostic *Cupressus* spp. pollen extract is not completely satisfactory in terms of allergenic potency further complicates the situation. The real incidence of this specific allergy may therefore be underestimated [11]. The need is therefore felt for a more potent cypress pollen extract.

One of the reasons why commercial cypress extracts are poorly effective may be that they contain large amounts of polymeric carbohydrates devoid of allergenic activity. Much work has been done to achieve a cypress pollen extraction procedure that eliminates, or at least reduces, these carbohydrates. Extracts from different cupressaceae pollens and different extraction procedures have been employed [12–17]. The extraction procedure based on precipitation of proteins with ammonium sulfate seems to be the most reliable in improving the allergenic potential [14]. Further improvement could come from the identification of a single *Cupressus* spp. pollen representative of the most common species in terms of diagnostic capabilities: *C. arizonica* pollen is a candidate [18]. Confirmation of both these aspects would considerably facilitate the preparation of an effective extract improving the diagnosis of this specific allergy and smoothing the path to the development of a vaccine.

The aims of our work are therefore: (1) to confirm the better IgE-binding capacity of a PPT mixture of pollen extracts (*C. arizonica*, *C. sempervirens* and *C. lusitanica*) compared to a non-PPT one; (2) to compare these three *Cupressus* spp. pollen extracts by different techniques in order to identify the most representative one in terms of allergenic potential; (3) to modify the selected extract (*C. arizonica*) and single purified allergen (Cup a 1) by a

chemical reaction with potassium cyanate to reduce their allergenic potential; (4) to characterize a chemically modified extract for its biological, immunological and physico-chemical features. The findings are discussed with the objective of developing specific diagnostic and therapeutic products.

Materials and Methods

Preparation of *Cupressus* spp. Pollen Extracts

C. arizonica, *C. lusitanica* and *C. sempervirens* pollens were kindly supplied by Dr. Raddi (Istituto per la Protezione delle Piante Forestali, CNR, Florence, Italy). Purity, checked by optical microscopy, was more than 99%. The pollens, as single species or as mixtures of equal amounts of each, were defatted before 5% (w/v) aqueous extraction in 0.125 M NH_4HCO_3 for 4 h at 4°C under stirring. The suspensions were centrifuged at 20,000 g for 1 h at 20°C and supernatants were extensively dialyzed against distilled water. The mixed or single pollen extracts were then treated or not with ammonium sulfate in order to obtain precipitated (PPT) single or mixed pollen extracts or a non-PPT mixture of pollen extracts.

To precipitate proteins, ammonium sulfate was slowly added to obtain a 80% saturated solution. After 4 h of stirring at 4°C, precipitated proteins from each extract (from the single or mixture of pollens) were recovered by centrifugation at 20,000 g for 1 h at 4°C, redissolved in 1/10 of the initial volume with water, dialyzed extensively against water to eliminate the residual salt and, last, against 0.05 M NH_4HCO_3 . The supernatant from the PPT mixture of pollen extracts was dialyzed in the same way and tested undiluted. Finally, the protein content of all extracts was determined according to Bradford [19] using the commercial BioRad Protein Assay Dye Reagent (BioRad, Milan, Italy) and BSA as reference standard. All extracts were then lyophilized and stored at 4°C.

Preparation of Solid Phases and Direct RAST

Polystyrene beads (6.4 mm diameter, Precision Plastic Balls, Chicago, Ill., USA) were coated with PPT mixture or supernatant mixture or non-PPT mixture or PPT single cypress pollen extracts or Cup a 1 allergen, according to the following procedure. Beads were pre-treated with glutaraldehyde 0.2 M in PBS for 5 h at room temperature, then incubated overnight in end-over agitation with 150 µl/bead of various samples, single or mixture extracts and Cup a 1 allergen, all at 10 µg/ml in PBS or with 150 µl/bead of undiluted supernatant from the PPT mixture of pollen extracts. After several washings with PBS, the beads were stabilized with NaHBO_3 0.37% in PBS for 1 h and, finally, saturated with 5% BSA in PBS for 24 h.

For direct RAST experiments, 50 µl of each undiluted serum were added to a coated bead and incubated overnight at room temperature. Bound specific IgE was detected by incubation for 8 h with a goat ^{125}I -labeled anti-human IgE (KLH, Celbio, Milan Italy) diluted in PBS-1% BSA in order to obtain 30,000 cpm/50 µl; after further washings, the residual radioactivity on each bead was measured with a gamma scintillation counter. The bound radioactivity was expressed as a percentage of the total radioactivity added. The RAST positivity class of sera was determined using a reference curve (Sferikit, Lofarma S.p.A., Milan, Italy).

RAST Inhibition Analysis

The concentration of all the extracts was equalized at 30 µg/ml, and three sets of RAST inhibition experiments were performed according to Ceska et al. [20] in order to compare the allergenic activity of (a) the three single *Cupressus* spp. extracts, (b) the native *C. arizonica* extract compared to the corresponding allergoid extract, (c) the native Cup a 1 allergen compared to the allergoid. Briefly, several two-fold dilutions of each sample in 1% BSA in PBS (PBS-BSA), were pre-incubated for 3 h with a pool of sera from patients allergic to cypress. Then one bead coated with PPT mixed pollen extract (case a), or with *C. arizonica* pollen extract (cases b and c), was added to each tube and incubated overnight. Bound IgE was detected with goat ¹²⁵I-labeled anti-human IgE (KLH, Celbio, Milan Italy) diluted as described for direct RAST. The residual radioactivity was measured in a gamma scintillation counter and inhibition was expressed as a percentage of the maximum binding without the inhibitor.

SDS-PAGE and Immunoblotting

Electrophoresis of *Cupressus* spp. extracts and native or modified Cup a 1 was done in a 10% polyacrylamide precast Nupage Bis-Tris gel according to the manufacturer's instructions (Novex, Prodotti Gianni, Milan, Italy) at 180 mA for 1 h. Thirty micrograms of each extract or 10 µg of purified allergen were loaded per centimeter of gel. The resolved proteins were stained with Coomassie Brilliant Blue. Protein bands from *C. arizonica* extract were transferred onto a nitrocellulose membrane according to Towbin et al. [21]. The membrane was saturated in Tris-buffered saline buffer containing 5% defatted dry milk (saturating buffer), before incubation with positive or negative single human sera diluted 1:2 in saturating buffer. Bound specific IgE was detected using 1:2,000 peroxidase-conjugated goat anti-human IgE antiserum (KPL, Celbio, Milan Italy) in saturating buffer, using the ECL Blotting Kit (Amersham, Milan, Italy) as substrate.

Purification of *C. arizonica* Major Allergen (Cup a 1)

Cup a 1 was obtained purified by anion-exchange chromatography using an Econopack High Q Cartridge (BioRad, Milan Italy), following the procedure described by Alisi et al. [22]. Four milligrams of the lyophilized PPT extract were reconstituted with 0.05 M Tris buffer pH 8 then loaded into the column, conditioned with the same buffer. After the unbound proteins had passed through, a 0–50% NaCl gradient was applied in 20 min to elute bound proteins. Fractions corresponding to the first eluting peak (at about 0.2 M NaCl) were dialyzed against water and analyzed by SDS-PAGE.

Chemical Modification of *C. arizonica* Extract and Cup a 1 Allergen

Lyophilized *C. arizonica* extract and purified Cup a 1 were reconstituted with 20 mM phosphate buffer pH 7.2 at 1 and 0.2 mg/ml, respectively, then 2.5 ml of each sample were passed through a Sephadex G25 column (PD10, Amersham Pharmacia Biotech, Milan, Italy) conditioned in the same buffer. Excluded peaks were collected and chemically modified as previously described [23]. Briefly, solid sodium tetraborate and potassium cyanate were added to 2.5 ml of gel-filtered samples to obtain final concentrations of 0.1 and 1 M. After 20 h at 40°C, the salts were eliminated by gel filtration on a G-25 column (PD10, Amersham Pharmacia Biotech, Milan Italy) and samples were characterized as IgE reactivity and molecular weight in comparison with the corresponding native counterpart.

Immunization Protocol

Three Balb/c mice (Charles River, Calco, Como, Italy) were injected subcutaneously with 20 µg each of chemically modified *C. arizonica* extract, emulsified with complete (first injection) or incomplete Freund's adjuvant (three injections at 1-week intervals). Three days after the last booster, the mice were bled from a tail vein and sera were tested by ELISA for their capacity to recognize native and modified *C. arizonica* extracts.

ELISA Procedure

Specific IgG antibody titer against native and modified *C. arizonica* extracts was determined by ELISA. Briefly, microtiter plates (Maxisorp, Nunc, Mascia Brunelli, Milan, Italy) were coated with 0.1 µg/well of native or modified extract and 100 µl of several two-fold dilutions of each serum in PBS-BSA were added to the wells. Bound specific IgG was detected by adding a 1:2,000 dilution of peroxidase-conjugated goat-anti-mouse IgG serum (Sigma, Milan, Italy); the subsequent colorimetric reaction was developed using TMB/H₂O₂ as substrate and the data were expressed as optical density at 450 nm.

Human Sera

Sera from patients allergic to cypress pollen were kindly provided by Dr. Ariano (Modulo di Allergologia, Bordighera Hospital, Imperia, Italy). Patients reporting clinical symptoms of allergy were investigated by a skin prick test and subsequently a specific nasal provocation test (table 1) with commercial products (Lofarma S.p.A., Milan, Italy), containing a mixture of *C. arizonica* and *C. sempervirens* pollen extracts. Sera from patients testing positive on both tests (allergic subjects) were harvested and used as single serum or as a pool (RAST inhibition). The patients were informed of the study and signed a written consent form before it started. Pooled sera from non-allergic patients were used as negative control.

Results

Biological Activity of PPT or non-PPT Mixed Cypress Pollen Extracts

This part of the study was done first as a basis for subsequent work. Direct RAST experiments using sera from subjects allergic to *Cupressus* pollen or controls showed that, compared to beads coated with non-PPT mixture, beads coated with PPT mixture extract gave a significantly higher percentage ($p < 0.01$ according to the Wilcoxon Signed Rank test) of bound IgE for all positive sera (data not shown). Control sera were negative for both extracts.

In addition, a check for allergenic activity in supernatant from the PPT mixture pollen extract by direct RAST using sera No. 2, 5 and 7 showed it was negligible, with less than 1.85% bound IgE on supernatant-coated beads (data not shown).

Fig. 1. Comparison of *C. arizonica*, *C. lusitanica* and *C. sempervirens* single extracts by direct RAST. 1, 2, 4–8, 10 = single sera from patients allergic to cypress; N = pool of normal sera.

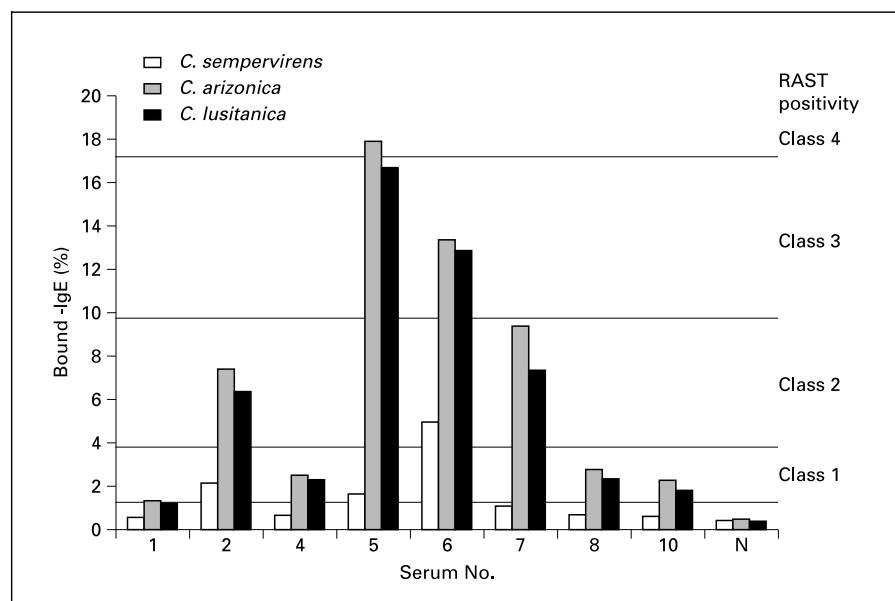


Table 1. Main clinical data of patients used in the study

Patient	Symptoms	SPT	NPT	Other positivities
1	conjunctivitis, rhinitis	2+	+	Dermatophagoides
2	conjunctivitis, rhinitis	3+	+	none
3	rhinitis	3+	+	none
4	rhinitis	2+	+	Parietaria
5	asthma	4+	+	none
6	rhinitis	3+	+	Dermatophagoides, Olea
8	conjunctivitis	3+	+	Dermatophagoides
9	conjunctivitis	3+	+	grass
10	asthma	2+	+	Dermatophagoides, Parietaria

SPT = Skin prick test performed with commercial mixed cypress pollen extract; NPT = nasal provocation test performed with commercial mixed cypress pollen extract.

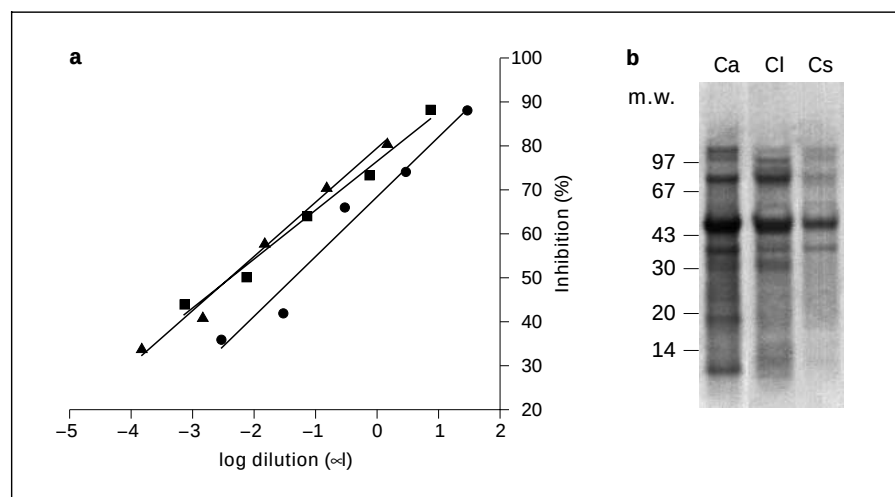
Characterization of *Cupressus* spp. Pollen Extracts

The protein content of PPT and non-PPT mixed pollen extracts was 115 and 13 µg/ml, respectively. This reflected the fact that the PPT extract was concentrated 10 times; the protein content of PPT *C. arizonica*, *lusitanica* and *sempervirens* pollen extracts was 238, 127 and 30 µg/ml, respectively. Comparative direct RAST data (fig. 1) using beads coated with single extracts indicated that *C. arizonica* extract had the most allergenic potential. In fact all positive sera showed specific IgE antibodies against it. With *C. sempervirens*-coated beads, we lost some positive sera (No.1, 4, 8 and 10), and the others showed lower positivity in any case. The results with

beads coated with *C. lusitanica* extract were similar to those with *C. arizonica* extract although the percentage of bound IgE was slightly lower. Wilcoxon's signed rank test confirmed a significant difference between *C. arizonica* and *C. sempervirens* ($p < 0.01$) but not between *C. arizonica* and *C. lusitanica*.

In RAST inhibition experiments (fig. 2a), the C_{50} (defined as the volume of extract able to inhibit IgE binding by 50% of *C. arizonica*, *lusitanica* and *sempervirens* extracts) were respectively 0.003, 0.006 and 0.013, indicating that *C. arizonica* extract showed the best allergenic potency. Subsequent SDS-PAGE analysis (fig. 2b) to compare the qualitative characteristics of the three differ-

Fig. 2. Comparison of pollen extracts from three cypress species. a RAST inhibition experiments: $\blacktriangle$ = *C. arizonica* (C_{50} = 0.003 μ l); $\blacksquare$ = *C. lusitanica* (C_{50} = 0.006 μ l); $\bullet$ = *C. sempervirens* (C_{50} = 0.013 μ l). b SDS-PAGE profile of *C. arizonica* (Ca), *C. lusitanica* (Cl), *C. sempervirens* (Cs).



ent extracts showed the protein profiles of *C. arizonica* and *C. lusitanica* extract were similar. Various components with molecular weights ranging from about 10 to 100 kD were evident, the most representative being the component at about 45 kD, known as the major allergen Cup a 1 [22]. The *C. sempervirens* extract gave a protein pattern with fewer bands, ranging from 35 to about 75 kD; components of lower molecular weights, including one at 30 kD, were lacking.

In immunoblotting experiments using single sera from patients allergic to cypress pollen or controls (pool) (fig. 3) all the sera from the first group reacted against *C. arizonica* pollen extract components, though with different patterns. Based on RAST results, sera No. 2, 5, 6, 7, and 9 yielded strong IgE reactivity, recognizing several bands of different molecular weights (30, 35, 45, 70, 100 and >100 kD); the other sera reacted with lower intensity, recognizing one or more allergens. All sera recognized the major allergen (Cup a 1). Pooled sera from controls recognized no band. These results strongly suggest that *C. arizonica* pollen extract can be considered representative of the three cypress species.

Purification of *C. arizonica* Major Allergen

When *C. arizonica* pollen extract was submitted to anion exchange chromatography, several fractions were obtained; some of them, pooled on the basis of SDS-PAGE analysis, contained only the band at about 45 kD, with a good degree of purity (fig. 4c, lane N). When tested by immunoblotting, this component was recognized as a single band by all *Cupressus*-positive sera (data not shown). Control serum recognized none of the bands.

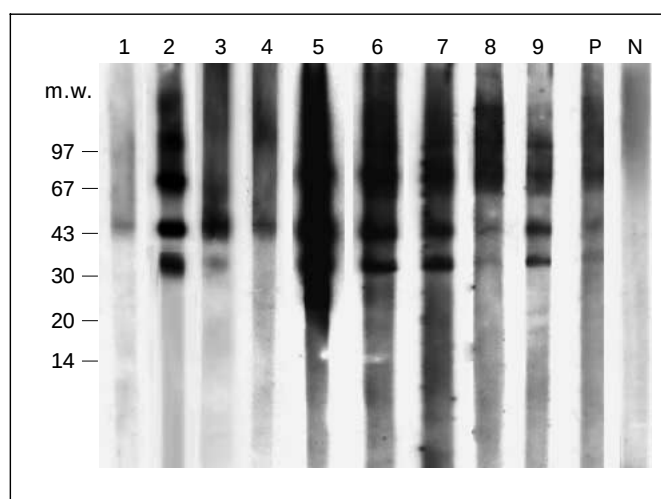
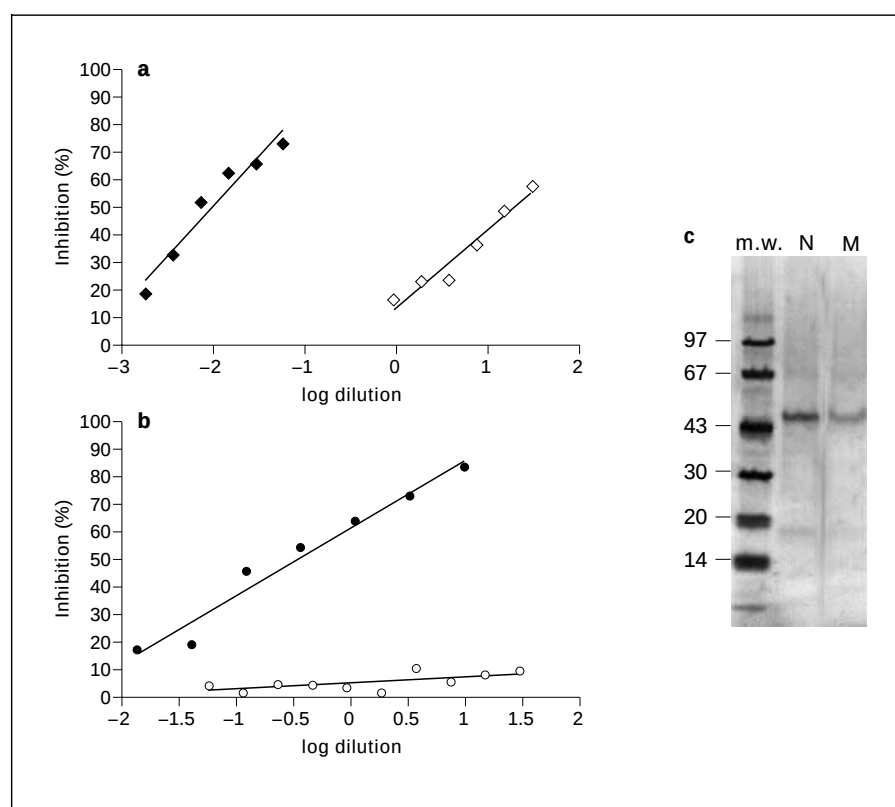


Fig. 3. Allergenic profile of *C. arizonica* pollen extract by immunoblotting. 1–9 = single sera from patients allergic to cypress; P = pool of positive sera; N = pool of sera from nonallergic individuals.

Allergenic Potency of Chemically Modified *C. arizonica* Extract and Cup a 1 Allergen

As we can deduce from RAST inhibition experiments (fig. 4a), the allergenic activity of modified *C. arizonica* pollen extract was significantly reduced, and the C_{50} value for the native *C. arizonica* extract was 0.005 μ l as against 22.3 μ l for the modified extract. Considering the purified major allergen, treatment with KCNO almost completely abolished its allergenic activity (fig. 4b), native Cup a 1 giving a C_{50} of 0.34 μ l whereas the C_{50} for the modified counterpart could not be established.

Fig. 4. Chemicophysical and immunological characterization of natural and modified products. a Comparisons of allergenic potency of native (◆) and modified (◇) *C. arizonica* extracts by RAST inhibition. b Comparisons of allergenic potency of native (●) and modified (○) Cup a 1 allergen by RAST inhibition experiments. c SDS-PAGE profile of native (N) and modified (M) Cup a 1.



SDS-PAGE experiments comparing the native and modified allergen Cup a 1 at the same concentration (fig. 4c) showed a single 45-kD band in lanes M and N, suggesting the molecular dimension of the modified allergen was not affected by the treatment with KCNO. The lower staining intensity of the band corresponding to the modified Cup a 1 might be due to the fact that the modification of the lysine residues induced by KCNO, decreasing the interaction between Coomassie stain and protein, makes it less colorable.

ELISA

In order to verify the capacity of the chemically modified *C. arizonica* extract to elicit an IgG response against its native, non-modified counterpart, mice were immunized with allergoid extract and their sera were tested by ELISA using native or modified extract as antigens. As shown in figure 5, all the sera showed an IgG response to both the natural and modified extract, although the specific antibody titers differed slightly, suggesting that modified *C. arizonica* extract maintained its immunogenic properties.

Discussion

Cupressus spp. pollens are a cause of allergic symptoms particularly among the peoples of the Mediterranean area [1–5]. It is a common opinion that recent increases in this pollinosis are due to extensive planting of cypress trees for different purposes. *C. sempervirens*, *C. arizonica* and *C. lusitanica* are the most common species; they have an early pollination period (from the end of November to February), but there is high IgE cross-reactivity among pollens from the Cupressaceae family (Thujoideae, Cupressoidae and Juniporoideae) and other taxonomically related families such as Taxodiaceae (i.e. *C. japonica*), so this can make a long season for sensitized patients who cross-react to the above pollens [7–24].

The early pollination period of *Cupressus* spp. trees induces symptoms often similar to those of seasonal illnesses such as the common cold or influenza, and the lack of a satisfactory commercial diagnostic cypress pollen extract makes a precise diagnosis difficult. The importance of cypress pollens is therefore an underestimated cause of specific allergy [11].

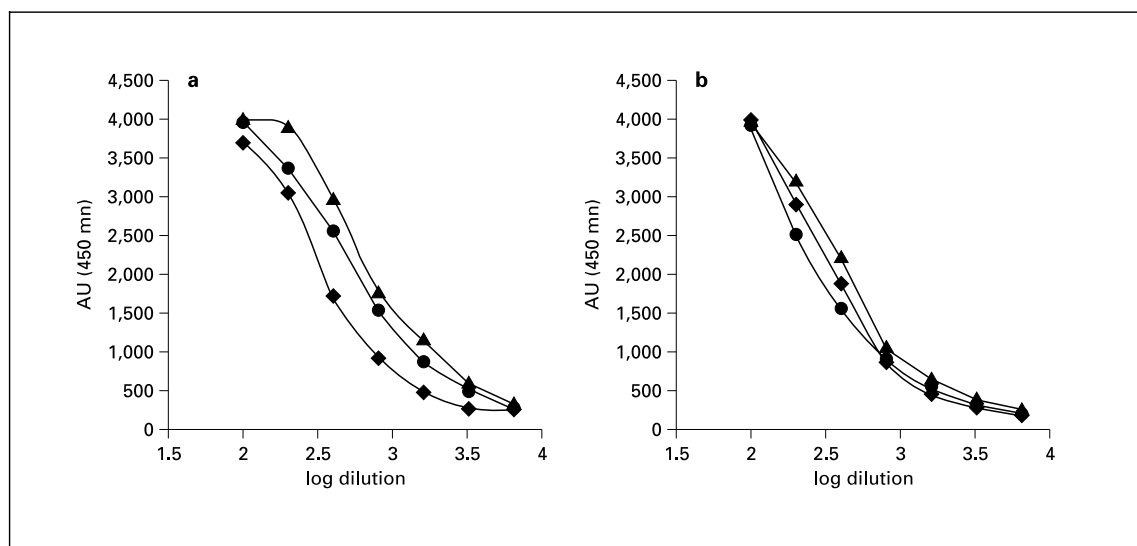


Fig. 5. ELISA experiments using sera of mice immunized with modified *C. arizonica* extract. Specific IgG reactivity against native (a) or modified (b) *C. arizonica* extracts. ▲ = Mouse serum No. 1; ◆ = mouse serum No. 2; ● = mouse serum No. 3.

One possible reason for the scarce diagnostic efficiency of commercial cypress pollen extracts may be their massive content of carbohydrates (almost 85%), proteins being only 2–3% [14]. While a certain proportion of these carbohydrates, bound to proteins to form glycoproteins and known as the cross-reactive carbohydrate determinant (CCD), seems to play an important role in explaining the IgE cross-reactivity among the Cupressaceae [25], most of them (i.e. polysaccharides) have negligible IgE-binding capacity and could have a deleterious effect in the preparation of high-quality *Cupressus* spp. extract.

The *Cupressus* spp. pollen extraction procedure based on precipitation of the proteins with 80% saturated $(\text{NH}_4)_2\text{SO}_4$, as described by Di Felice et al. [14], might be a simple way to prepare a useful diagnostic extract. Our preliminary study by direct comparative RAST experiments (data not shown) comparing a PPT and a non-PPT mixture (*C. arizonica*, *C. sempervirens*, *C. lusitanica*) of pollen extracts was designed to confirm this; compared to the non-PPT mixture, the PPT extract maintained its biological activity (IgE-binding capacity) after a significant reduction (more than 50%, data not shown) of its total carbohydrate content as determined by the method of Dubois et al. [26]. This procedure not only most probably eliminates the polymeric non-IgE-binding carbohydrates, but also has the important advantage that it can be concentrated a number of times without any problematic

increase in the viscosity of the solution, unlike the non-PPT extract. So the use of PPT extract for our subsequent experiments is largely justified.

As mentioned above, *C. arizonica*, *C. lusitanica* and *C. sempervirens* pollens dominate the aeroallergen scene in the Mediterranean area and there appears to be strong cross-reactivity at least within Cupressaceae species [1]. A similar situation has already been seen for grass pollen extracts which include several different species. In view of this high cross-reactivity, it has been proposed that at least for diagnostic purposes a single or at most a mixture of two different grass pollens might well be enough to prepare an efficacious extract [27].

Recent observations seem to indicate that *C. arizonica* can be considered the most reliable species for the diagnosis of Cupressaceae allergy [18]. As this has practical implications for allergen manufacturers, simplifying the production of these extracts, a wide comparative analysis of the three most common cypress pollens was a further aim of our study. Our experiments concerning direct RAST, RAST inhibition and SDS-PAGE, point to *C. arizonica* pollen extract as having the greatest allergenic potential (direct RAST and RAST inhibition data), probably as a result of the larger number of protein components (SDS-PAGE data). In any case, the yield in terms of proteins differs dramatically between *C. arizonica*, *C. lusitanica* and *C. sempervirens* extracts. Further charac-

terization of *C. arizonica* extract by immunoblotting analysis using single sera from allergic patients evidenced a major allergen, Cup a 1, which can be easily purified by anion-exchange chromatography.

These observations suggest that a diagnostic product based on PPT *C. arizonica* pollen alone should reveal *Cupressus* spp. pollen allergy, whereas the use of *C. sempervirens* pollen extract might be associated with the risk of false-negative results.

The development of an effective diagnostic product is the first step on the way to the preparation of a product for immunotherapy of patients allergic to *Cupressus* spp. pollens. The risk of side effects with injected allergenic extracts [28] has led allergists to explore safer therapeutic modalities. Chemical modification of allergens with KCNO, under conditions that promote the selective substitution of the ϵ -amino groups of the lysine residues of the protein molecules, has proved ideal for developing monomeric allergoids [23]. Our comparative RAST inhibition experiments indicated that KCNO significantly reduces the allergenic potential of the modified allergens, whether they are in a mixture (*C. arizonica* extract) or well defined like Cup a 1 (fig. 5a, b). This means that the KCNO, reacting with the ϵ -amino groups of the lysine residues of the *C. arizonica* pollen proteins, influences the interaction between specific IgE and B cell epitopes on *C. arizonica* proteins. Lastly, our ELISA experiments using sera of animals immunized with *C. arizonica* allergoid extract showed that this extract can induce the formation of specific IgG antibodies able to recognize the native,

nonmodified extract, so it would appear that the allergoid extract maintains its immunogenic properties.

In addition to the preservation of its immunogenic properties, *C. arizonica* pollen allergoid extract also offers another important feature, the monomeric dimension of the native extract, as confirmed by SDS-PAGE experiments with Cup a 1 (fig. 4c). We used a single allergen in these experiments in order to assess any change in molecular size induced by KCNO more accurately; with a raw *C. arizonica* extract, containing different molecular weight components, it would have been harder to interpret any polymerization caused by KCNO.

The preservation of the native monomeric nature of *C. arizonica* allergoid extract is a very important feature with a view to developing a vaccine to be administered by the sublingual/oromucosal route. Other monomeric allergoids have already been used in clinical practice [29–32] and absorption of the allergen through the mucosal membrane seems to be important in the expression of the allergoid's therapeutic effects [33]. Polymerized allergoids, such as those obtained by reacting an extract with aldehydes would certainly have more difficulty crossing the mucosa, presumably on account of the high molecular dimension of their components. This is probably the reason why polymerized allergoids have only been given by subcutaneous injection in clinical trials up to now.

Studies are now planned to assess the in vivo diagnostic efficacy of PPT *C. arizonica* pollen extract and its potential benefit in local immunotherapy of patients allergic to cypress pollen.

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