

Arizona cypress (*Cupressus arizonica*) pollen allergens. Identification of cross-reactive periodate-resistant and -sensitive epitopes with monoclonal antibodies

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Species of the Cupressaceae family are a worldwide cause of respiratory allergies. We used monoclonal antibodies (mAbs) to investigate the presence and the nature of cross-reacting epitopes shared by various components within *Cupressus arizonica* pollen extract (CaE) or by CaE and pollen extract from *C. sempervirens* (CsE). mAbs were produced in mice immunized with whole CaE (4A6 and 5E6) or with the major allergen components (2D5). Their reactivity was investigated by ELISA and immunoblotting before and after CaE periodate treatment. Cross-reactivity was evaluated by ELISA inhibition and immunoblotting. mAbs 2D5 and 4A6 recognized periodate-resistant epitopes, whereas the mAb 5E6 reacted with a periodate-sensitive determinant. The former mAbs recognized epitopes present on CaE major allergen and also shared by other components. mAb 5E6 showed a spread reactivity on CaE, with exclusion of the major allergen. When the three mAbs were tested with CsE, a restricted pattern of reactivity to mAbs 2D5 and 4A6 was obtained, whereas mAb 5E6 maintained a spread reactivity. The CaE major allergen is represented by two components recognized by human IgE and sharing common epitopes, as proven by mAbs reactivity. The use of these mAbs demonstrates that cross-reactivity within CaE components and between CaE and CsE is due to the presence of periodate-sensitive as well as -resistant epitopes.

Key words: alkaline treatment; cross-reactivity; *Cupressus arizonica*; *Cupressus sempervirens*; monoclonal antibodies; periodate treatment.

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Human allergy to Cupressaceae pollens is a worldwide problem (1–4). The increased diffusion of species belonging mainly to the genus *Cupressus*, in particular, *C. arizonica* and *C. sempervirens*, has been reported from wide areas of some Mediterranean countries (2, 5–8). So far, one of the major problems encountered in the diagnosis of such

pollinosis has been related to the poor quality of extracts available for *in vitro* and *in vivo* testing (9). Recently, characterization of allergenic pollen extracts from the *Cupressus* genus has been reported for *C. sempervirens* (10, 11) and *C. arizonica* (5). Such studies showed a very low protein amount in these extracts as compared with the high carbohydrate content (12), whose role in IgE binding has been recently studied. Studies suggesting that sugar moieties can be involved in the binding of human IgE are available for allergens from *Cryptomeria japonica* (13), *Cynodon dactylon* (14), and *Olea europaea* (15), and from unrelated sources, such as *Candida albicans* and *Pityrosporum orbiculare* (16). However, the role of carbohydrate moieties in pollen extracts is an important problem.

Abbreviations: CaE: *Cupressus arizonica* pollen extract; CFA: complete Freund's adjuvant; CsE: *Cupressus sempervirens* pollen extract; DMSO: dimethyl sulfoxide; IFA: incomplete Freund's adjuvant; mAb: monoclonal antibody; MW: molecular weight; NC: nitrocellulose; OD: optical densities; PBS: phosphate-buffered saline; r.t.: room temperature; SAS ppt: saturated ammonium sulfate precipitated; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sIgE: specific IgE.

since sugar moieties may be responsible for widespread cross-reactivities between extracts (17).

Besides cross-reactivity between extracts, a further level of cross-reactivity, which could strongly influence many standardization approaches, has been described (18–20) for other allergenic extracts, and has been demonstrated to be associated with the presence of structures shared by several components within the extract. Information has not been acquired until now for Cupressaceae, since the only available data have been obtained with polyclonal antibodies. In this context, monoclonal antibodies (mAbs) do represent the reagent of choice.

Allergen-specific mAbs have been successfully used in many instances, such as characterization studies (18, 21–23), standardization (24–27), and cross-reactivity (13, 28, 29) evaluation of allergenic extracts. mAbs specific for Cupressaceae extracts (*Juniperus ashei*) have been described and employed to assess the presence of *J. ashei* allergen in nonpollen tree parts (30).

The present paper deals with an investigation of the presence and nature of cross-reacting epitopes shared by various components of CaE, or by CaE and the pollen extract from the closely related species *C. sempervirens*. This goal has been accomplished by the production of mAbs specific for allergenic components of CaE.

Material and methods

Pollen extracts from *C. arizonica* (CaE) and *C. sempervirens* (CsE)

Pollens from *C. arizonica* and *C. sempervirens* were purchased from Allergon, Angelholm, Sweden. The extracts were prepared as previously described (5). Protein content was measured according to Bradford (31).

Human sera

Human sera were obtained from patients sensitive to Cupressaceae, as proven by skin prick test with commercial extracts (Dome/Hollister-Stier; Bayropharm, Milan, Italy) and by the specific IgE (sIgE) detection test (CAP System, Pharmacia, Uppsala, Sweden). Sera from four highly positive subjects (class 4 with commercial sIgE detection methods for *C. sempervirens*, optical densities [OD] range 1.8–2.8 in enzyme-linked immunosorbent assay [ELISA] for *C. arizonica*) were pooled, aliquoted, and stored at -30°C until use. Subjects were free from allergen-specific immunotherapy and their informed consent to participate in the study was obtained.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransfer

SDS–PAGE and electrotransfer were performed essentially as previously described (12). The electrophoresis was run under constant amperage (8 mA in the stacking gel and 15 mA in the separating gel). CaE and CsE (10 μg protein/well) were reduced by 5% v/v 2-mercaptoethanol before application to the 15% w/v polyacrylamide gel. The gel was stained with 0.05% Coomassie brilliant blue in water:methanol:acetic acid (50:40:10). The separated proteins were transferred from the SDS gel to a nitrocellulose (NC) membrane overnight under constant amperage (80 mA).

Monoclonal antibodies (mAbs)

mAbs were prepared and characterized as previously described (18). This study was performed according to Italian decree no. 116 (1992) for the protection and welfare of laboratory animals, under control of the Safety and Quality Animal Experimentation Department of our institution. This decree supersedes the European guideline no. 609/86. Two different immunizations were carried out. mAbs 4A6 and 5E6 were obtained from Balb/c mice immunized three times with whole CaE in complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) (100 μg of protein for each injection). mAb 2D5 was obtained from mice immunized with the CaE major allergenic component separated by SDS–PAGE and transferred onto NC. The NC portion containing the bands of interest was cut out, dissolved in a small amount of dimethyl sulfoxide (DMSO), and then emulsified with CFA.

After the fusion, cells were seeded in selecting medium, and growing hybrids were tested by ELISA and immunoblotting was performed as described below. Hybrids of interest were cloned twice by limiting dilution and expanded *in vitro*. Supernatants were 10-fold concentrated by precipitation at 50% ammonium sulfate saturation (SAS ppt).

The isotype of mAbs was determined by ISO-Strip kit (Boehringer Mannheim, Germany), according to the manufacturer's instructions.

mAbs from supernatants were affinity purified by protein A column chromatography (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. ELISA plates were coated for 3 h at 37°C and overnight at 4°C with twofold dilution of purified mAbs, starting from 20–1.25 $\mu\text{g}/\text{ml}$ (100 $\mu\text{l}/\text{well}$), and blocked by adding 200 $\mu\text{l}/\text{well}$ of phosphate-buffered saline (PBS), pH 7.2, containing 0.1% Tween for 1 h at room tem-

perature (r.t.). The plates were incubated for 2 h with 20 µg protein/ml of CaE in PBS containing Tween 20 0.05% v/v (PBS-Tween). After washings, 100 µl/well of the pool of human allergic subjects, diluted 1:10, was added and incubated for 3 h. The IgE binding to the CaE was developed by adding peroxidase-labeled rabbit antihuman IgE (Bio-Rad).

ELISA

ELISA was performed essentially according to Mucci et al. (18). CaE was used as antigen at a concentration of 20 µg/ml. Hybridoma supernatants were used undiluted in the preliminary screening of growing hybrids and clones. SAS ppt of selected mAbs was titrated on untreated or periodate-treated CaE, starting from 1:250 to 1:8000 (2D5), or from 1:500 to 1:512 000 (4A6 and 5E6).

Immunoblotting

After blocking for 1 h at r.t. in PBS containing 3% gelatin, the blotted NC strips were incubated overnight at r.t. with 1 ml of each mAb SAS-ppt supernatant diluted 1:20 in PBS-Tween. After having been washed five times with PBS-Tween, strips were incubated with peroxidase-labeled goat antimouse IgG (Bio-Rad) diluted 1:1000 in PBS-Tween for 3 h at r.t. The colorimetric reaction was developed with 4-chloro-1-naphthol, according to the instructions of the manufacturer.

Specific IgE detection was carried out by incubating overnight at r.t. the strips with the human sera pool diluted 1:2 in PBS-Tween and then with ¹²⁵I-labeled rabbit antihuman IgE (Bio-Allergy, Rome, Italy). The reactive bands were detected by autoradiography at -80°C for 3 days with radiograph film (Kodak Diagnostic film X-Omat AR; Eastman Kodak Company, Rochester, NY, USA).

Periodate treatment on solid-phase adsorbed CaE

For further characterization of the nature of the epitopes recognized by mAbs on cypress pollen extracts, CaE was subjected to NaIO₄ oxidation after either absorption on ELISA plates or blotting onto NC, according to the method described by Woodward et al. (32).

ELISA. CaE adsorbed on the plate was washed five times with PBS-Tween and then rinsed with 50 mM sodium acetate buffer (pH 4.5). Experimental wells were exposed to 50 mM NaIO₄ for 1 h at r.t. in the dark, whereas control wells (mock) were treated with the buffer only. Control and experi-

mental wells were then rinsed with sodium acetate buffer and exposed to 50 mM NaBH₄ in PBS for 30 min.

Immunoblotting. Blotted strips were blocked with PBS-Tween for 30 min and then rinsed with sodium acetate buffer. Control strips (mock) were incubated in the same buffer for 1 h, whereas experimental strips were exposed to 30 mM NaIO₄ in the sodium acetate buffer for 1 h at r.t. in the dark. Control and experimental strips were then rinsed with sodium acetate buffer and exposed to 50 mM NaBH₄ in PBS for 30 min.

NaOH treatment

As a means to destroy the protein structure, 9 mg CaE was dissolved in 100 µl 3 M NaOH/0.1 M NaBH₄ overnight at r.t. The reaction was neutralized with 10 M hydrochloric acid and the solution stored in aliquots at -20°C until used.

ELISA inhibition

ELISA inhibition was performed essentially according to the procedure previously developed in our laboratory (24). The working dilution of SAS-ppt antibodies was calculated on the basis of the values giving approximately 80% maximum binding in direct ELISA. On the basis of preliminary experiments, mAbs 4A6 and 5E6 were prediluted 1:1000, and mAb 2D5 was prediluted 1:250 in PBS-Tween. mAbs were incubated for 1 h at r.t. with twofold serial dilution of inhibitors (CaE, CsE, NaOH-treated CaE). The optimal amount of inhibitors was chosen for each inhibition system on the basis of preliminary experiments. Doses higher than 7 mg/ml extract or 200 µg/ml protein were never tested because of solubility problems with the extracts. In ELISA inhibition employing NaOH-treated extract, the amount of the inhibitors was calculated on the basis of the weight of the lyophilized extracts. The mixtures were then transferred to the test plate. Incubation times were 1 h at r.t. for the antibody-inhibitor mixtures and 1 h at r.t. for peroxidase-labeled goat antimouse IgG (Bio-Rad) diluted 1:2000.

Percent inhibition was calculated in relation to the values obtained in the absence of inhibitor in the system. The amounts of inhibitor giving 50% inhibition were determined by graphic interpolation.

Results

The protein profile of CaE was characterized by the presence of a major component (90% as

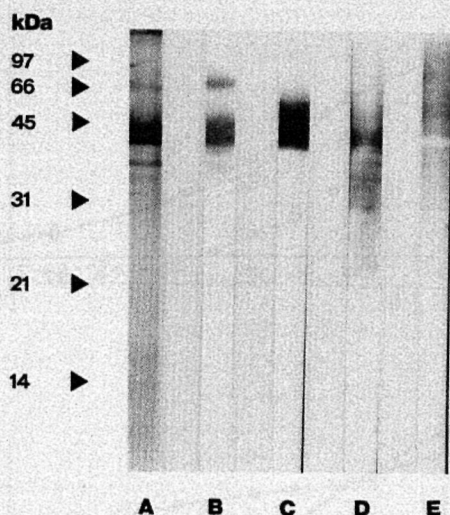


Fig. 1. CaE after separation by SDS-PAGE in reducing conditions. Coomassie brilliant blue staining (A), immunoblotting developed with pool of sera from subjects allergic to Cupressaceae (B), and with mAbs 2D5 (C), 4A6 (D), and 5E6 (E).

assessed by densitometric evaluation) in the range 40–45 kDa, as demonstrated by SDS-PAGE analysis and Coomassie brilliant blue staining (Fig. 1A). This component, previously identified as a single band corresponding to the CaE major allergen (5), was now resolved by using a different concentration of acrylamide in the separating gel, 15% instead of 12%, in a couple of bands with slightly different MW (41–43 kDa). Immunoblotting analysis developed with a pool of sera from Cupressaceae-allergic subjects (Fig. 1B) indicated both bands to be allergens and identified at least two other allergenic components displaying MW of about 70 and 36 kDa, respectively. None of the subjects individually tested reacted to only one of the two components (data not shown).

Cell fusion between the myeloma cell line and spleen cells of Balb/c mice immunized either with the whole CaE or with the NC-blotted major allergen component (41–43 kDa) resulted in a series of hybridomas secreting antibodies against CaE components. The reactivity of these antibodies was determined with ELISA and immunoblotting, and positive hybridomas were cloned. Three clones were selected on the basis of their immunoblotting pattern: mAbs 4A6 and 5E6 obtained by immunization with the whole CaE, and 2D5 obtained by immunoblotting with the major allergenic component. The isotype of all the three mAbs was IgG1 with the κ -light chain. mAb 4A6 recognized an epitope represented on the CaE major allergen but also shared by other components of 39, 36, 33, and 31 kDa and by a series of poorly resolved high-MW bands (approximately 100 kDa) (Fig. 1, lane D). When 2D5

was tested, its reactivity pattern was restricted to components of 45–39 kDa (Fig. 1, lane C). mAb 5E6 showed a diffuse reactivity ranging from 100 down to 20 kDa without sharp bands (Fig. 1, lane E); the 41–43-kDa region was not recognized by this mAb.

The mAbs 4A6 and 2D5 could recognize allergenic components, as indicated by comparative analysis of the SDS-PAGE and immunoblotting data. To further explore this issue, we developed a capturing ELISA procedure, using CaE. Data obtained indicate that protein-A-purified mAbs (4A6 and 2D5) could bind CaE components and present them to allow binding by IgE of a pool of sera from Cupressaceae-allergic subjects (Fig. 2), thus confirming the results obtained in Western blotting. No binding of the IgE to the CaE could be detected in the experimental conditions used in the absence of mAbs or in the presence of an irrelevant mAb.

The reactivity of these mAbs was further characterized to determine whether they could recognize periodate-sensitive (“carbohydrate”) or periodate-resistant (“protein”) epitopes on CaE. Periodate treatment of CaE adsorbed on the ELISA plate indicated that mAbs 2D5 (Fig. 3A) and 4A6 (Fig. 3B) recognized periodate-resistant epitopes, whereas mAb 5E6 (Fig. 3C) reacted to a periodate-sensitive epitope. ELISA inhibition of the three mAbs was then performed by using either native CaE or NaOH-CaE as inhibitors. Binding inhibition of mAbs 2D5 and 4A6 (Fig. 4A and B) never reached 50% with NaOH-CaE, thus confirming their specificity for “protein” epitopes. Fifty percent inhibition of mAb 4A6 binding, with native CaE as inhibitor, was obtained with an amount 280-fold lower than 2D5 (0.025 mg extract/ml vs about 7 mg extract/ml). Binding of mAb 5E6 (Fig. 4C) was inhibited by NaOH-CaE similarly to native CaE, confirming its recognition of a “non-protein” epitope. However, native CaE produced 50% inhibition of the binding of this mAb at a concentration 10-fold lower than NaOH-CaE (Fig. 4C), although both inhibitors gave the same values at the higher concentrations.

The different reactivity of the three mAbs was used in immunoblotting to characterize CaE further at the level of separated components. Fig. 5 reports the immunoblotting pattern of the three mAbs tested against untreated CaE (lanes A, B, and C), periodate-treated CaE (lanes D, E, and F), and mock CaE (lanes G, H, and I). Since periodate-treatment of the antigen eliminated the diffuse reactivity pattern of mAb 5E6 (Fig. 5, lane F), the results obtained in ELISA with unseparated CaE were confirmed. The same treatment did not affect the recognition pattern of mAbs 2D5 and 4A6 (Fig. 5, lanes D and E).

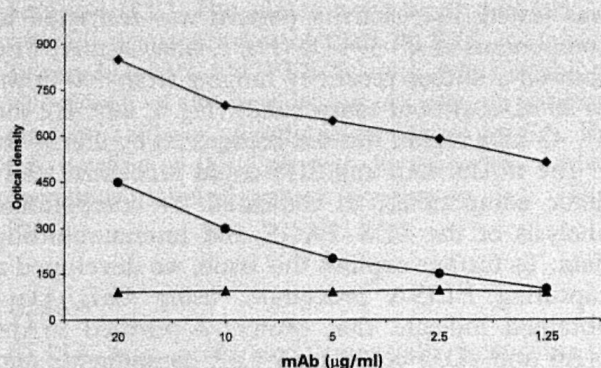


Fig. 2. Capturing ELISA of CaE by mAbs 2D5 (circles), 4A6 (diamonds), and irrelevant mAb (triangles).

The presence of cross-reacting components shared by CaE and CsE and identified by the three mAbs was evaluated by immunoblotting and ELISA inhibition. Immunoblotting analysis of the reactivity of the three mAbs against CsE is shown in Fig. 6. mAbs 4A6 (lane D) and 2D5 (lane C) identified two bands with MWs of 47 and 42 kDa. mAb 5E6 (lane E) recognized a broad pattern of components with MWs ranging from 100 to 36 kDa.

ELISA inhibition experiments with CaE and CsE as inhibitors vs CaE adsorbed on the plate are reported in Fig. 7. The homologous extract was found to be a better inhibitor than CsE when mAb 4A6 was tested (Fig. 7B). The antigen amount giving 50% inhibition was 2 µg/ml for CaE, whereas an amount 100-fold higher of CsE was needed. In testing mAb 2D5 (Fig. 7A), CsE proved a better inhibitor (50% inhibition at 6.5 µg/ml) than CaE, which reached a maximum value of 50% at the highest amount of inhibitor used. CaE and CsE inhibited in a similar way (Fig. 7C) the binding of mAb 5E6 to CaE, giving 50% inhibition at similar concentrations (about 0.2 µg/ml).

Discussion

The *C. arizonica* major allergen was also identified as a molecule displaying a MW of about 43 kDa, which is recognized by most sera reactive in immunoblotting (5, 12). In this study, appropriate electrophoretic conditions (i.e., increasing the concentration of the acrylamide gel from 12% to 15%) allowed us to resolve the CaE major allergen in two different bands with MWs of 43 and 41 kDa. A clear cross-reactivity between CaE and CsE using both specific polyclonal rabbit antisera and human IgE has been recently demonstrated (12), although no information concerning the distribution and the nature of such cross-reactive structures was acquired. In this study, the use of mAbs allowed us to demonstrate two levels of cross-

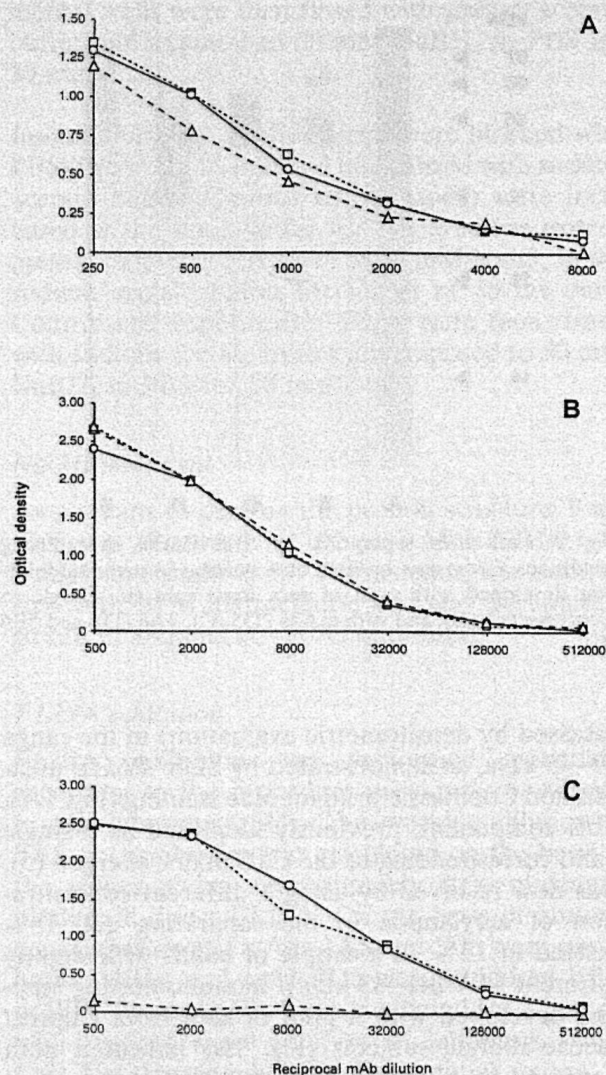


Fig. 3. ELISA reactivity of mAbs 2D5 (A), 4A6 (B), and 5E6 (C) to native CaE (circles), periodate-treated CaE (triangles), and mock CaE (squares).

reactivity, related to the presence of epitopes shared by several allergenic components within the CaE and between CaE and CsE, and to study the nature of such epitopes. The group of mAbs produced by immunizing mice with the whole native CaE (mAbs 4A6 and 5E6) showed a broad reactivity to different components of CaE, never restricted to the major allergen only, whereas the injection of purified major allergen led to the production of mAbs with a sharp reactivity to the two components of the allergen.

The three mAbs selected showed different recognition patterns of the CaE components. mAb 4A6 reacted to an epitope present on the CaE major allergen but also shared by other components, whereas mAb 2D5 showed a blotting pattern

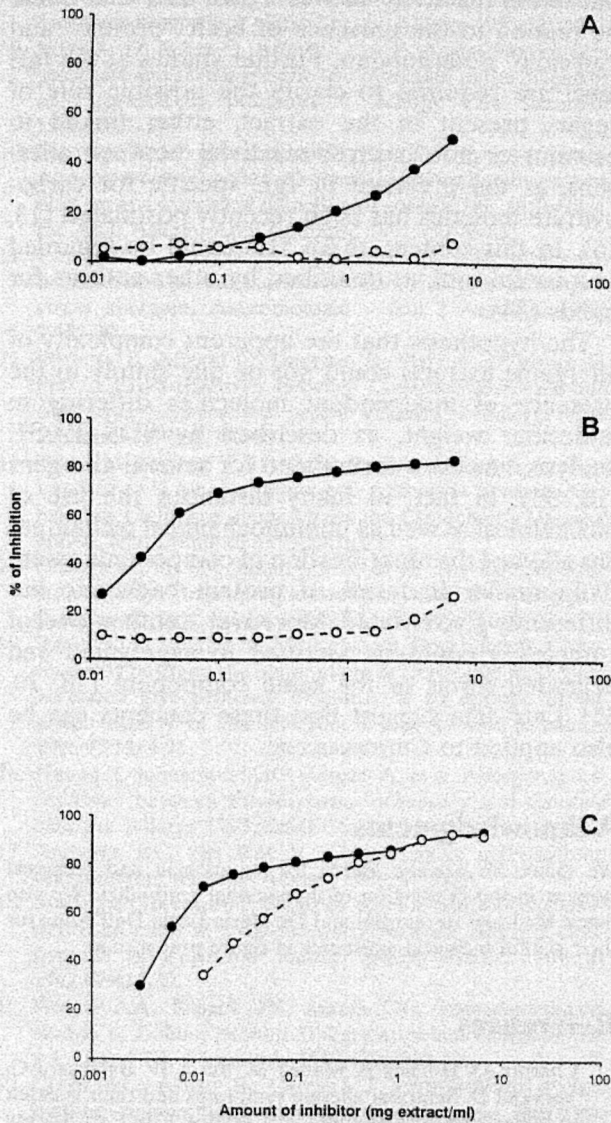


Fig. 4. ELISA inhibition of mAbs 2D5 (A), 4A6 (B), and 5E6 (C) against native CaE. Inhibitors were native CaE (filled circles) and sodium hydroxide-treated CaE (open circles).

restricted to bands of 45–39 kDa. Taken together, these data suggest that unique epitopes, as well as cross-reactive ones, were present on the major allergen. The third mAb, 5E6, showed a spread reactivity on CaE, with the exclusion of the major allergen. ELISA inhibition results suggest that the epitopes recognized by mAb 4A6 and mAb 5E6 are represented in CaE in a higher amount than the epitope detected by mAb 2D5.

After periodate treatment on CaE bound to the solid phase, mAbs 4A6 and 2D5 were still able to give a positive reaction, indicating that they recognized periodate-resistant epitopes. On the other hand, mAb 5E6 reacted to periodate-sensitive determinants, supporting the idea that a great

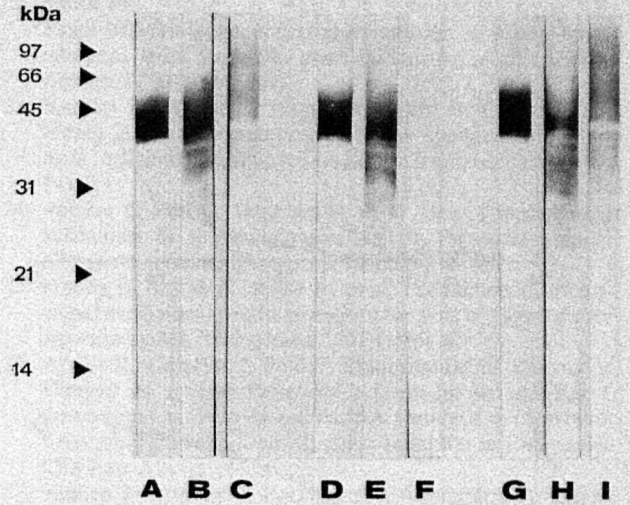


Fig. 5. Immunoblotting pattern of mAbs binding to untreated CaE (lanes A, B, and C), periodate-treated CaE (lanes D, E, and F), and mock CaE (lanes G, H, and I). Lanes A, D, and G) mAb 2D5; B, E, and H) 4A6; C, F, and I) 5E6.

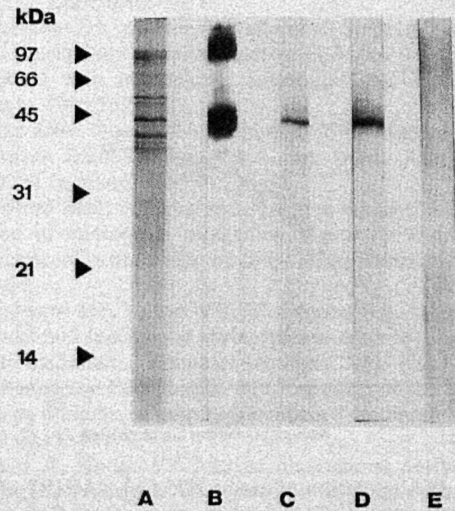


Fig. 6. CsE after separation by SDS-PAGE in reducing conditions. Coomassie brilliant blue staining (lane A), immunoblotting developed with pool of sera from subjects allergic to Cupressaceae (lane B), and with mAbs 2D5 (lane C), 4A6 (lane D), and 5E6 (lane E).

heterogeneity of molecules sharing at least one glycidic epitope does exist. The binding of 4A6 and 2D5 mAbs to the deglycosylated extract suggests that periodate treatment, performed in mild conditions at pH 4.5, is essentially unable to damage proteins, as previously reported (15, 33).

A clear cross-reactivity between CaE and CsE has been previously reported (12). The results of ELISA inhibition and immunoblotting assay confirm the presence of common epitopes between CaE and CsE, which were represented in different amounts on the pollen extracts from the two

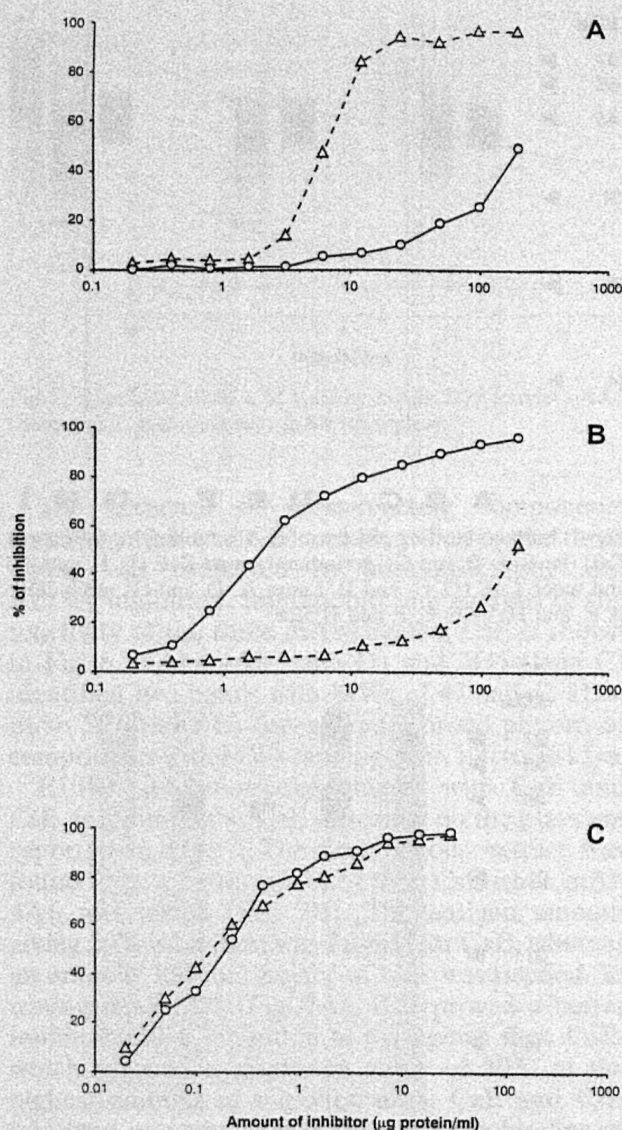


Fig. 7. ELISA inhibition of mAbs 2D5 (A), 4A6 (B), and 5E6 (C) against native CaE. Inhibitors were native CaE (circles) and CsE (triangles).

different species. Interestingly, ELISA inhibition showed that mAb 2D5 recognized an epitope more represented on CsE than on CaE, whereas mAb 4A6 was specific for an epitope mainly present on CaE. Periodate-sensitive epitope recognized by mAb 5E6 seemed to be equally represented in both extracts.

Immunoblotting results indicate that mAbs 4A6 and 2D5 recognize two epitopes on CsE, shared by components with the same MW and also identified by sIgE (12). The broad reactivity displayed by mAb 5E6 on CsE as well as on CaE supports the hypothesis that "glycidic" epitopes could also play a role in the cross-reactivity within the Cupressaceae family. Taken together, these data suggest

that cross-reactivity between CaE and CsE could be related to the presence of both "proteic" and "glycidic" determinants. Further studies at the IgE level are required to clarify the possible role of sugars present in the extract, either linked to proteins or not, in cross-reactivity between allergens, as the presence of IgE specific for carbohydrate moieties has been recently postulated (13, 16). In this context, mAb 5E6 could be regarded as a useful tool, as described by other authors for molds (34).

The hypothesis that the apparent complexity of allergenic extracts could not be due simply to the presence of independent molecules differing in molecular weight, as described by SDS-PAGE analysis, has been formulated for several allergens (18, 35). In fact, in many instances, the use of biochemical as well as immunochemical techniques has allowed the identification of components essentially similar in terms of protein backbone but differently glycosylated. Moreover, another level of complexity could be ascribed to aggregated and degraded forms of the same component (18, 20, 35). Our data suggest that these concepts can be also applied to Cupressaceae.

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