

Cross-reactivity between *Cupressus arizonica* and *Cupressus sempervirens* pollen extracts

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Background: *Cupressus arizonica* and *C. sempervirens*, two species belonging to the Cupressaceae family, are recognized as an important cause of respiratory allergies in countries with a Mediterranean climate.

Objective: The relationship between pollen extracts from these two species was studied by evaluating the reactivity with polyclonal rabbit antisera and human IgE.

Methods: The two extracts were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis. Cross-reactivity was evaluated by ELISA and immunoblotting inhibition experiments.

Results: The electrophoretic patterns of the two extracts are quite different, although some components display identical molecular weights. The immunoblotting developed with human IgE from subjects allergic to members of the Cupressaceae family indicated that two major IgE-reactive components, displaying molecular weights of about 43,000 and 36,000 d, were similarly detected in both extracts. Inhibition experiments showed a high degree of cross-reactivity between the two extracts when tested with rabbit polyclonal antibodies against *C. arizonica* and *C. sempervirens*. When tested with human IgE inhibition methods, both extracts were able to reciprocally inhibit all of the IgE-reactive bands, although *C. arizonica* extract was always a better inhibitor.

Conclusions: *C. arizonica* and *C. sempervirens* extracts are highly cross-reactive at the IgE level and share a number of common epitopes also identified by polyclonal rabbit antisera. (*J Allergy Clin Immunol* 1996;98:797-804.)

Key words: *Cupressus arizonica*, *Cupressus sempervirens*, allergens, characterization, cross-reactivity, diagnosis, IgE, blotting inhibition, ELISA inhibition

Human allergy to Cupressaceae was reported for the first time more than 50 years ago. However, its clear-cut role in the so-called “winter pollinosis” has only recently been obtained.¹⁻⁷ Species of the Cupressaceae family, mainly *Cupressus sempervirens*, have been identified as responsible for an increasing number of cases of allergy over the winter period.⁸⁻¹⁵ Several indications suggest that in addition to *C. sempervirens*, *C. arizonica* can also play a role in those countries with a Mediterranean climate, related in part to its increasing use not only in reforestation programs but also in gardens

Abbreviations used

CaE:	<i>Cupressus arizonica</i> pollen extract
CsE:	<i>Cupressus sempervirens</i> pollen extract
MW:	Molecular weight
PBS:	Phosphate-buffered saline
RT:	Room temperature
SDS-PAGE:	Sodium dodecylsulfate–polyacrylamide gel electrophoresis
sIgE:	Specific IgE

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and parks. In fact, despite their recent introduction, *C. arizonica* plants can account for a great part of the *Cupressus* species currently spread in the Mediterranean area¹⁴ (Mari. Unpublished data). One of the major problems encountered in the diagnosis of such pollinosis has been related to the poor quality of extracts available for in vivo and in vitro testing. This problem was clearly addressed

by Ford et al.,⁶ who prepared and characterized a *C. sempervirens* pollen extract (CsE), which allowed the identification of the major allergenic components able to bind IgE from sera of allergic subjects. Because of the increasing importance of *C. arizonica*, we have recently focused our attention on the preparation and characterization of extracts from this species,¹⁶ identifying the IgE-reactive components including a major allergen with a molecular weight (MW) of 43,000 d. This species was then used in in vivo comparative studies, carried out with CsE and *C. arizonica* pollen extract (CaE), which were either home-made or commercially available. The results of this study¹⁷ have clearly indicated that CaE always provides better results in skin prick tests, as compared with CsE, in terms of a greater cutaneous response and a larger number of subjects identified as allergic to "cypress." These results raised two questions: Do any quantitative or qualitative differences exist between the two species, and what is their role in the induction of sensitization?

The aim of this study was to evaluate the degree of cross-reactivity between CsE and CaE by means of different in vitro approaches with the use of both polyclonal rabbit antisera and human IgE from patients allergic to cypress.

METHODS

Pollen extracts (CaE and CsE)

Pollen from *C. arizonica* and *C. sempervirens* were purchased from Allergon (Angelholm, Sweden). The extracts were prepared as previously described.¹⁶ Protein content was measured according to the method of Bradford.¹⁸

Antisera

Rabbit antisera. Two rabbits were immunized four times at 2-week intervals with CaE and CsE by injection of 300 µg of protein of the freeze-dried extracts, dissolved in 0.5 ml of phosphate-buffered saline (PBS) and mixed with an equal volume of Freund's adjuvant.

Human sera. Human sera were obtained from patients sensitive to members of the Cupressaceae family, as proven by results of skin prick tests with *C. sempervirens* and *C. arizonica* and results of specific IgE detection tests. Sera from four subjects with highly positive results (class 4 with commercial specific IgE detection methods for *C. sempervirens*; optical density range, 1.8 to 2.8 in ELISA for *C. arizonica*), containing IgE able to recognize most of the components in both extracts as proven by preliminary immunoblotting, were pooled and stored in 0.5 ml aliquots. Subjects were free from allergen-specific immunotherapy, and their informed consent to participate in the study was obtained. Serum samples to be used as negative controls were collected from two nonatopic volunteers.

Polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransfer

SDS-PAGE and electrotransfer were carried out, as previously described,^{16,19} in a mini-electrophoresis and blotting system (Bio-Rad, Richmond, Calif.). CaE and CsE (10 µg protein/well) were reduced by 5% vol/vol 2-mercaptoethanol before application to the 12% wt/vol polyacrylamide gel. The gel was stained with 0.05% Coomassie Brilliant Blue (Imperial Chemical Industries, Ltd., Macclesfield, Cheshire, U.K.) in water/methanol/acetic acid (50:40:10).

ELISA inhibition

ELISA inhibition was performed essentially according to the procedure previously developed in our laboratory.²⁰ CaE or CsE was used as antigen at a concentration of 20 µg/ml. In both inhibition systems, the working antibody dilution was calculated on the basis of the values producing approximately 80% maximum binding.

Rabbit antibody inhibition. Serum from rabbit anti-CaE was prediluted 1:5000 in PBS-Tween 0.05% vol/vol in the system involving CaE as antigen on the plate and prediluted 1:3000 in the system involving CsE. Serum from rabbit anti-CsE was prediluted 1:10,000 or 1:1000 when tested against CaE and CsE, respectively. Prediluted sera were incubated for 1 hour at room temperature (RT) with twofold serial dilutions of inhibitors. The optimal amount of inhibitors (ranging from 0.03 µg protein/ml to 80 µg protein/ml) was chosen for each inhibition system on the basis of preliminary experiments. Incubation times were 1 hour at RT for the mixtures of antibody and inhibitor and 1 hour at RT with peroxidase-labeled goat anti-rabbit IgG (Bio-Rad) diluted 1:3000.

Human IgE inhibition. Inhibition of specific IgE was carried out by incubating the human sera pool, prediluted 1:5 in PBS-Tween and 1% wt/vol gelatin, with twofold serial dilutions of inhibitors for 3 hours at RT. Amount of inhibitors ranged from 0.015 µg protein/ml to 20 µg protein/ml. Plates were previously blocked for 1 hour at RT with 3% gelatin in PBS and incubated for 3 hours at RT with the mixtures, followed by overnight incubation with peroxidase-labeled rabbit anti-human IgE (KPL, Gaithersburg, Md.) diluted 1:1000 in PBS-Tween-1% gelatin.

Percent of inhibition was calculated in relation to the values obtained in the absence of inhibitor in the system. Linear regression analysis and evaluation of the amounts of inhibitor producing 50% inhibition were performed after logarithmic transformation, as previously reported.²⁰

Immunoblotting inhibition

Immunoblotting inhibition was performed essentially according to the methods reported by Johansson et al.²¹ and Huang et al.²²

Rabbit antibody inhibition. The same protocol was applied to all immunoblotting experiments, independently of the rabbit serum used. Eight different final

concentrations of CaE and CsE (50, 25, 10, 5, 2.5, 1, 0.5, and 0.25 $\mu\text{g/ml}$ protein) were obtained after mixing 0.5 ml of inhibitor solution in PBS-Tween with an equal volume of rabbit serum prediluted 1:1000 in PBS-Tween. The mixtures were incubated for 3 hours at RT and then transferred to the strips and further incubated for 3 hours at RT. The strips were incubated overnight at RT with peroxidase-labeled goat anti-rabbit IgG (Bio-Rad), diluted 1:1000 in PBS-Tween, and developed with 4-chloro-1-naphthol according to the Bio-Rad instructions.

Human IgE inhibition. Inhibition of specific IgE was carried out by overnight RT incubation of the human sera pool diluted 1:2 in PBS-Tween with 50, 25, 5, 1, 0.25, 0.05, 0.01, and 0.005 $\mu\text{g/ml}$ protein of CaE or CsE. The strips were incubated overnight at RT with the mixtures and then overnight with iodine 125-labeled rabbit anti-human IgE (RAST I; Pharmacia, Uppsala, Sweden). Strips were developed by exposing the blots to x-ray film (Kodak Diagnostic Film X-Omat AR; Eastman Kodak Company, Rochester, N.Y.).

The results of inhibition experiments were analyzed by densitometric scanning of the strips (Zeineh Video Densitometer; Biomed Instruments, Fullerton, Calif.). Values of 50% inhibition were graphically calculated on the inhibition curves.

RESULTS

Electrophoretic profile of CaE and CsE

After the extraction procedures, a protein yield of about 3% (wt/wt) for both CaE and CsE was obtained. Carbohydrate content ranged from 65% to 85%. The electrophoretic pattern of the two extracts stained by Coomassie Brilliant Blue, together with the immunoblotting pattern developed with a pool of sera from subjects allergic to *Cupressus* species or from normal donors, are shown in Fig. 1. The protein profile (Fig. 1, *a* and *b*) of the two extracts show different patterns. CaE is characterized by a major component with an MW of about 43,000 d and some minor bands with MWs of about 36,000 d and 70,000 d. CsE is characterized by a series of bands with MWs ranging from 97,000 to 14,000 d, all essentially equivalent in terms of intensity. The immunoblotting of the two extracts developed with human specific IgE confirms the differences in the reactivity pattern of CsE and CaE (Fig. 1, *c* and *d*). Most of the components detected by Coomassie Brilliant Blue staining in the two extracts are also recognized by human IgE. The most reactive component in CaE has an MW of 43,000 d and corresponds to the *C. arizonica* major allergen previously identified.¹⁶ Two other bands, displaying MWs of about 36,000 d and 70,000 d were

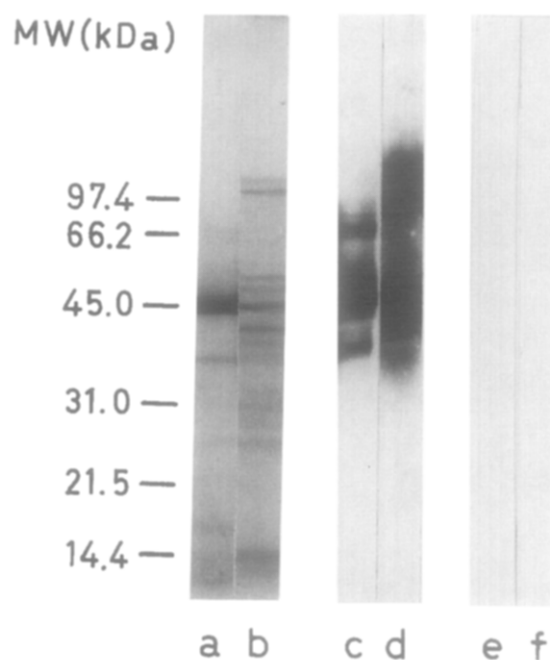


FIG. 1. CaE (*a*, *c*, *e*) and CsE (*b*, *d*, *f*) pollen extracts after separation by SDS-PAGE in reducing conditions. Coomassie Brilliant Blue staining (*a*, *b*), immunoblotting developed with a pool of sera from subjects allergic to Cupressaceae (*c*, *d*), and immunoblotting developed with a pool of sera from normal subjects (*e*, *f*).

also detected. When the pool was tested with CsE, two bands, displaying MWs comparable with that of the major allergen (43,000 d) and that of the 36,000 d component of *C. arizonica* were detected. However, a family of closely related bands with MWs of about 100,000 d were clearly reactive in CsE only.

Inhibition experiments

Inhibition of polyclonal rabbit antisera. The reactivity of rabbit anti-CaE and anti-CsE was evaluated by ELISA and immunoblotting. Both sera were able to react with the homologous, as well as with the heterologous, extracts as assessed by ELISA (data not shown) and immunoblotting (Fig. 2, lane 17). The two sera were then used in ELISA inhibition experiments. As shown in Fig. 3, CsE was able to inhibit the binding of the anti-CaE antiserum when tested against CaE coated on the plate (Fig. 3, *A*), although the amount of CsE required to produce 50% inhibition was approximately six times higher than the amount of the homologous (CaE) inhibitor (4.26 vs 0.7 $\mu\text{g/ml}$). Interestingly, such a difference was not evident when CsE was used as antigen on the plate (1.0

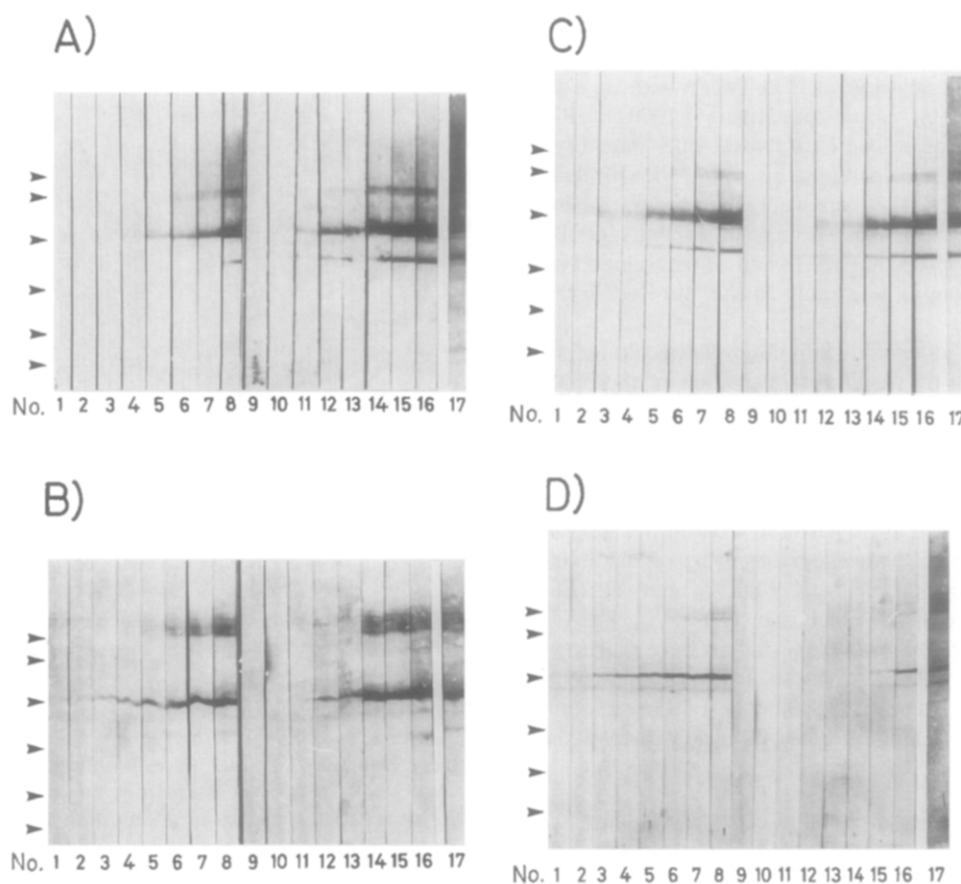


FIG. 2. Immunoblotting inhibition pattern of anti-*C. arizonica* rabbit antiserum binding to CaE (**A**) and CsE (**B**), inhibited with CaE (lanes 1 to 8) or CsE (lanes 9 to 16); immunoblotting inhibition pattern of anti-*C. sempervirens* rabbit antiserum binding to CaE (**C**) and CsE (**D**), inhibited with CaE (lanes 1 to 8) or CsE (lanes 9 to 16). Amounts of inhibitors are indicated in Methods. Lane 17 shows the antiserum binding to blotted antigens in the absence of inhibitor.

and 1.48 $\mu\text{g/ml}$ for CaE and CsE, respectively, Fig. 3, B), supporting the assumption that all the epitopes detected by anti-CaE antiserum on CsE are quantitatively represented in a similar way in both extracts. When the anti-CsE antiserum was tested by using CsE absorbed on the plate (Fig. 3, D), the homologous extract was found to be a much more powerful inhibitor than CaE. In fact, the amount producing 50% inhibition was 0.39 $\mu\text{g/ml}$ for CsE, whereas only a negligible inhibition was observed with CaE (approximately 25% inhibition at 40 $\mu\text{g/ml}$). Interestingly, when CaE was used as antigen on the plate (Fig. 3, C), the CaE became a better inhibitor than CsE (50% inhibition values: 0.62 $\mu\text{g/ml}$ and 5.87 $\mu\text{g/ml}$, respectively).

Results of blotting inhibition experiments obtained with CaE and CsE probed with anti-CaE rabbit antiserum are shown in Fig. 2 (A and B, respectively). When rabbit antibodies were tested against CaE (Fig. 2, A, lane 17), five bands were

clearly identified, together with several minor bands. Three of them were also semiquantitatively analyzed by densitometric scanning, and values producing 50% inhibition were calculated. All components were inhibited almost completely by both extracts, although to a different extent; the components with MWs of 36,000 d and 43,000 d (*C. arizonica* major allergen) were more efficiently inhibited by the homologous extract. The component displaying an MW of 70,000 d could be inhibited by comparable amounts of both extracts. When the same antisera were tested against CsE (Fig. 2, B, lane 17), five components were resolved together with a series of high MW bands (approximately 100,000 d). Although CsE and CaE behave in a similar way, CsE produces the 100% inhibition for both components at a lower concentration, whereas CaE is a slightly better inhibitor as compared with CsE in terms of 50% inhibition.

Results obtained with CaE and CsE probed in immunoblotting with anti-CsE rabbit antiserum

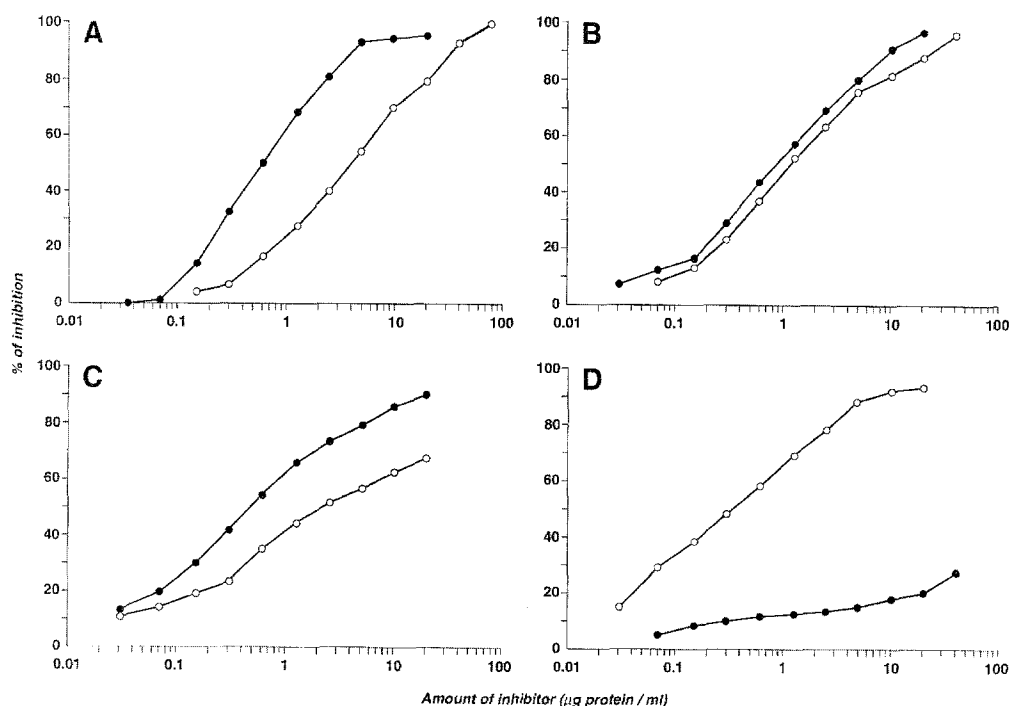


FIG. 3. ELISA inhibition of anti-*C. arizonica* rabbit antiserum tested against CaE (A) and CsE (B) and of anti-*C. sempervirens* rabbit antiserum tested against CaE (C) and CsE (D). Inhibitors were CaE (filled circles) and CsE (open circles).

are shown in Fig. 2 (C and D). At least five separate components could be detected in CaE (Fig. 2, C, lane 17), whereas at least nine bands were clearly identified together with several minor bands, when the rabbit antibodies were tested against CsE (Fig. 2, D, lane 17). Three major bands of CsE (39,000 d, 43,000 d, and the 100,000 d MW family of components) in the homologous inhibition system were quantified by densitometric analysis. All the components could be more efficiently inhibited by CsE, whereas the 50% inhibition by CaE was obtained with much higher inhibitor concentrations.

Inhibition of human IgE. ELISA results are shown in Fig. 4. IgE antibodies are directed toward epitopes mainly present on CaE, because CaE is always a better inhibitor independently of the antigen adsorbed on the plate. In fact, 0.14 μg/ml CaE was required to produce 50% inhibition of IgE binding to CaE adsorbed on the plate, whereas CsE reached a maximum value of 30% at the highest amount of inhibitor used (20 μg/ml). These differences were greatly reduced when CsE was used as antigen on the plate. In this case, concentrations of 0.1 μg/ml CaE and 1.33 μg/ml CsE were required to produce 50% inhibition.

In blotting inhibition experiments, CaE is a

better inhibitor for all the major IgE-binding components, independently of the extract transferred onto the blotting paper (Fig. 5). These results were also confirmed by densitometric analysis performed on the most reactive bands in the two systems considered.

DISCUSSION

The coincidence of the Cupressaceae pollination period with the winter season, when upper respiratory tract diseases are common, together with the low quality and low biologic activity of the *in vivo* and *in vitro* diagnostics prepared from *C. sempervirens* pollen,¹⁷ have always hampered the clinical diagnosis of cypress allergy.¹² In this context, the low protein content and the very high carbohydrate amount, which characterize such extracts, represent two crucial aspects that influence the preparation of a reagent suitable for diagnostic purposes. Recently, the development of ad hoc procedures has allowed the preparation of extracts from *C. arizonica* and *C. sempervirens* pollen,¹⁶ which have been used in both *in vivo* and *in vitro* assays to demonstrate specific IgE in sera from patients with cypress allergy.¹⁷ The two extracts, prepared by identical procedures, do not substantially differ either in terms of total protein and

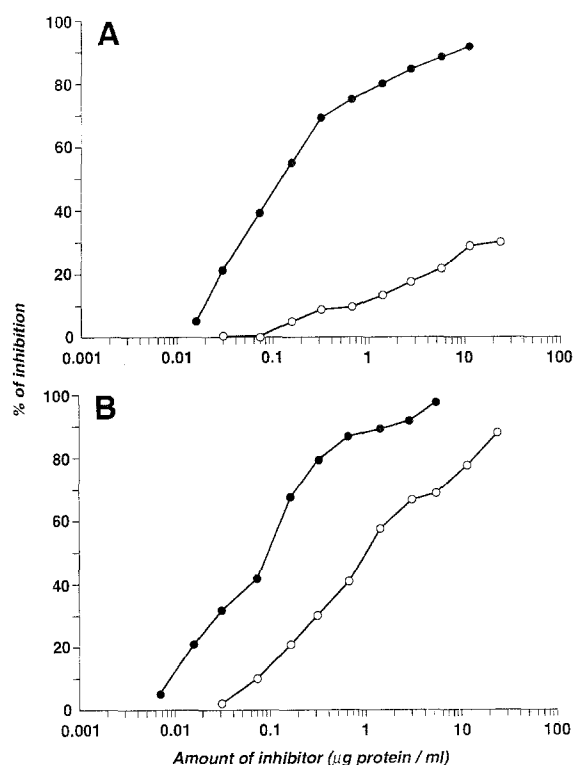


FIG. 4. ELISA inhibition of pooled human sera from patients allergic to Cupressaceae tested against CaE (A) and CsE (B). Inhibitors were CaE (filled circles) and CsE (open circles).

carbohydrate content or in terms of dry weight of extract that can be obtained from the same amount of dry pollen (Barletta. Unpublished data). However, the data obtained strongly support the idea that CsE remains less reactive than CaE in terms of number of positive subjects detected.

Despite the fact that the two species belong to the same genus of the same family, the protein pattern obtained by SDS-PAGE analysis showed some differences between CaE and CsE. These data are essentially in agreement with previously published studies by us for CaE,¹⁶ and by Ford et al.⁶ and Huang et al.²² for CsE, although in the latter case the MW of the major allergen was found to be equal to 42,000 d.

The ELISA results obtained with rabbits immunized with the two *Cupressus* species indicate that cross-reactive epitopes are shared by the two extracts, although it appears that unique epitopes are also present. These data also suggest that the two extracts have unique and cross-reactive epitopes recognized by polyclonal rabbit antibodies and that the cross-reactive ones are represented in a higher amount in CaE. The use of rabbit antibodies

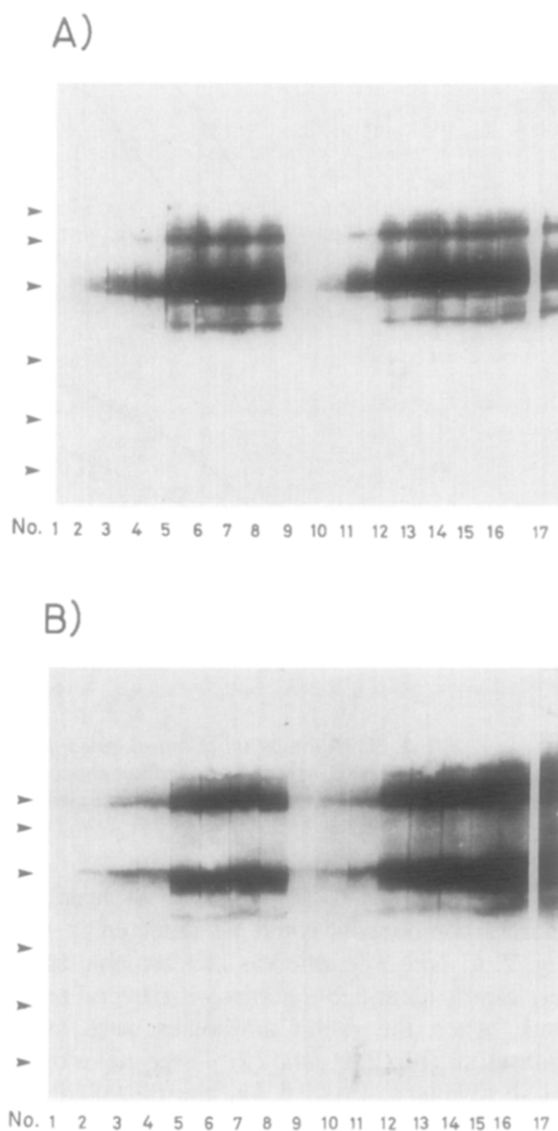


FIG. 5. Immunoblotting inhibition pattern of IgE binding to components of CaE (A) and CsE (B) inhibited with CaE (lanes 1 to 8) or CsE (lanes 9 to 16). Amounts of inhibitors are indicated in Methods. Lane 17 shows IgE binding to blotted antigens in the absence of inhibitor.

yielded a great advantage over human IgE obtained from patients allergic to *Cupressus* species after in vivo natural exposure to both extracts and that cannot therefore be regarded as specifically or even preferentially exposed to one of the two species.

The ELISA results are essentially confirmed by blotting inhibition assays, because all the major components detectable in the two extracts are able to reciprocally inhibit the binding of the two rabbit antisera, independently of the antigen blotted onto the nitrocellulose filter.

The clear cross-reactivity demonstrated with polyclonal rabbit antisera was confirmed by using human IgE, although the IgE system indicates a preferential recognition of CaE by human sera, which is not found with polyclonal rabbit antibodies. However, when large amounts of inhibitors are used in the IgE blotting system, a complete inhibition of IgE binding to CaE with CsE is achieved, thus supporting the conclusion that the pool of cross-reacting epitopes is qualitatively the same but markedly different from the quantitative point of view.

Although some of the components recognized by human IgE in the two extracts display different MWs, the cross-inhibition with the two extracts is always achieved for all the bands detected in immunoblotting, indicating that cross-reactive epitopes are shared by components differing in terms of MW. This finding can be explained by assuming that most IgE-reactive and polyclonal antisera-reactive epitopes are essentially represented on three highly stable components in CaE (i.e., the major allergen with a MW of 43,000 d and the 70,000 d and 36,000 d MW components), which can also be preferentially recovered after the extraction procedure. On the contrary, the reactive components in CsE might be present in various forms as a result of degradation or aggregation, phenomena often observed in pollen extracts.²⁴⁻²⁶ Indeed, the low stability of pollen and extracts have been reported specifically for *C. sempervirens*,²⁷ leading to the recommendation of using fresh pollen for the preparation of good quality CsE. On the other hand, the presence of IgE specific for carbohydrate moieties, which behave as cross-reactive epitopes spread along several protein components or which may react with IgE as independent molecules, cannot be ruled out for these carbohydrate-rich extracts, also on the basis of other data available for the allergens Cry j 1 and Cry j 2 from *Cryptomeria japonica*²⁸ and allergens from *Candida albicans* and *Pityrosporum orbiculare*.²² The role of glycidic epitopes in the cross-reactivity within the Cupressaceae family needs further investigation, taking into account that the results obtained from the analysis of the fine specificity of IgE antibodies might be influenced by the criteria used in selecting sera and areas for pollen collection.²⁹

Data on cross-reactivities within the Cupressaceae family and between Cupressaceae and closely related families (Taxodiaceae and Podocarpaceae) or taxonomically distinct pollens³⁰ have been obtained with either monoclonal antibodies

or human IgE. Such data refer to *C. sempervirens* and *Cryptomeria japonica*^{28,31} and to *C. sempervirens* and *Callitris glaucophylla*²³ or *Callitris verrucosa*.³² No data were available concerning CaE and CsE, which, despite the close taxonomic relationship, behave quite differently when used both in vivo and in vitro. The data reported in this study suggest that CaE and CsE are highly cross-reactive at the IgE level and that they share a number of common epitopes also identified by polyclonal rabbit antisera. However, it also seems clear that CaE always behaves better in all the immunochemical tests used, thus suggesting that CaE also provides better results in in vivo testing¹⁷ because the components that are important for IgE binding can be present or extracted in a higher amount from *C. arizonica* than from *C. sempervirens* pollen.

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