Juniperus oxycedrus: A new allergenic pollen from the Cupressaceae family

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Background: Cupressaceae allergy is a worldwide pollinosis caused by several species. Some species in limited geographic areas pollinate in fall and winter. *Juniperus oxycedrus* matches these features.
Objective: We sought to define the immunochemical, allergologic, and environmental aspects of *J. oxycedrus* pollen.
Methods: Pollen extract from *J. oxycedrus* was prepared and characterized by biochemical analysis and human specific IgE binding by means of ELISA and immunoblotting. A 3-year phenological study was conducted to define the pollinating period of *J. oxycedrus*. Forty consecutive patients allergic to cypress were recruited in two areas and divided into two groups according to their exposure to *J. oxycedrus* pollen. Clinical evaluation, skin prick tests, and specific IgE determination with *J. oxycedrus*, *J. ashei*, and *Cupressus arizonica* extracts were carried out on both groups.
Results: *J. oxycedrus* pollen extract was obtained, and it showed specific IgE binding and wide cross-reactivity with other Cupressaceae species. The extract caused a positive skin test response in all the patients tested, with about 80% of them having detectable specific IgE. Symptoms related to *J. oxycedrus* pollen exposure were recorded in 72% of the directly exposed patients and occasionally in 9% of the nonexposed patients. In the Mediterranean coastal area considered, *J. oxycedrus* was the first Cupressaceae species that started to pollinate at the beginning of November and ended in the first part of December.
Conclusions: *J. oxycedrus* represents a newly characterized pollen species of the Cupressaceae family that cross-reacts with other members of the same family. Subjects with cypress allergy have in vivo and in vitro positive test responses for *J. oxycedrus* and can show symptoms when exposed to its pollen. Finally, the most important feature of *J. oxycedrus* is its early pollinating period in southern Europe (Italy), causing a further extension of the cypress pollen season in areas where other Cupressaceae species are present. (J Allergy Clin Immunol 1998;101:755-61.)

Key words: *Cupressaceae, cypress, juniper, allergen characterization, cross-reactivity, fall-to-winter pollinosis*

Abbreviations used

CaE:	*Cupressus arizonica* extract (Arizona cypress)
JaE:	*Juniperus ashei* extract (*J. sabinoides*, Mountain cedar)
JoE:	*Juniperus oxycedrus* extract
MW:	Molecular weight
SDS-PAGE:	Sodium dodecylsulfate–polyacrylamide gel electrophoresis

Cypress pollinosis, an almost worldwide respiratory allergy,[1-7] is gaining more attention because of its status as a cause of autumn and winter pollinosis[8-10] and because of the widespread use of these plants for human needs.[4, 11-13] An increasing number of species belonging to the Cupressaceae family or to closely related cross-reactive families are described as sensitizers.[7, 11, 14-19] Moreover, a potential underestimation of the real prevalence of cypress allergy could be related to the low potency of commercially available diagnostic extracts.[20] The species already described as sensitizers and elicitors of allergic symptoms are only a few of the hundreds of species belonging to the Cupressaceae, Podocarpaceae, Taxaceae, and Taxodiaceae families. Clinical manifestations may be caused by exposure to cross-reactive species different from those eliciting the initial sensitization.

Juniperus oxycedrus, a species belonging to the Cupressaceae family (Juniperoideae subfamily) grows along the coast of almost all the Mediterranean countries and in internal woody areas.[22, 23] A preliminary observation in a coastal area identified the pollen of *J. oxycedrus* as a possible cause of allergic respiratory symptoms in exposed subjects in a period when other Cupressaceae species do not pollinate. Therefore we set out to investigate the pollinating period of *J. oxycedrus,* to prepare a *J. oxycedrus* pollen extract, and to study its capability to sensitize and to elicit allergic symptoms. We also investigated cross-reactions with other pollens belonging to the same family.

METHODS

Pollen extracts

Pollen from *J. oxycedrus* was collected in November from plants growing along the coastal area of the Circeo National Park in central Italy. Source plants were identified by means of botanical criteria.[22] *J. oxycedrus* was the only species (Cupressaceae or not) pollinating in the collection area during the considered period. Pollen was collected from the mature flow-

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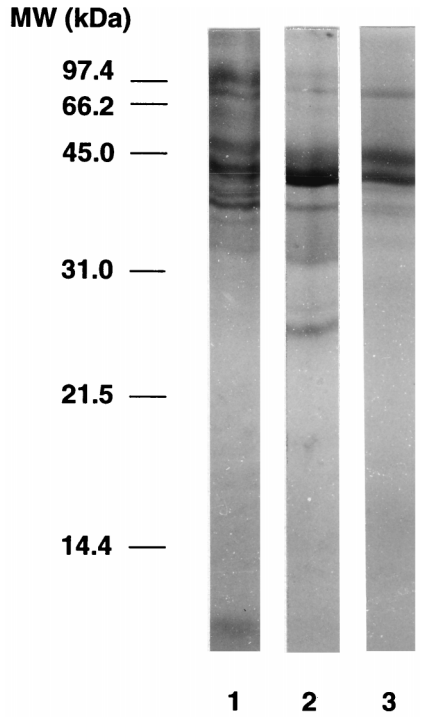

FIG. 1. Coomassie Brilliant Blue staining of JoE *(lane 1)*, JaE *(lane 2)*, and CaE *(lane 3)* after separation by SDS-PAGE in reduced condition.

ering plants by using, at a close distance, a filter-equipped vacuum device to avoid the collection of particles other than pollen. Purity was checked by microscopic analysis (>99%). Twelve grams of pollen were extracted soon after collection in 160 ml of 125 mmol/L NH_4HCO_3 at 4° C for 12 hours with slow stirring followed by centrifugation (13,000 *g* for 1 hour at 4° C), filtering, and dialysis (48 hours against distilled water). *J. oxycedrus* extract (JoE) was then lyophilized and stored at 4° C under vacuum.[24, 25] Pollens from *J. ashei* and *Cupressus arizonica* were purchased from Allergon (Angelholm, Sweden). Twelve grams of *J. ashei* pollen were extracted in 120 ml of 125 mmol/L NH_4HCO_3 following the same protocol used for *J. oxycedrus*. Pollen from *C. arizonica* (300 gm) was extracted as previously described.[26] Protein content of JoE, *J. ashei* extract (JaE), and *C. arizonica* extract (CaE) was assayed according to the method of Bradford.[27] Total carbohydrate content was assayed according to the method of Dubois et al.[28]

Human sera

Forty patients sensitive to cypress were selected as previously described.[29] Subjects were not receiving specific immunotherapy, and their informed consent to participate was obtained. Sera were collected, aliquoted, and stored at –20° C until used. Six of these 40 sera were selected on the basis of their JoE ELISA individual values (greater than 1.0 optical $density_{490}$) and of their broad reactivity when tested in JoE immunoblotting and pooled. The pool was used for ELISA and immunoblotting inhibition.

Six sera from normal subjects (defined by negative history and absence of clinical signs or symptoms suggesting a possible respiratory allergy, total IgE below 10 IU/ml, negative skin prick test response to the same panel of allergens used to screen the patients, and negative specific IgE to a restricted panel of allergens relevant for the population studied) were used to define the negative cutoff point of the ELISA. Sera from patients sensitive to other pollen species were tested as internal laboratory controls, found to be negative, and not reported as results in the article.

Skin testing

Patients were skin prick tested by using a standard method[20] with JoE, JaE, and CaE 50% glycerinated water solutions at a final concentration of 500 μg protein/ml. Extracts were tested according to the European Guidelines and the European Pharmacopoeia Monograph on Allergen Products[30] before they were used on human beings.

ELISA and ELISA inhibition

IgE specific for JoE, JaE, and CaE were detected by ELISA in the sera from the 40 patients according to the procedure developed in our laboratory[26] by using an antigen concentration of 20 μg protein/ml for coating the plates. Mean value (+3 SD) of the mean optical density, obtained with a group of six normal subjects selected as negative control subjects, was regarded as the negative cutoff.

Inhibition of specific IgE binding was carried out as previously described for *C. arizonica* and *C. sempervirens*.[29] Pooled sera (see above) were prediluted 1:2.5 and incubated with twofold serial dilutions of the various inhibitors (JoE, JaE, and CaE) at a concentration ranging from 10 μg protein/ml to 0.01 μg protein/ml. Concentrations causing 50% inhibition were calculated after graphic interpolation on the inhibition curves.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis and electrotransfer

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransfer were carried out as previously described.[26, 29] JoE, JaE, and CaE (10 μg protein/well) were reduced by 5% vol/vol 2-mercaptoethanol before application to the 15% wt/vol polyacrylamide gel. The gel was stained with 0.05% Coomassie Brilliant Blue (Imperial Chemical Industries, Ltd., Macclesfield, U.K.) in water/methanol/acetic acid (50:40:10).

Immunoblotting and immunoblotting inhibition

Pooled sera and inhibitors were the same used in the ELISA-inhibition assay. Immunoblotting and inhibition of the specific IgE-binding was carried out as previously described.[29] Inhibitors were used at 50, 15, 5, 1, and 0.1 μg protein/ml.

Cupressaceae phenology and environmental distribution

Because the microscopic morphology of pollen grains from Cupressaceae and closely related trees does not distinguish between species, genera, or families,[31] botanical phenological criteria[32] were used to define the beginning, the magnitude, and the end of the pollinating period of the Cupressaceae species most represented in the observed area (*J. oxycedrus*, *J. phoenicea*, *C. arizonica*, *C. sempervirens*, and *Thuja orientalis*).[22] Weekly observations were carried out from October to April over 3 years, and the percent of pollinating plants in the study area was considered. Following the scale proposed by Arrigoni et al.[33] and taking into consideration the pollen dispersal from plants in a previously defined area, the observed species were scored as 1 when less than 25% of individuals were pollinating,

as 2 when the number of pollinating individuals was between 25% and 50%, and as 3 when more than 50% of individuals were pollinating. In the area chosen for the study, *J. oxycedrus* plants spontaneously grow for 20 km in a band about 100 to 200 meters wide along the coastal dune at a short distance from coastal towns. Another Juniperoide species, *J. phoenicea,* is less common in the same area than *J. oxycedrus,* whereas, to our knowledge, the presence of *J. ashei* has not been recorded in Italy.[22] Other nonnative Cupressaceae species *(C. arizonica, C. sempervirens,* and *T. orientalis)* are typically planted in urban and rural areas, facing the coast.[4]

RESULTS

Forty consecutive adult patients found to be hypersensitive to cypress by skin prick tests and specific IgE assays were divided into two groups according to mean distance from their living or working area and the source of *J. oxycedrus* pollen. The first group of 18 subjects (group A), living in an area close to the pollen source (1 to 10 km), was directly exposed for most of the *J. oxycedrus* pollen season. The remaining 22 (group B) lived at a distance ranging from 20 to 70 km from the nearest source of the *J. oxycedrus* pollen in an urban area where no cypress-like pollens were sampled by spore traps during November and the first part of December in a 5-year period (Mari, personal observation). Patients had never been exposed to *J. ashei,* whereas *C. arizonica* pollen has been recorded in both urban and coastal areas. A retrospective study, as well as a 3-year follow-up clinical study, was carried out to assess the presence of conjunctival, nasal, or bronchial symptoms during the *J. oxycedrus* pollinating period and during the winter.

Biochemistry of pollen extracts

Protein content of JoE was 2% of the freeze-dried extract, whereas carbohydrates represent 43% of the dry weight of the extract. In comparison, protein content of JaE and CaE was, respectively, 2.5% and 3.3%, and carbohydrate content was 37% and 59%. Several components, displaying a relative molecular weight (MW) ranging from 10,000 d to 100,000 d, could be detected in the JoE when analyzed by SDS-PAGE Coomassie Blue staining (Fig. 1, *lane 1*), although the large part of the Coomassie reactivity is associated with components in the 35,000 to 100,000 d range. A more restricted pattern was obtained with the closely related species JaE, which showed the presence of several components ranging from 25,000 d to 100,000 d (Fig. 1, *lane 2*). However, the majority of the proteins of the extract consist of a major component displaying a relative MW of 40,000 d, thus confirming the results of other studies.[24, 25] The pattern obtained for CaE (Fig. 1, *lane 3*), confirms the presence of two major bands, with a relative MW of 41,000 and 43,000 d, corresponding to the CaE major allergen (submitted manuscript).

Specific IgE ELISA and ELISA inhibition

About 80% of the 40 sera from both exposed and unexposed patients showed IgE reactivity for JoE, whereas more than 90% of them were reactive with JaE and CaE (Table I). At least one of the three allergens was positive in every serum.

TABLE I. Skin prick tests, specific IgE, and *J. oxycedrus*-related symptoms in patients with cypress allergy

		Skin prick test*			Specific IgE*			*J. oxycedrus*-related symptoms†
	N	JoE	JaE	CaE	JoE	JaE	CaE	
Group A	18	100	100	100	78	95	90	72
Group B	22	100	100	100	76	97	93	9‡

Group A, Patients living or working close to the area of *J. oxycedrus* pollen source; *Group B,* patients not directly exposed to the pollen of *J. oxycedrus*.
*Expressed as percent of positive results.
†Percent of patients with clinical symptoms during *J. oxycedrus* pollinating period.
‡Two patients reported rhinitis and conjunctivitis during November weekends in the area studied.

When the serum pool with reactivity to JoE was used in the ELISA-inhibition experiments, all three extracts were able to produce almost overlapping inhibition curves in all combinations investigated (i.e., JoE, JaE, and CaE either on the solid phase or as inhibitors). Fifty percent inhibition was reached with concentrations of the pollen extracts ranging from 0.13 μg protein/ml to 0.4 μg protein/ml, without major differences between the various coating antigens and inhibitors used. In more detail, maximum inhibition values close to 100% were obtained with all three extracts used as inhibitors when JoE and JaE were used as antigens on the plate. On the other hand, in the system involving CaE on the solid phase, only the homologous antigen was able to approach 100% inhibition, being the maximum value obtained by both JoE and JaE equal to 75% (data not shown).

Immunoblotting and immunoblotting inhibition

When pooled sera were tested against the three extracts in the absence of any inhibitor, different patterns of reactivity could be observed. In particular, one major band was clearly identified with JoE (41,000 d), together with a number of closely related bands with a relative MW of about 70,000 d (Fig. 2, *lane 1*). Moreover, several very minor bands, displaying relative MWs of 29,000 d, 32,000 d, and 39,000 d, were also present. Most of these allergenic components could be also identified in the JaE (Fig. 2, *lane 2*), which showed a reactive component with a low MW of 19,000 d, whereas in the CaE (Fig. 2, *lane 3*) a clear reactivity could be detected only with the components displaying a relative MW of 70,000 d, 43,000 d, and 41,000 d.

The results of immunoblotting inhibition are shown in Fig. 3. When the two *Juniperus* species (JoE and JaE) were used as blotted antigens (Fig. 3, *A* and *B*, respectively), none of the inhibitors was able to cause a total IgE inhibition. In fact, IgE binding to JoE components

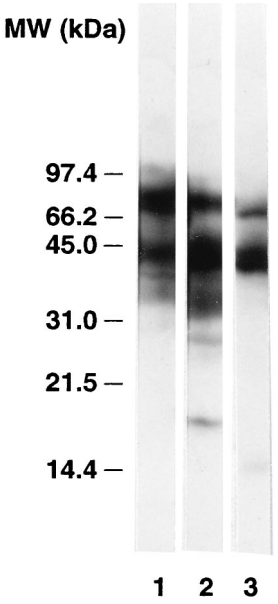

FIG. 2. Immunoblotting developed with pooled human sera from subjects allergic to cypress on transferred JoE *(lane 1)*, JaE *(lane 2)*, and CaE *(lane 3)*.

at 70,000 d and 41,000 d was never completely inhibited. Similarly, one component (40,000 d) of the blotted JaE was never completely inhibited by all three extracts tested up to a concentration of 50 μg protein/ml. Higher inhibitor concentrations could not be tested because of solubility problems and high unspecific background. When the antigen transferred onto the blotting paper was CaE (Fig. 3, *C*), homologous (CaE) and heterologous extracts (JaE and JoE) were able to cause a total inhibition of IgE binding, starting from 5 μg protein/ml.

Clinical evaluation

Each of the 40 patients had a positive skin prick test response to JoE, JaE, and CaE, whether they were regularly exposed to *J. oxycedrus*. Slightly different values in the percent positivity in specific IgE ELISA with the three extracts were reported for the two groups of subjects (Table I).

During the follow-up study, 13 of 18 patients from group A reported early-November conjunctival and nasal symptoms, which responded to common antiallergic drugs. None of them reported asthma or asthma-like symptoms. The remaining five lived at the longest distance from the source or had a low level of clinical symptoms during previous cypress pollen seasons. One patient recorded more severe rhinitis and conjunctivitis in November than during the winter pollinating period. Ten of 18 subjects reported more than 5 years of respiratory symptoms during the fall, without an allergic cause being suspected by either the family physicians or themselves. Treatment in previous years with antibiotics and antiphlogistic drugs had not been helpful.

As expected, none of the patients in group B reported

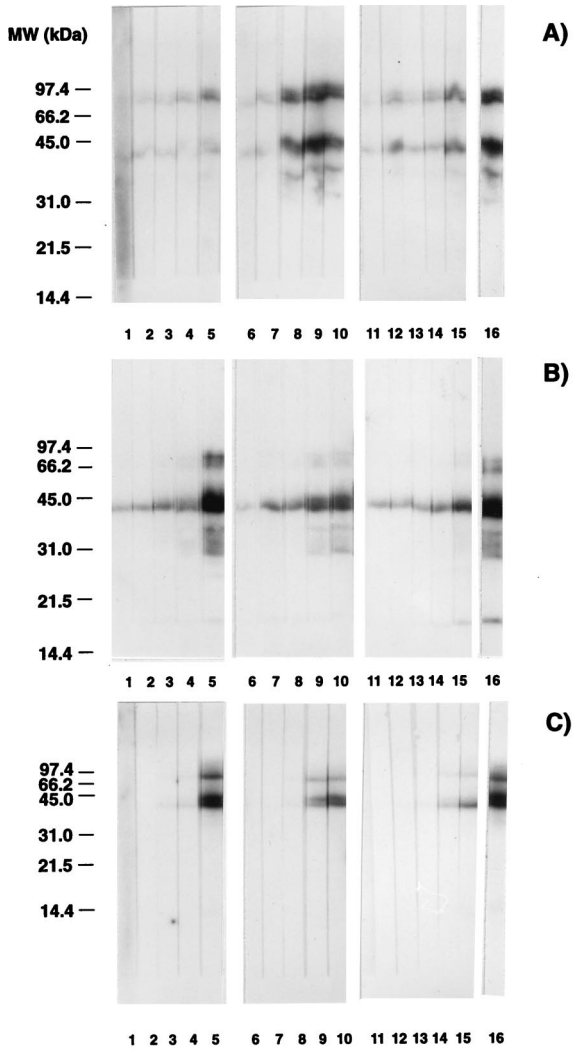

FIG. 3. Immunoblotting inhibition pattern of IgE binding to components of JoE **(A),** JaE **(B),** and CaE **(C)** inhibited with JoE (*lanes 1* to *5*), JaE (*lanes 6* to *10*), and CaE (*lanes 11* to *15*). Inhibitors were used at 50, 15, 5, 1, and 0.1 μg protein/ml. *Lane 16* shows IgE binding to blotted antigens in absence of inhibitor.

symptoms in November, even though two of them experienced rhinitis and conjunctivitis on several occasions during weekends spent in the coastal area considered in this study.

Cupressaceae phenology

Three years of cumulative data of Cupressaceae phenological survey are showed in Fig. 4. The presence of *J. oxycedrus* mature pollinating male cones was recorded at the beginning of November and almost all the plants started to release pollen within 2 weeks, declining and ending in mid-December. The presence of *J. oxycedrus* advances the beginning of the Cupressaceae pollen in mid-fall, followed by *J. phoenicea* and *T. orientalis* in December and January. The two Cupressoideae species, *C. arizonica* and *C. sempervirens,* define the Cupres-

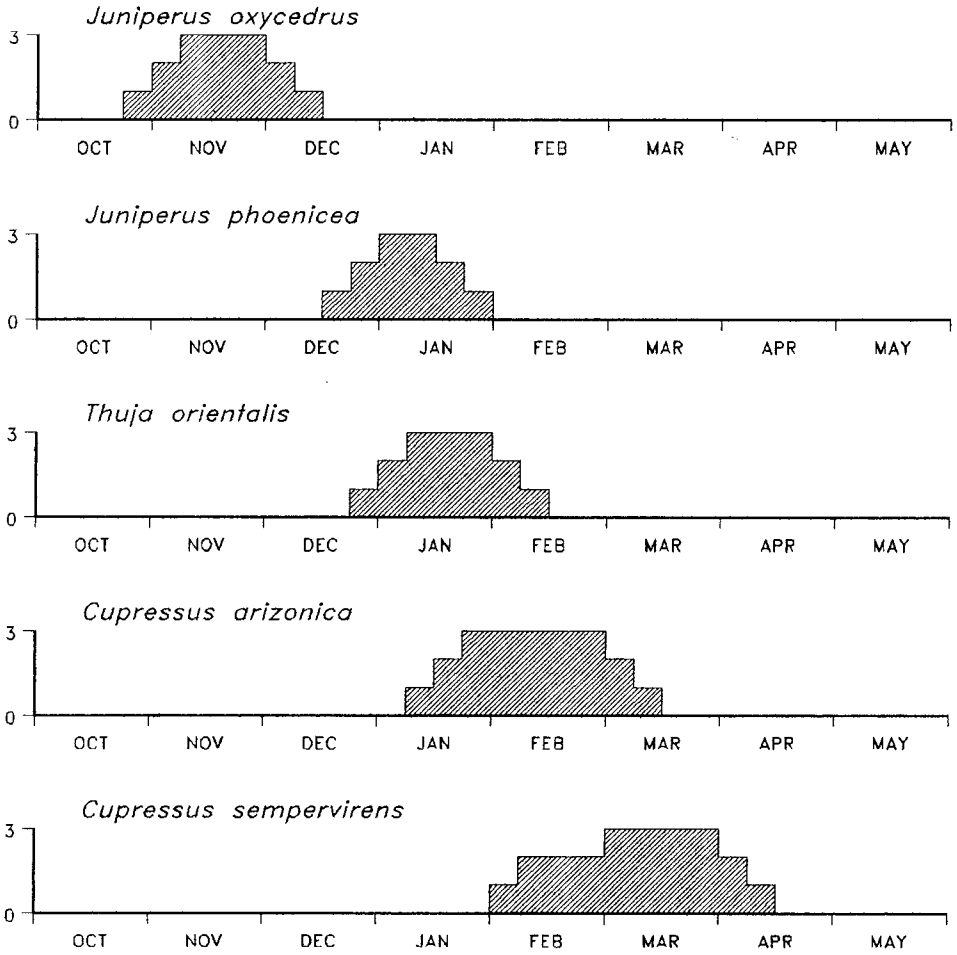

FIG. 4. Phenological data. Weekly observations in 3-year period considering pollen dispersal from five different Cupressaceae species and scored as 1 when less than 25% of individuals were pollinating, as 2 when number of pollinating individuals was between 25% and 50%, and as 3 when more than 50% of individuals were pollinating.

saceae winter pollen season, which ends at the beginning of April in the area considered.

DISCUSSION

The number of pollen species from Cupressaceae and closely related families *(Taxodiaceae* and *Podocarpaceae),* which are documented as respiratory sensitizers, is growing,[7, 11, 14-19] supporting an important role of these families in late autumn and winter pollinosis. This report deals with the characterization of the extract obtained from pollen of *J. oxycedrus,* an autochthonous Cupressaceae species present in Italy and in the Mediterranean area, the allergenic importance of which has not been investigated before.

The biochemical characterization of JoE revealed that, as reported for other Cupressaceae,[26, 34] the protein content of the extract was low, thus suggesting that pollen from this and other related families might have peculiarities different from other pollens. However, the percentage of proteins present in JoE and JaE, which were prepared by a classical extraction procedure,[24, 25] was comparable to that obtained with *C. arizonica* pollen, which requires a specific and complex extraction procedure involving ammonium sulfate precipitation.[26] Similarly, total carbohydrate content was comparable in the three extracts, although a detailed characterization of sugar composition has not yet been carried out. The SDS-PAGE analysis of JoE found components with a relative MW similar to that of components present in JaE and CaE. However, JoE seems to show a broader pattern of equally stained bands without a clearly identified major component, some of which are probably shared with JaE and CaE, which show the greatest qualitative and quantitative reciprocal similarities.

The ELISA results indicate that most of the sera tested have antibodies reactive with all extracts, although JaE appears to be slightly more reactive as compared with the others. The presence of cross-reactive epitopes has been supported by the ELISA-inhibition experiments, which have clearly demonstrated that

the three extracts share epitopes almost equivalently represented. Immunoblotting inhibition further confirmed that there is a close relationship between *J. oxycedrus* and other species belonging to the Cupressaceae family.[20] However, in the system involving JaE and JoE as blotted antigens, several bands detected by IgE could never be completely inhibited by either the homologous or the heterologous inhibitors. These data are in disagreement with those obtained in the ELISA inhibition, in which 100% inhibition is approached in all systems making use of these two extracts as antigens adsorbed onto the plate.

This discrepancy between ELISA-inhibition and blotting-inhibition results cannot be explained on the basis of an insufficient amount of inhibitor used in immunoblotting. In fact, it appears that an increase in the amount of inhibitors does not produce a comparable inhibition increase, suggesting that the system has reached a plateau. The observed discrepancy could be alternatively explained by assuming that the epitopes involved in the two assays are different. In particular, in ELISA both the antigen on the plate and the inhibitors are maintained in comparable conditions (aqueous buffers and presence of mild detergents such as Tween 20), and therefore the latter should efficiently compete for IgE binding. On the other hand, blotting-inhibition experiments are based on the competition between SDS- and 2-mercaptoethanol–treated samples (blotted antigens) and untreated samples diluted in aqueous buffer (inhibitors). Therefore JoE and JaE appear to have some epitopes recognized by the IgE only after SDS and 2-mercaptoethanol treatment, which are essentially absent or poorly represented in all three native extracts used as inhibitors. This feature is not shared by the CaE, which does not display newly formed epitopes available for IgE binding after SDS and 2-mercaptoethanol treatment.

When CaE was used as an antigen, a complete blotting inhibition could be obtained with all inhibitors, being the homologous extract that serves as the best inhibitor. These results are in disagreement with those obtained in the ELISA inhibition, in which the homologous inhibitor was able to produce only complete inhibition. A possible explanation for this finding could be that some CaE allergens involved in the binding in native conditions (i.e., when the extracts are used as antigens in ELISA or as inhibitors in both systems) and absent in JoE and JaE are not able to migrate when submitted to SDS-PAGE assay. This might be due to the presence of aggregates or of large glycidic moieties, the latter explanation being supported by a series of observations suggesting that sugars might play an important role in IgE binding.[35-37]

JoE elicited cutaneous reactivity in sensitized patients, and positive test results are obtained in exposed and nonexposed subjects, as already reported for other related pollens.[7, 20, 38] Data obtained from patients enrolled in this study strongly support the appearance of a pollen-related allergy in a late fall season. An article describing *J. pinchotii* as an allergen reported that this species pollinates in the fall in North America.[39] Examining other fall-to-winter Cupressaceae pollen calendars, it is possible to argue that pollen-related respiratory symptoms could be present and not identified in areas where *J. oxycedrus* or other related species pollinate.[23, 40] As demonstrated for winter pollinosis,[8] the allergy diagnosis may be overlooked and followed by unsuccessful treatment.

In conclusion, *J. oxycedrus* is a newly characterized allergenic pollen of the Cupressaceae family. Because *J. oxycedrus* pollinates during November, Cupressaceae pollinosis can last in some areas for 3 to 5 months.

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