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Allergens of Arizona cypress (*Cupressus arizonica*) pollen: Characterization of the pollen extract and identification of the allergenic components

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Abstract

Species of the Cupressaceae family are an important cause of respiratory allergies in countries with a Mediterranean climate. An allergenic extract from *Cupressus arizonica* pollen was prepared with two extraction steps followed by ammonium sulfate precipitation, giving a protein yield of about 3%. *Cupressus arizonica* pollen extract was also characterized by means of sodium dodecylsulfate–polyacrylamide gel electrophoresis, followed by IgE and IgG immunoblotting and lectin blotting. IgE reactivity was restricted to six components, whereas IgG binding showed a more complex pattern. A 43 kd component, predominant both in its intensity and frequency of recognition by human IgE antibodies, was identified as the major allergen of *C. arizonica*. Four of the six IgE-binding components, including the major allergen, seem to be glycoproteins, as confirmed by the lectin blotting analysis. The extract produced inhouse was used to set up an immunoenzymatic test to evaluate the specific IgE binding in a panel of sera from 33 immunotherapy-free subjects who were monosensitized to cypress pollen. The percent of positivity obtained was much higher than that reported in the literature for commercial immunoassays. (J ALLERGY CLIN IMMUNOL 1994;94:547-55.)

BSL	<i>Bandeiraea simplicifolia</i> lectin
CaE	<i>Cupressus arizonica</i> pollen extract
Con A	Concanavalin A
LCA	<i>Lens culinaris</i> agglutinin
MW	Molecular weight
OD₄₉₅	Optical density at 495 nm
PHAe	<i>Phaseolus vulgaris</i> erythroagglutinin
PNA	Peanut agglutinin
SDS-PAGE	Sodium dodecylsulfate–polyacrylamide gel electrophoresis
SJA	<i>Sophora japonica</i> agglutinin
SPT	Skin prick test
UEA	<i>Ulex europaea</i> agglutinin

Key Words: [Cupressus arizonica](#), [allergens](#), [characterization](#), [diagnosis](#), [IgE](#), [IgG](#), [lectins](#), [ELISA](#)

Human allergy to Cupressaceae pollens has been reported since 1945.¹ Species of the Cupressaceae family are a very important cause of respiratory allergies in North America (*Juniperus sabinoidea*s or mountain cedar, in Texas and the southwestern states²⁻⁴) and Australia (*Cupressus sempervirens*⁵). In Japan the major pollen allergen source is the Japanese cedar (*Cryptomeria japonica*⁶⁻⁸), a member of the closely related family of Taxodiaceae.

This pollinosis constitutes a significant problem in some countries of the Mediterranean area, including France,^{9, 10} Italy,¹¹ and Israel,¹² because of the diffusion in wide areas of these countries of species belonging mainly to the genus *Cupressus*, in particular, *C. sempervirens* and *C. arizonica*. Pollen extracts of these Cupressaceae species are currently used for diagnosis and immunotherapy of Cupressaceae pollinosis, but commercially available in vitro diagnostic tests frequently provide equivocal or false-negative results in patients showing symptoms, as compared with the clinical evaluation and results of skin prick tests (SPTs).^{5, 13} This finding, which could account for the relative underestimation of this pollinosis, may be related to the quality of the extracts used in the coating of the solid phase (i.e., disks or wells for immunoassays), as suggested by Ford et al.,⁵ and reflects the need for improvement in the characterization and standardization of the active components of Cupressaceae pollen extracts.

Because of the increasing number of *C. arizonica* plants used for gardening and reforestation purposes and because of the probable increasing epidemiologic impact of the relevant pollinosis,¹¹ we prepared a *C. arizonica* pollen extract (CaE) in house and characterized this extract by means of sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by blotting, to investigate the various components and their allergenic importance. This extract was used to set up an immunoenzymatic test to evaluate the specific IgE binding in a panel of sera from subjects diagnosed, on the basis of clinic history and SPT results, as allergic to Cupressaceae pollen only.

Methods

Human sera

Serum samples were obtained from 33 subjects (mean age, 36.2 years; age range, 19 to 64 years) from the area of Bari, Southern Italy, where *C. arizonica* is regarded as the species mainly responsible of

Cupressaceae pollinosis. These subjects, who have never received specific immunotherapy, were diagnosed, on the basis of clinical history and SPT results, as being allergic to Cupressaceae pollen only. They all had oculorhinitis, and 9 of 33 (27.3%) also had asthma. SPTs were performed in a quantitative fashion by using a commercial cypress extract (Dome Hollister Stier, Miles-Bayer, Bridgend, U.K.). A mean wheal diameter of greater than 3 mm, 15 minutes after pricking, was considered a positive result. The allergen panel included the following commercially available extracts: *Phleum pratense*, *Parietaria judaica*, *Olea europaea*, *C. sempervirens*, *Artemisia vulgaris*, *Dermatophagoides pteronyssinus*, *D. farinae*, *Cladosporium herbarum*, *Felis domesticus*, and *Canis familiaris*. Serum samples to be used as negative controls were collected from 10 nonatopic volunteers from the same sera and tested with the same allergen panel.

C. arizonica pollen extract (CaE) preparation

C. arizonica pollen was collected in February 1992 in a Cupressaceae woody area south of Bari, Southern Italy. Source plants were identified by means of rigorous botanical criteria, and pollen was collected under controlled conditions to avoid contamination. Pollen purity (>99%) was assessed by microscopic analysis performed by a well-trained specialist.

Three hundred grams of pollen were defatted with ether and then extracted in 3000 ml of 1 mmol/L NH_4HCO_3 , pH 8, with 1 mmol/L phenylmethylsulfonyl fluoride and 0.1% wt/vol sodium azide, at 4° C for 48 hours with stirring. After extraction, the pollen was separated from the supernatant by centrifugation (13,000 g for 30 minutes at 4° C) and again extracted in 2000 ml of 125 mmol/L NH_4HCO_3 , pH 8, with 1 mmol/L phenylmethylsulfonyl fluoride and 0.1% wt/vol sodium azide, at 4° C for 48 hours with slow stirring. After centrifugation under the same conditions as in the first extraction, the two extracts were pooled and extensively dialyzed against 2 mmol/L NH_4HCO_3 and then against double distilled water.

Saturated $(\text{NH}_4)_2\text{SO}_4$ was slowly added to the pooled extract to 80% saturation. The suspension was stirred overnight at 4° C, then centrifuged (10,000 g for 30 minutes at 4° C), and the resultant pellet was resuspended in 125 mmol/L NH_4HCO_3 . After extensive dialysis against 125 mmol/L NH_4HCO_3 and then against double distilled water, the extract was lyophilized and stored at 4° C under vacuum. Protein content of the freeze-dried extract was assayed according to the method of Bradford.¹⁴

Polyacrylamide gel electrophoresis

SDS-PAGE¹⁵ was carried out as previously described.¹⁶ Briefly, CaE (100 µg/well) was reduced by 5% vol/vol 2-mercaptoethanol before application to the 13% wt/vol polyacrylamide gel. Low molecular weight markers (Bio-Rad, Richmond, Calif.) were included in each gel to calculate the relative molecular weight (MW) of the various components. The run was carried out in a refrigerated system at 8° C (Bio-Rad) under constant amperage (15 mA in the stacking gel and 25 mA in the separating gel), and the gel was stained with 0.05% wt/vol Coomassie Brilliant Blue (Imperial Chemical Industries, Ltd., Macclesfield, Cheshire, U.K.) in water:methanol:acetic acid (50:40:10).

IgE and IgG immunoblotting

Immunoblotting was performed according to the method of Towbin et al.¹⁷ in a Bio-Rad apparatus after SDS-PAGE separation. The blotted nitrocellulose strips were incubated overnight with 2 ml of individual serum diluted 1:2 in phosphate-buffered saline–Tween 20 0.05% vol/vol, pH 7.2 (PBS-Tween), at room temperature, washed five times with PBS-Tween, and then incubated overnight with iodine 125–labeled rabbit anti-human IgE (RAST I; Pharmacia, Uppsala, Sweden) (approximately 500,000 cpm/strips) diluted in PBS-Tween 20. The strips were then washed five times, and the reactive bands were detected by autoradiography at –80° C for 3 days with the use of x-ray film (Kodak Diagnostic Film X-Omat AR; Eastman Kodak Company, Rochester, N.Y.). IgG antibodies bound to the blotted strips were detected by overnight incubation with a peroxidase-labeled, affinity-purified goat antibody to human IgG (KPL, Gaithersburg, Md.), diluted 1:500 in PBS-Tween. The colorimetric reaction was developed with 4-chloro-1-naphthol according to the Bio-Rad instructions. Strips incubated with PBS-Tween instead of serum samples were used as negative controls.

Lectin immunoblotting

The specific binding of lectins to carbohydrate moieties in the allergenic components of CaE was studied by using the Vectastain Lectin Kit (Vector Laboratories, Burlingame, Calif.), according to the manufacturer's instructions. After SDS-PAGE and electroblotting of CaE, performed as described in the previous sections, the nitrocellulose-blotted strips were incubated with the following biotinylated lectins at the final concentration of 15 µg/ml for 1 hour at room temperature: concanavalin A (Con A), *Lens culinaris* agglutinin (LCA), *Sophora japonica* agglutinin (SJA), peanut agglutinin (PNA), *Ulex europaea* agglutinin (UEA), *Bandeiraea simplicifolia* lectin (BSL), *Phaseolus vulgaris* erythroagglutinin (PHAe). Strips were then incubated, after washings, with Vectastain ABC–Immunoperoxidase Reagent, for 30 minutes at room temperature. The colorimetric reaction was developed as described for IgG immunoblotting.

ELISA for specific IgE detection

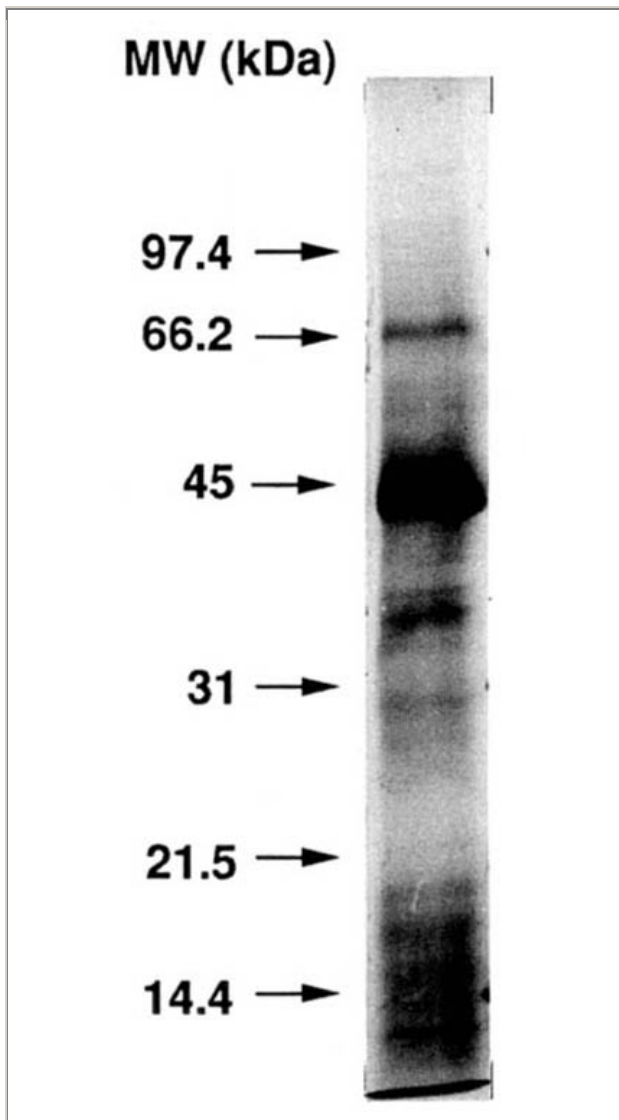
ELISA was performed essentially as described by De Cesare et al.¹⁶ Briefly, polyvinyl chloride plates (Falcon 3912; Becton Dickinson, Oxnard, Calif.) were coated with 0.2 ml/well of 20 µg/ml of CaE in 0.05 mol/L carbonate buffer, pH 9.6, for 3 hours at 37° C and overnight at 4° C. After washing four times with PBS-Tween, the plates were blocked with 3% gelatin in PBS for 1 hour at room temperature (200 µl/well). IgE detection was carried out by incubating 0.2 ml/well of the individual sera, 1:5 diluted in PBS–1% wt/vol gelatin, for 3 hours at room temperature. After further washings, 0.2 ml/well of the peroxidase-labeled rabbit anti-human IgE (KPL), diluted 1:2000 in PBS–Tween–1% gelatin, were added and incubated overnight at room temperature. The colorimetric reaction was developed by adding 0.2 ml/well of ortho-phenylenediamine solution (0.5 mg/ml in 0.1 mol/L citrate–0.2 mol/L phosphate buffer, pH 7.2, containing 0.01% hydrogen peroxide), for 30 minutes at room temperature. After stopping the reaction with 50 µl/well of 0.5 N sulfuric acid, the plates were read at 495 nm in a Microplate Reader (model 3550, Bio-Rad). Mean value of optical density at 495 nm (OD₄₉₅) plus 3 standard deviations of the mean, calculated for sera from a group of 10 normal subjects tested as negative controls, was regarded as the negativity cutoff. Thus all the sera producing O.D.₄₉₅ higher than 0.065 were scored as positive for the presence of *C. arizonica*–specific IgE.

Results

Production of *C. arizonica* extract (CaE)

Three hundred grams of dry Arizona cypress pollen, after two extraction procedures and ammonium sulfate precipitation, produced a crude extract, which after extensive dialysis and lyophilization, contained approximately 10 gm of dry weight material with a protein content of 300 mg (3%). SDS-PAGE in reducing conditions of CaE, after Coomassie Brilliant Blue staining, revealed about 30 bands in the MW range of 5 to 100 kd (Fig. 1), with graded density. The components that were present in the greatest quantity in the extract, as assessed by visual evaluation, were assigned MWs of 43, 70, 36, and about 17 kd, respectively.

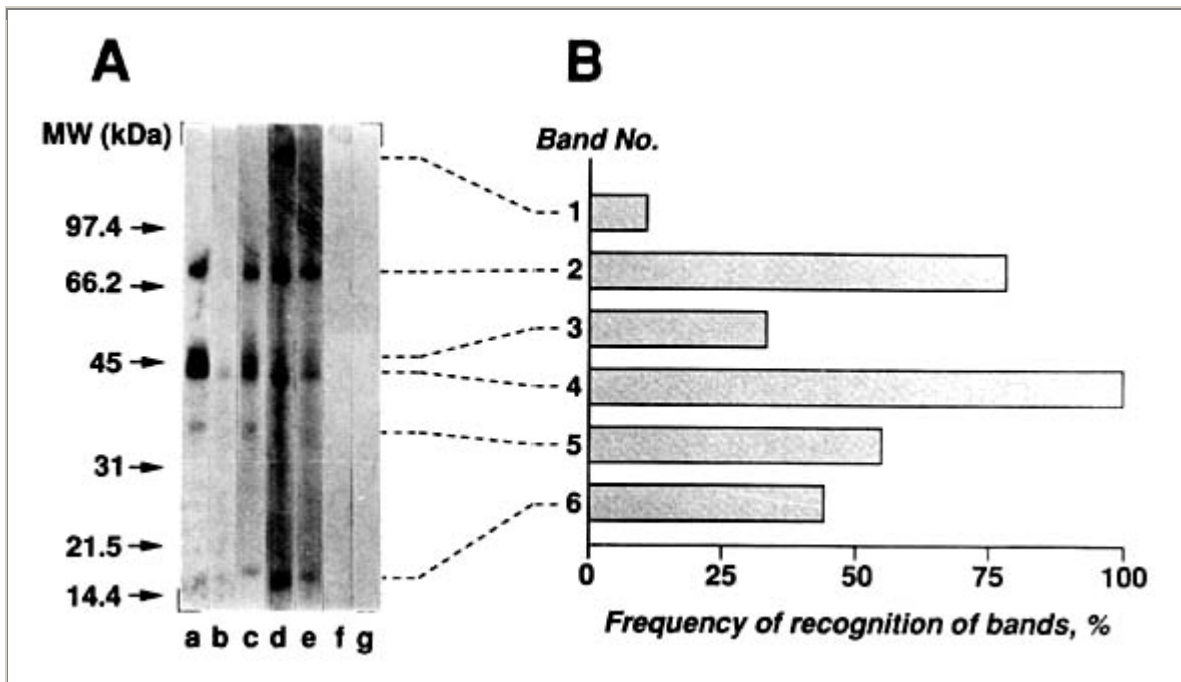
FIG. 1. *C. arizonica* pollen extract after separation by SDS-PAGE in reducing conditions and Coomassie Brilliant Blue staining.



IgE binding on CaE

Serum samples from 33 patients who were monosensitized to Cupressaceae pollen were tested for IgE binding to CaE after SDS-PAGE and electroblotting onto nitrocellulose membranes. Nine of the 33 sera tested bound to at least one component of the extract (27%) and were then regarded as positive in IgE immunoblotting. The other 24 sera did not show any IgE binding to CaE. Six protein components in the electroblotted CaE with MWs of greater than 100, 70, 46, 43, 36, and 19 kd were recognized by IgE antibodies, as shown in Fig. 2, A, on five representative sera (lanes a to e). Serum samples from 10 normal subjects, tested as negative controls, did not react with any of the separated components: two representative sera are shown in Fig. 2, A (lanes f and g). All of the nine sera reacting with at least one component were able to recognize band 4, with MW of 43 kd (Fig. 2, B). Moreover, this band showed the greatest intensity of reaction. Therefore this component can be regarded as a major allergenic fraction of *C. arizonica* pollen extract. Band 2 (70 kd) and band 5 (36 kd) bound to IgE from seven and five of the nine patients' sera, respectively (Fig. 2, B).

FIG. 2. A, IgE antibody-binding patterns of *C. arizonica* pollen extract in immunoblotting. *Strips a* to *e*: sera from five representative patients monosensitized to Cupressaceae pollen. *Strips f* and *g*: sera from two representative normal subjects. **B**, Frequency of recognition of the six IgE-binding components in *C. arizonica* pollen extract, calculated on the nine positive sera. Numbers 1 to 6 indicate the six bands recognized by human IgE (>100, 70, 46, 43, 36, and 19 kd).



IgG binding on CaE

The same panel of sera was also tested for IgG binding to CaE components electroblotted onto nitrocellulose membranes. The pattern of bands recognized by human IgG showed a higher degree of heterogeneity, with MW ranging from 70 to 16.5 kd. Sixteen components bound to IgG from 26 of the 33 patients' sera tested, with graded intensity of reaction, as shown in Fig. 3, A, for five representative sera (lanes a to e). The band with an MW of 37.5 kd was the component recognized by the highest proportion of IgG-positive sera (22 of 26, corresponding to 85%), followed by the band with an MW of 16.5 kd (18 of 26, 69%). The component with an MW of 43 kd, identified as the most reactive component on the basis of IgE recognition, was bound by IgG from only one serum of 26 (4%) (Table I). However, five of the six bands recognized by IgE (MWs of 70, 46, 43, 36, and 19 kd) also bound IgG from at least one serum. Sera from 10 normal subjects, added as a control group, contained IgG able to bind some components of CaE, in particular the bands with MWs of 16.5, 17, 17.5, 37.5, and 38 kd (Table I); two representative sera are shown in Fig. 3, A (lanes f and g).

FIG. 3. A, IgG antibody-binding patterns of *C. arizonica* pollen extract in immunoblotting. *Strips a to e:* sera from five representative patients monosensitized to Cupressaceae pollen. *Strips f and g:* sera from two representative normal subjects. **B,** Frequency of recognition of the 16 IgG-binding components in *C. arizonica* pollen extract, calculated on the 26 positive sera. Numbers 1 to 16 indicate the 16 bands recognized by human IgG (ranging from 70 to 16.5 kd). Bands 1, 3, 4, 10, and 19 correspond to five of the six bands recognized by human IgE.

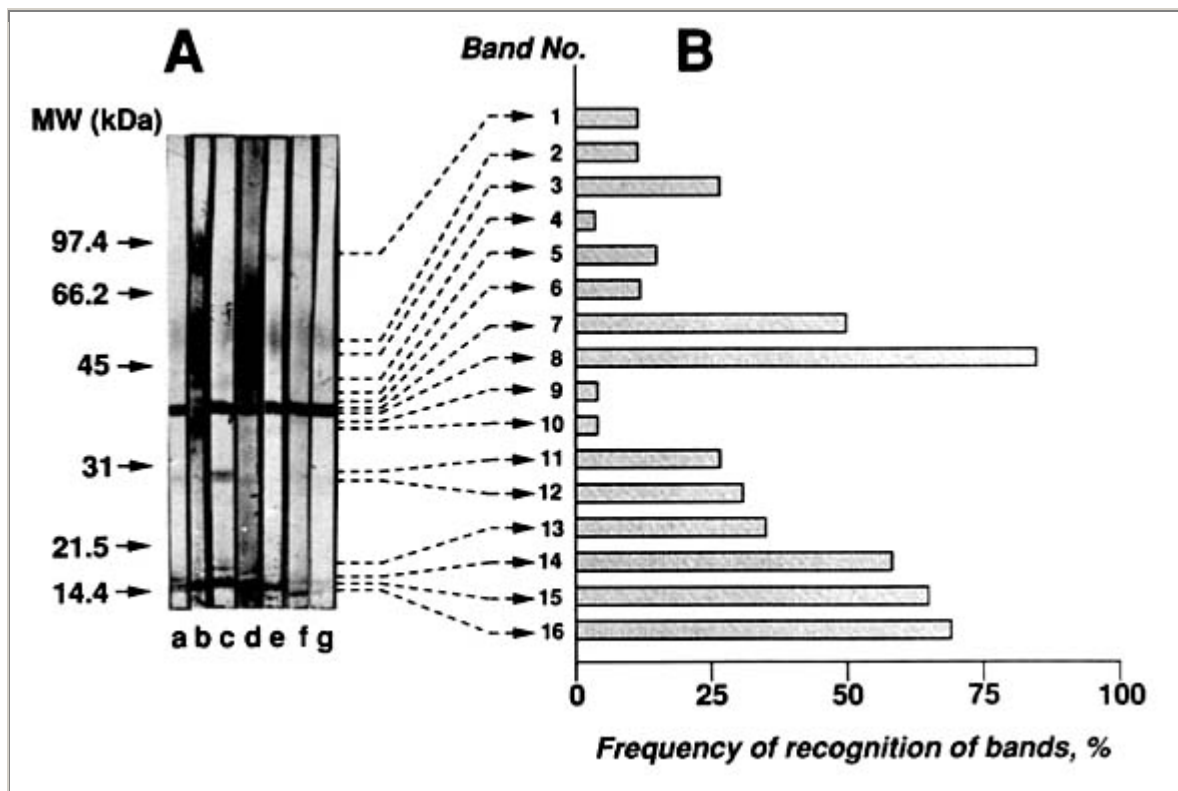


TABLE I. Molecular weight of components detected by IgE and IgG and number of subjects' sera reacting with each component

MW (kd)	<u>Allergic subjects</u>		<u>Normal subjects</u>
	IgE	IgG	IgG
>100	1 (11)*		
70	7 (77)	3 (12)†	3 (30)‡
50		3 (12)	3 (30)
46	3 (33)	7 (27)	5 (50)
43	9 (100)	1 (4)	2 (20)
41		4 (15)	3 (30)
39		3 (12)	3 (30)
38		13 (50)	6 (60)
37.5		22 (85)	10 (100)
37		1 (4)	
36	5 (55)	1 (4)	1 (10)
30.5		7 (27)	4 (40)
29.5		8 (31)	4 (40)
19	4 (44)	9 (35)	3 (30)
17.5		15 (58)	7 (70)
17		17 (65)	6 (60)

*Frequency (percent) calculated on 9 sera reacting with at least one band.

†Frequency (percent) calculated on 26 sera reacting with at least one band.

‡Frequency (percent) calculated on 10 sera reacting with at least one band.

Lectin binding

The presence of carbohydrate moieties in the CaE components was studied by using the following lectins: Con A, LCA, SJA, PNA, UEA, BSL, PHAe. Sugar specificities bound by each lectin are reported in [Table II](#), [18](#) together with the CaE bands recognized by the seven lectins. Twenty-two different bands, with MW ranging from 100 to 37 kd, reacted with the lectins tested. LCA and Con A lectins, which bind to the same panel of carbohydrates (i.e., α -D-mannose, α -D-glucose, D-fructose), recognized a high number of bands in the MW range of 90 to 37 kd with very similar patterns ([Fig. 4](#)), thus indicating that most of the CaE glycoproteins contain these glycosidic residues. Reaction with UEA lectin, specific for L-fucose and N-acetylglucosamine, detected 10 CaE components in a similar MW range. These were different from those bound by LCA and Con A, with the exception of two components with MWs of 43 and 37 kd, the former corresponding to the major allergen of *C. arizonica*. Moreover, these two bands were recognized by five lectins and by all seven lectins, respectively ([Fig. 4](#)), thus displaying the highest degree of heterogeneity in their carbohydrate content. Interestingly, at least four of the six components recognized by human IgE, with MWs of greater than 100, 70, 46, and 43 kd were able to bind to at least one of the seven lectins tested, thus indicating that most of the allergenic components of CaE are glycosylated.

FIG. 4. Lectin-binding patterns of *C. arizonica* pollen extract in blotting.

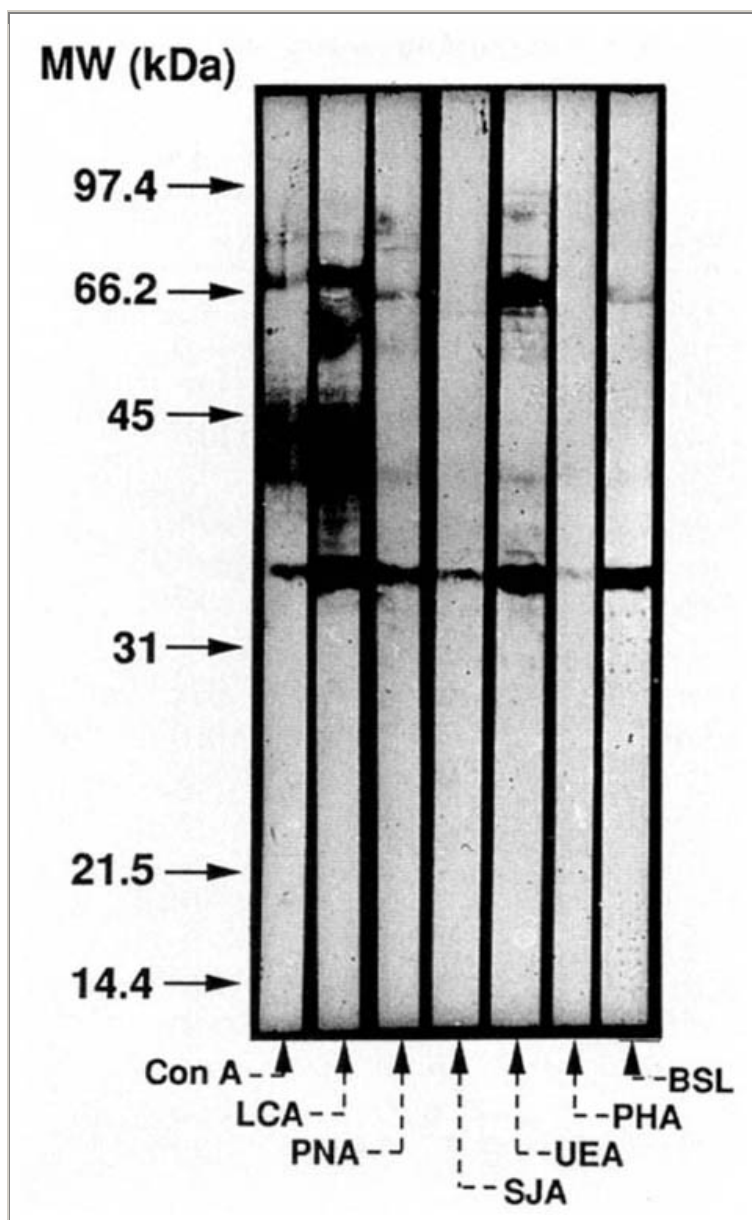


TABLE II. Lectin binding by CaE components after SDS-PAGE and blotting

Lectin	Sugar specificity	Bands recognized*
Con A	α -D-Man	9
α -D-Glc		
D-Fru		
LCA	α -D-Man	12
α -D-Glc		
D-Fru		
PNA	Gal- β (1-3)-GalNAc	5
SJA	α -D-GalNAc	2
α -D-Gal		
UEA	L-Fuc	10

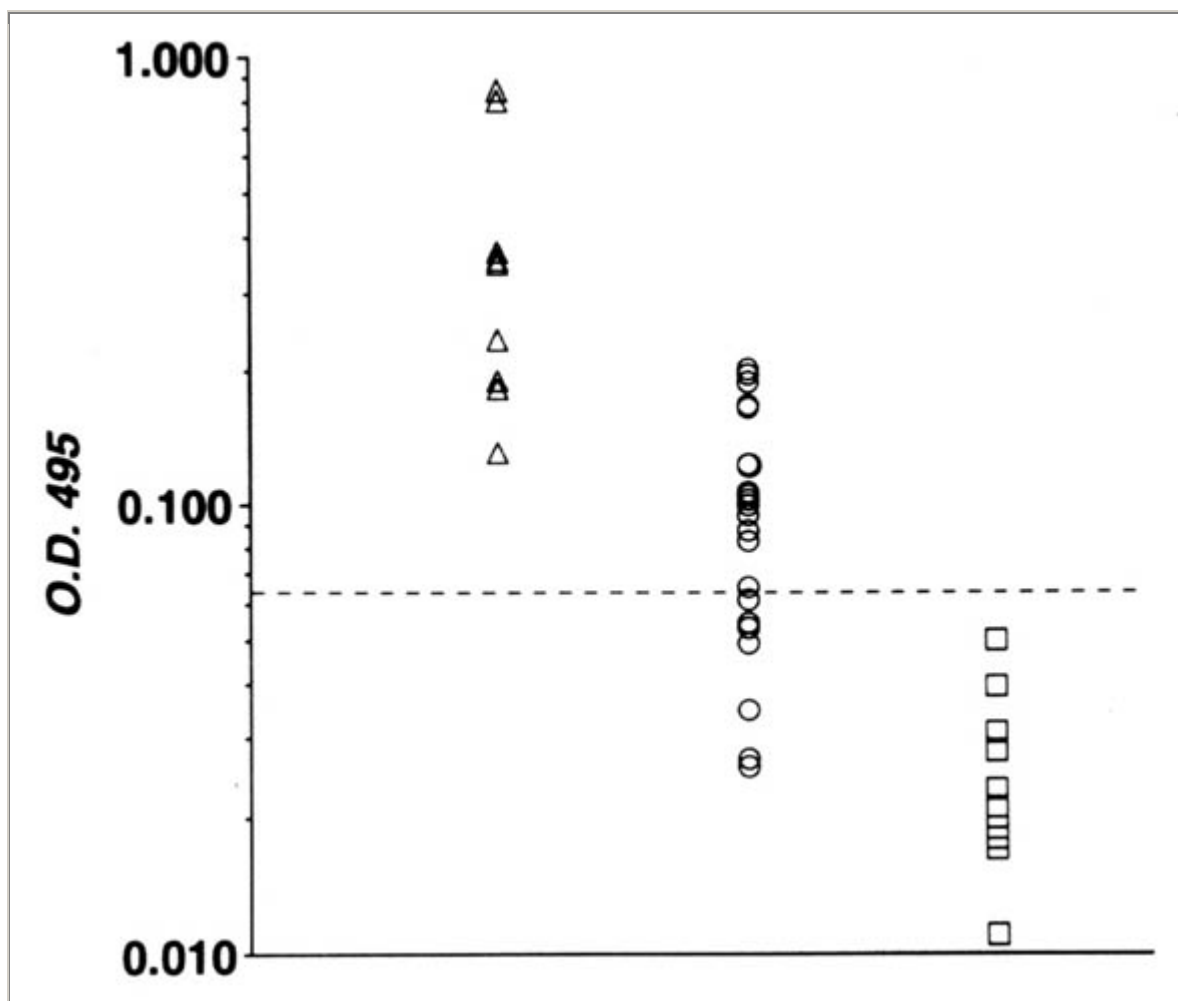
D-GlcNAc		
PHA	D-GalNAc	1
BSL	α -D-Gal	4

*The numbers indicate how many bands each lectin recognizes.

ELISA detection of CaE-specific human IgE

Results of the CaE-specific IgE ELISA of the 33 sera from patients who were monosensitized to Cupressaceae, as diagnosed by clinic history and SPT results, are reported in ■Fig. 5. Serum samples from 10 nonatopic volunteers were also tested as negative controls. Allergic patients have been grouped according to the positivity in IgE immunoblotting, and differences between groups were evaluated by analysis of variance. The group of nine sera positive in IgE immunoblotting displayed OD₄₉₅ values significantly higher than the values shown by both patients with negative IgE immunoblotting results ($p < 0.001$) and normal subjects ($p < 0.001$). OD₄₉₅ values in the group of patients with negative IgE immunoblotting results were significantly higher than the values reported for the normal group ($p < 0.001$). Mean OD₄₉₅ value plus 3 SDs of the mean, calculated for the normal group, was 0.065 (dashed line in the graph). According to these positivity criteria, 25 of 33 sera (75.7%) were positive.

FIG. 5. ELISA detection of *C. arizonica*–specific human IgE in sera of 33 individual patients monosensitized to Cupressaceae, using the in-house prepared *C. arizonica* pollen extract. Patients were divided into two groups: 9 with positive IgE immunoblotting results (open triangles) and 24 with negative IgE immunoblotting results (open circles). Ten normal subjects' sera have been tested as negative controls (open squares). The dashed line indicates the mean OD₄₉₅ value plus 3 SDs of the mean (0.065), calculated for the normal group, which was regarded as the negativity cutoff.



Discussion

TOP

The airborne pollens of Cupressaceae family, including *C. arizonica* and *C. sempervirens*, have been recognized as a cause of respiratory allergy for many years in the Mediterranean area, as well as in extra-Mediterranean countries with a Mediterranean climate. Because of the increasing diffusion of *C. arizonica* trees for reforestation purposes and garden use, this Cupressaceae species is inspiring a growing aerobiologic interest.

This study describes the preparation and characterization of an extract from the pollen of *C. arizonica* and the use of this extract on an IgE-based ELISA.

In preliminary experiments the optimal extraction conditions of *C. arizonica* pollen were established. The final protocol was chosen on the basis of the highest protein yield of about 3%. Even if this value seems to be rather low, it is comparable to the yield obtained with other Cupressaceae pollens.⁵ This finding raises the question of how the nonprotein contents of the extract can be identified. Preliminary results obtained by high-performance liquid chromatography sugar analysis and the phenol–sulfuric acid method indicate that freeze-dried *C. arizonica* pollen extracts contain 85% (wt/wt) of sugars (Di Felice et al. Unpublished data). The protein-bound carbohydrate moieties in CaE were further characterized by lectin blotting analysis on the SDS-PAGE-separated extract. The results suggest that most of the CaE components contain glycosidic residues, mainly mannose, glucose, fucose, fructose, Nacetyl-glucosamine, and N-acetyl-galactosamine. The presence of such a proportion of carbohydrate could be of great relevance in regard to the high degree of cross-reactivity at the IgE level reported for the conifer pollens.^{2, 19, 20} Indeed, IgE cross-reactions caused by the sharing of glycosidic epitopes have been described between pollens of closely related plants^{21, 22} and even between quite unrelated allergens.^{23, 24} Moreover, cross-reactions may extend to the presence of carbohydrate structures shared by several components of the same pollen extract.^{25, 26}

SDS-PAGE analysis of the CaE demonstrated about 30 different protein components, whereas IgE binding recognized six components. Of these bands, the MW of 43 kd was predominant both in the intensity of its reaction and in the frequency of its recognition by human allergic sera. Therefore this component can be regarded as a major allergenic component of *C. arizonica* pollen. This finding resembles that reported by Ford et al.,⁵ who identified the major allergen in the pollen extract of the closely related species, *C. sempervirens*, as a 42 kd component. The number of sera positive in IgE immunoblotting is rather low when compared with that found by Ford et al.⁵ A possible explanation for this discrepancy is based on the different cypress species studied and on the different geographic areas from which both sera and pollen were collected. This concept has been recently underlined by Bousquet et al.²⁷ However, differences in the sensitivity of the IgE immunoblotting techniques cannot be ruled out.

Four of the six IgE-binding components of CaE seem to be glycoproteins, because they are able to bind to at least one of the seven lectins tested in blotting. In particular, the major allergen is recognized by Con A, LCA, PNA, UEA, and BSL, indicating the presence of a variety of glycosidic residues (i.e., mannose, glucose, fucose, galactose, N-acetyl-glucosamine, N-acetyl-galactosamine) on the protein frame of the molecule. This finding is in agreement with that reported for the major allergen of the related species *Cryptomeria japonica*.²⁸

On the other hand, the 43 kd allergen was recognized by IgG antibodies in the serum of only one allergic subject of the 33 tested, even though the IgG recognition pattern of the CaE components was much more complex than that of IgE. Interference or competitive effects exerted by IgE could be ruled out because in untreated subjects with Cupressaceae allergy considered in this study, IgE levels are always quite low. Furthermore, the only serum showing IgG recognition of the 43 kd allergen, also contains IgE specific for this component in immunoblotting. The wide IgG reactivity found against CaE components is significant, taking into account that none of the subjects screened in immunoblotting have received specific immunotherapy. Moreover, the normal subjects' sera tested as control also contained IgG specific for some CaE components, five of which were also recognized by IgE from allergic subjects. These findings are in line with those obtained in a great number of studies dealing with IgG response against inhalant and food allergens by atopic and normal individuals.²⁹⁻³¹

CaE produced and characterized in house was used to set up a specific IgE ELISA test, intended for screening of the sera from the 33 subjects who were monosensitized to Cupressaceae. The percent of positivity obtained (about 76%) was clearly higher than that obtained in IgE immunoblotting (27%). However, it should be considered that nine sera, which scored as positive in immunoblotting, showed the highest reactivity in ELISA. The percent of subjects with positive results (76%) was quite different when compared with the finding that in a panel of sera from subjects living in the same area, the percent of subjects with positive results ranged from 0¹³ to 14 (unpublished results) when commercial immunoassays with allergen-coated disks were used. Comparable results have been reported by Ford et al.⁵ with in-house *C. sempervirens* pollen extract. These findings suggest that the high incidence of false-negative results obtained with commercial immunoassays may be due to either poor quality of the extract used for the coating of the solid phase or to chemical modifications introduced by the coating procedure.

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