

Cross-reactivity among conifer pollens

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Background: There are increasing reports of Cupressaceae pollinosis from various geographic areas. Cross-reactivity among a limited number of species within the Cupressaceae family has been suggested. *Juniperus ashei* (mountain cedar) is the leading cause of respiratory allergy in South Texas.

Objective: This study examines in vivo and in vitro cross-reactivity among 12 Cupressaceae species, one Taxodiaceae species, one Pinaceae species, and an angiosperm.

Methods: Cross-reactivity among pollen extracts of mountain cedar (MC) and the other 14 trees was investigated by: (1) prick skin testing of each tree pollen extract in ten patients with MC pollinosis. (2) Ouchterlony gel immunodiffusion employing rabbit antisera to MC. (3) IgE immunoblotting using high-titer MC pooled human sera, and immunoblot inhibition after pre-incubation with MC protein. (4) Monoclonal antibody immunoblotting using a murine monoclonal antibody with strong affinity for the gp40 major allergen of MC.

Results: Positive skin wheal-and-flare responses occurred to all 12 Cupressaceae and Japanese cedar (the Taxodiaceae), but not to the Pinaceae or the angiosperm. In Ouchterlony gels, lines of identity or partial identity formed between MC and all pollens except the Pinaceae and the angiosperm. Immunoblots demonstrated IgE binding to the 40 to 42 kD protein in each Cupressaceae, and to a parallel band in Japanese cedar at 43 to 46 kD. Immunoblot inhibition by MC pollen was complete for all trees. The monoclonal bound both the 40 to 42 kD protein in 11 of 12 Cupressaceae species and the 46 kD band in Japanese cedar, but bound no protein bands in the Pinaceae or the angiosperm.

Conclusion: Pollen proteins of the 12 Cupressaceae (including MC) and the Taxodiaceae (Japanese cedar) are extensively cross-reactive. In particular, the MC major allergen, gp40, is cross-reactive with 40 to 42 kD proteins of the other Cupressaceae and with the Japanese cedar major allergen of 46 kD. Component-based immunotherapy may someday allow a standard treatment for both Juniper-allergic and *C. japonica*-allergic patients.

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INTRODUCTION

Allergenic cross-reactivity between pollens is a well-recognized phenomenon

in the plant kingdom that can present problems in the diagnosis and treatment of respiratory allergy. Knowledge of cross-reactivity allows for more informed treatment of patients in our increasingly mobile population. Since closely related plants have more shared antigens, knowledge of cross-reactivity can contribute to plant taxonomy.

The gymnosperm order Coniferales (Fig 1) contains several species that are of interest to allergists. Families Cu-

pressaceae and Taxodiaceae have several distinctive characteristics that have been recognized in several different countries, including an isolated pollination season in winter or early spring. Cupressaceae pollinosis is a significant cause of winter rhinitis in Spain and Italy.¹⁻³ Although Cupressaceae are usually found in mild climates, a Taxodiaceae, Japanese cedar (*Cryptomeria japonica*), is the major winter allergen in Japan, affecting nearly 10% of exposed populations.⁴ Cupressaceae allergy often develops during adulthood and may include previously "nonallergic" patients.^{3,5} Low protein and high carbohydrate content is a pollen characteristic in several species.^{6,7}

Juniperus ashei (sometimes incorrectly identified as *Juniperus sabi-noides*)⁸ is a Cupressaceae commonly known as mountain cedar (MC), the leading cause of respiratory allergy in South Texas from December to March. The cross-reactivity of MC pollen with Port Orford Cypress (*Chamaecyparis lawsonia*) of California and Oregon was suggested as early as 1929 by Black using skin tests and outcomes of hyposensitization.⁹ Kahn and Grothaus used similar techniques in 1931 to demonstrate MC cross-reactivity with red cedar (*Juniperus virginiana*).¹⁰ Cross-reactivity among several Cupressaceae antigens in rabbits and in man was reported by Yoo et al.¹¹ Panzani et al showed antigenic similarity between the common cypress of southern France (*Cupressus sempervirens*, also known as Italian cypress) and Japanese cedar.¹² This significant cross-antigenicity was the first to be demonstrated between two Coniferales families so geographically diverse. Subsequently, studies with sera of European patients allergic to *C. sempervirens* were shown to also recognize the protein antigens of *Callitris glau-*

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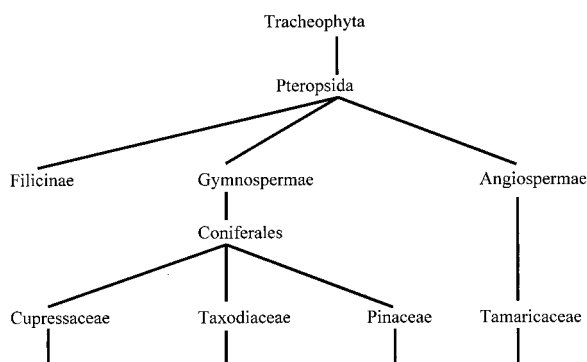
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1. *J. ashei*, 2. *J. californica*, 3. *J. chinensis*, 4. *J. scapulorum*, 5. *J. osteosperma*, 6. *J. monosperma*, 7. *J. pinchotti*, 8. *J. occidentalis*, 9. *J. virginiana*, 10. *L. decurrens*, 11. *C. Arizona*, 12. *C. lawsonia*, 13. *C. japonica*, 14. *C. deodora*, 15. *Tamarix gallica*

Figure 1. The phylum Tracheophyta includes all plants above simple mosses. The subphylum Pteropsida consists of three classes: gymnosperms (conifers); angiosperms (flowering plants), and the Filicinae (ferns). Allergenic gymnosperms are found in three of the six Coniferales families: Pinaceae [pines, firs, spruces], Cupressaceae [cedars, junipers], and Taxodiaceae [sequoia; Japanese cedar]. *Tamarix gallica*, an angiosperm, was included in the study as a control.

cophylla, a winter pollinating conifer of rural Australia.¹³ Many conifer species grow naturally or are cultivated as ornamental plants throughout the world. Recent reports suggest significant cross-reactivity between common native *C. sempervirens* and ornamental *Cupressus arizonica*, in the Mediterranean.¹⁴ *C. arizonica* has become a prominent *Cupressus* species throughout the Mediterranean due to planting as part of reforestation programs and use in gardens and parks.¹

Major allergens are usually abundant pollen proteins or glycoproteins, often with a molecular weight range of 20,000 to 40,000 daltons.¹⁵ Gross et al found that a relatively small fraction of crude MC pollen extract accounted for the pollen's major allergenicity. They identified the MC prominent allergen as a 50-kD glycoprotein and analyzed its N-terminal sequence.¹⁶ Later this major allergen of MC was shown to be a protein closer to 40 kD in molecular weight.^{17,18} Among other Cupressaceae, a 42-kD protein component of *C. sempervirens* bound patient IgE both most strongly and in 81% of subjects tested.⁷ In *C. arizonica*, a 43-kD protein, predominant in its intensity and frequency of recognition by human IgE, was identified as the major allergen.¹⁹ The major allergen (sugi ba-

sic protein, Cry j I) of the Taxodiaceae, Japanese cedar, has been found to be a basic glycoprotein of approximately 41 to 46 kD (by SDS-PAGE) with four subfractions that are antigenically and allergenically identical.²⁰

This study was undertaken to establish the degree of cross-reactivity among fourteen geographically diverse Coniferales: twelve Cupressaceae family members, one Taxodiaceae family member, and one Pinaceae family member. An angiosperm served as control. Common and botanical names are listed in Table 1.

METHODS AND MATERIALS

Preparation of Pollen Extracts

Study trees' genus and species were confirmed by botanists prior to pollen collection. Raw pollen from each tree was supplied by Center Laboratories (Port Washington, NY). Extracts were prepared in a manner previously reported for MC.¹⁷ Pollen protein extracts were prepared as 1:10 wt/vol raw pollen stirred in a solution of 0.125 M NH_4HCO_3 for 16 hours at 4°C. Extracts were centrifuged, and the supernatants were passed through 0.45- μ pore-size filters. The filtrates were dialyzed against distilled water overnight at 4°C in 3500-dalton dialysis tubing.

The resulting extracts were lyophilized and stored at 4°C. Extract protein concentrations were determined by a modification of the Lowry technique.²¹

Sera

Human sera were obtained from 23 south Texas patients sensitive only to MC as determined by seasonal pollinosis symptoms and prick skin testing. Out of a panel of 45 aeroallergens, these 23 patients reacted only to MC. None had received immunotherapy. As previously described, serum MC-specific IgE for each patient was determined by enzyme linked immunosorbent assay (ELISA), from which a pooled high-titer sera was prepared and stored in aliquots at -60°C.²²

Rabbit antisera to MC was raised in New Zealand white rabbits utilizing 500 μ g of MC extract protein in complete Freund's adjuvant. Antibody response was boosted subsequently using Freund's incomplete adjuvant. Antisera were pooled and used for Ouchterlony immunodiffusion against MC and the other trees. The care and use of animals followed the principles set forth in National Institutes of Health (NIH) Publication No. 86-23, Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act of 1966, as amended.

MC Monoclonal Antibodies

Monoclonal antibodies to MC had been prepared as previously described.²³ All monoclonals were murine isotype and subclass IgG1. Monoclonal MC2 demonstrated the highest affinity and most exclusive binding to the MC major allergen, gp40, on direct ELISA and immunoblot assays. In a MC ELISA inhibition assay, MC2 binding was inhibited an average of 74% by sera from MC hypersensitive subjects. Conversely, in a human specific-IgE mountain cedar ELISA inhibition assay, IgE binding was inhibited an average of 21% by MC2 binding to its single antigenic epitope. A monoclonal antibody to an irrelevant antigen (and of the same murine isotype and subclass) served as a control in all monoclonal experiments.

Table 1. Botanical and Common Names of Studied Trees. Numbers Correspond to Those Used in Double Immunodiffusion and Immunoblot Figures

Family	Genus/Species	Common Name
Cupressaceae	1. <i>Juniperus ashei</i>	Mountain cedar
	2. <i>Juniperus californica</i>	California juniper
	3. <i>Juniperus chinensis</i>	Chinese juniper
	4. <i>Juniperus scapulorum</i>	Rocky Mountain cedar
	5. <i>Juniperus osteosperma</i>	Utah juniper
	6. <i>Juniperus monosperma</i>	One-seed juniper
	7. <i>Juniperus pinchotti</i>	Pinchot juniper
	8. <i>Juniperus occidentalis</i>	Hooker western juniper
	9. <i>Juniperus virginiana</i>	Eastern red cedar
	10. <i>Libocedrus decurrens</i>	Incense Cedar
	11. <i>Cupressus arizonica</i>	Arizona cypress
	12. <i>Chamaecyparis lawsonia</i>	Port Orford Cedar
Taxodiaceae	13. <i>Cryptomeria japonica</i>	Japanese cedar
Pinaceae	14. <i>Cedrus deodora</i>	Deodar cedar
Tamaricaceae	15. <i>Tamarix gallica</i>	Salt cedar (an angiosperm)

Double Immunodiffusion

Double immunodiffusion precipitin assays for all tree extracts were accomplished on agarose plates according to the method of Ouchterlony using rabbit polyclonal antisera raised against MC pollen extract.²⁴ The hexagonal array of outer wells, containing MC antigen alternating with extracts of other trees, surrounded the center antisera well. Plates were incubated at room temperature and read at 24 and 48 hours.

Skin Tests

The patients who were skin tested to all trees had resided in south Texas for at least 3 years. None had traveled to each of the geographic areas in which study trees were native. Ten patients with MC seasonal allergic rhinitis, sensitive only to MC by skin testing⁵ were prick tested to all trees using a concentration of 1.0 mg pollen protein/mL phenol-saline. Skin tests were read at 15 minutes. Positive reactions were defined as a wheal 3 mm or greater than saline control, with a flare of 10 mm or greater. A nonallergic patient was skin tested as a control.

Polyacrylamide Gel Electrophoresis

Pollen extract proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described.¹⁷ Approximately 50 to 100 µg of pollen

protein per well was electrophoresed under reduced conditions on Pharmacia PAA 4/30 gradient gels along with low molecular weight protein controls (Pharmacia, Piscataway, NJ) which provided six calibration points (phosphorylase B, 94,000 D; albumin, 67,000 D; ovalbumin, 43,000 D; carbonic anhydrase, 30,000 D; trypsin inhibitor 20,100 D; and alpha-lactalbumin, 14,400 D).

Gels were stained with 0.1% Coomassie Blue R-250 in 7% acetic acid. The molecular weight of each stained protein was calculated by comparison of the protein's gel position to a standard curve constructed from the molecular weight standards.

Immunoblots and sIgE

Autoradiography

Immunoblots were accomplished as previously described.^{17,23,25} In short, tree extract proteins were transferred from unstained SDS-PAGE gels to nitrocellulose membranes. After transfer, the molecular weight standards were cut from the nitrocellulose membrane and stained with Coomassie Blue. The nitrocellulose membranes were cut into individual strips, washed, blocked, and incubated overnight at room temperature either in (1) a 1:6000 dilution of mountain cedar monoclonal antibody MC2 (or control monoclonal) in 10% fetal calf serum or in (2) the hu-

man MC high-titer pooled serum (or human cord serum control), diluted 1:10 in 10% fetal calf serum. Monoclonal assay membranes were incubated overnight in alkaline phosphatase conjugated goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:2000 in 10% fetal calf serum and were subsequently developed. Detection of human MC sIgE on immunoblot membranes utilized autoradiography. Following incubation with MC high-titer sIgE, strips were incubated 24 hours with ¹²⁵I-labeled antihuman IgE. After washing, the strips were loaded into a Kodak X-Omatic film-containing cassette (Eastman Kodak Co, Rochester, NY). The cassette was sealed to prevent evaporation and stored for seven days at -70°C before development. Results were quantitated using a densitometer. Immunoblot inhibition was carried out as above except that diluted MC high-titer pooled serum was first pre-incubated overnight with 1.0 mg of MC protein/mL.

RESULTS

Table 2 lists the skin test results. There were no positive responses to either the Pinaceae (*C. deodora*) or to the angiosperm (*T. gallica*). The remaining conifers elicited positive responses from most patients. Ten of ten patients were positive to eight trees, all *Juniperus* species. Nine of ten were positive to *J. occidentalis*. Other Cupressaceae genera elicited positive tests in eight of ten (*L. decurrens* and *C. lawsonia*) or nine of ten patients (*C. arizonica*). The Taxodiaceae, *C. japonica*, elicited a wheal and flare response in nine of ten patients. The nonatopic control did not react to any tree tested.

Figure 2 depicts the double immunodiffusion results. The center well of each plate contained rabbit antisera; the six surrounding wells contained MC antigen, alternating with other study trees. Within the Cupressaceae family, lines of identity formed with all *Juniperus* species (1 to 9); lines of partial identity formed with trees of other Cupressaceae genera (10 to 12). *C. japonica* (13), of the Taxodiaceae

Table 2. Prick Skin Test Results for Ten Mountain Cedar-Allergic Patients

Tree	Patient Number									
	1	2	3	4	5	6	7	8	9	10
<i>J. ashei</i>	+	+	+	+	+	+	+	+	+	+
<i>J. californica</i>	+	+	+	+	+	+	+	+	+	+
<i>J. chinensis</i>	+	+	+	+	+	+	+	+	+	+
<i>J. scapularum</i>	+	+	+	+	+	+	+	+	+	+
<i>J. osteosperma</i>	+	+	+	+	+	+	+	+	+	+
<i>J. monosperma</i>	+	+	+	+	+	+	+	+	+	+
<i>J. pinchotti</i>	+	+	+	+	+	+	+	+	+	+
<i>J. occidentalis</i>	+	+	0	+	+	+	+	+	+	+
<i>J. virginiana</i>	+	+	+	+	+	+	+	+	+	+
<i>L. decurrens</i>	+	+	0	+	+	+	+	+	+	0
<i>C. arizonica</i>	+	+	+	+	+	+	+	+	+	0
<i>C. lawsonia</i>	+	0	+	+	0	+	+	+	+	+
<i>C. japonica</i>	+	+	0	+	+	+	+	+	+	+
<i>C. deodora</i>	0	0	0	0	0	0	0	0	0	0
<i>T. gallica</i>	0	0	0	0	0	0	0	0	0	0

+ = positive skin test and 0 = negative skin test; a control patient was negative for all 15 trees.

family, demonstrated partial identity with MC. No lines of identity or partial identity formed between MC and the Pinaceae (14) or the angiosperm (15).

Polyacrilamide gel electrophoresis of the 15 trees demonstrated proteins ranging from less than 14 kD to 94 kD, with graded densities (data not shown). All 12 Cupressaceae contained a dense protein band at 40 to 42 kD and Japanese cedar (*C. japonica*) at 43 to 46 kD. This density was most uniform among the Juniperus species. The Pinaceae and angiosperm contained no prominent bands at these molecular weights.

IgE immunoblot autoradiography results are depicted in Figs 3 and 4. Immunoblots a, b, and c for each tree display the results of uninhibited human MC-hypersensitive sera, sera inhibited by MC pollen protein, and con-

trol (human cord) sera, respectively. Trees are numbered from 1 to 15 as in Table 1. The MC high-titer sIgE bound tree extract proteins primarily within the 30 to 94 kD molecular weight range. For all trees, bound IgE was completely inhibited by preincubation of sera with MC pollen extract. The most radiodense and most significant binding to MC and the other Cupressaceae occurred at 40 to 42 kD, with a parallel band in Japanese cedar (*C. japonica*) at 43 to 46 kD. A band of variable density was also present in all Cupressaceae and in the Japanese cedar at approximately 74 kD. Surprisingly, several proteins of the Pinaceae and angiosperm were also bound by pooled MC specific-sera, albeit with much less intensity. No binding was demonstrable with cord blood.

Monoclonal immunoblots showed strong murine monoclonal MC2 binding to only the 40 kD protein band in each of the Juniperus species (1 to 9) and *C. arizonica* (11), and weak binding to the 40 kD band in *L. decurrens* (10) (data not shown). Strong binding of MC2 was seen to a single 46 kD band in *C. japonica* (13). Monoclonal MC2 bound no protein bands in *C. lawsonia* (12), the Pinaceae (14), or the angiosperm (15). The irrelevant control monoclonal bound no proteins in any tree pollen extract.

DISCUSSION

Our immunodiffusion and skin test results, demonstrating cross-reactivity among species of the Cupressaceae family, parallel and extend those of Yoo et al.¹¹ Mountain cedar antisera

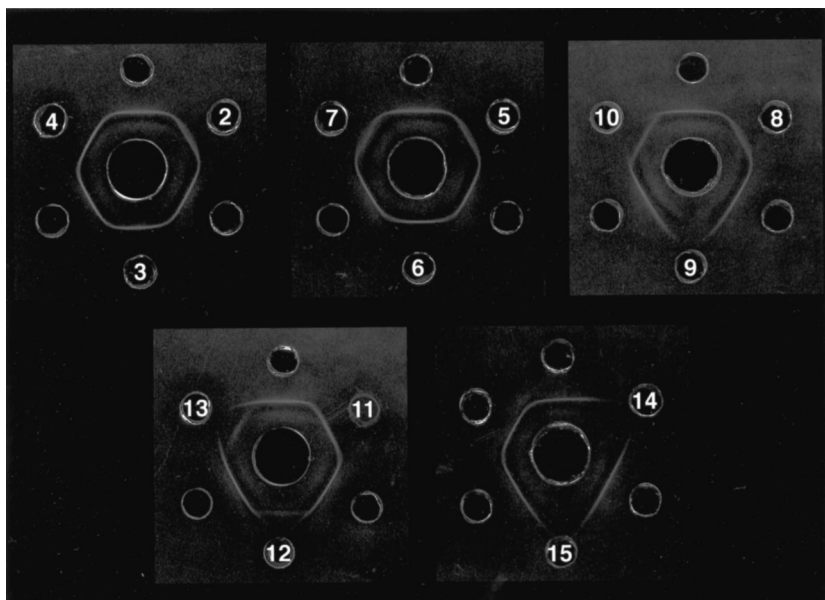


Figure 2. Double immunodiffusion results. Five plates: each center well contains rabbit MC antiserum; surrounding wells contain either a study tree pollen extract (see Table 1 for numbering) or MC pollen extract (in the three unnumbered wells).

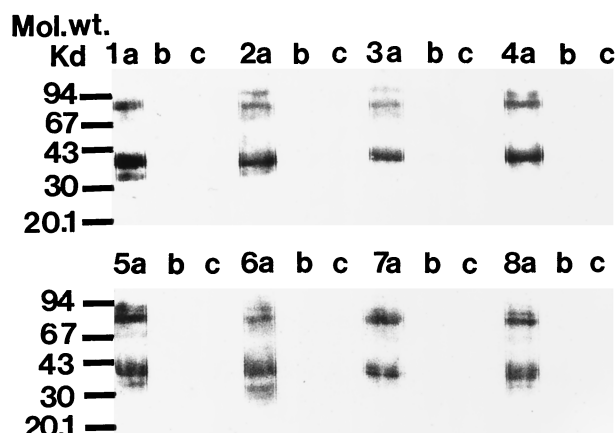


Figure 3. Human IgE immunoblots. Autoradiographic detection of IgE bound to tree pollen extract proteins following SDS-PAGE. Trees are numbered as in Table 1 (1. *Juniperus ashei*, 2. *Juniperus californica*, 3. *Juniperus chinensis*, 4. *Juniperus scapulorum*, 5. *Juniperus osteosperma*, 6. *Juniperus monosperma*, 7. *Juniperus pinchotti*, and 8. *Juniperus occidentalis*). Lane "a" is uninhibited human MC-hypersensitive sera; lane "b" is sera inhibited by mountain cedar extract; and lane "c" is human cord sera control.

strongly recognized all the *Juniperus* species, as well as partially recognizing the other Cupressaceae genera and the one Taxodiaceae species tested. These immunodiffusion results validate current plant taxonomy. In a closely parallel manner, 80% to 100% of MC-allergic patients were skin test

positive to each of Cupressaceae trees, and to the Taxodiaceae (*C. japonica*). Immunodiffusion and skin tests were uniformly negative to the Pinaceae and the angiosperm, suggesting antigenic dissimilarity. The skin test results are notable since the pollens tested are quite geographically diverse,²⁶ allow-

ing little chance of patient exposure to many or all species.

Immunoblots corroborated the skin test and immunodiffusion cross-reactivity results, but with more detailed and discriminating comparisons. The IgE of individuals sensitized to MC pollen is directed against a spectrum of pollen proteins. The densest IgE binding bands in MC pollen were at 40, 74, and 36 kD. The 40 and 74 kD allergen bands of MC were closely mimicked in all the Cupressaceae trees and in Japanese cedar, where the 43 to 46 kD band is most probably the major allergen Cry j I.²⁷ The monoclonal immunoblot results confirmed the antigenic similarity of epitopes on the 40 to 42 kD Cupressaceae and 43 to 46 kD Japanese cedar allergens. As with the immunodiffusion and skin test finding, these results suggest a strong clinical allergen cross-reactivity among the Cupressaceae trees and Japanese cedar. A parallel to the MC 36 kD allergen was less frequently evident in the Cupressaceae immunoblots, but was present in Japanese cedar and is most probably the 37 kD second major allergen, Cry j II.²⁸ Interestingly, gp 74, though faint on SDS-PAGE (data not shown), demonstrated significant IgE binding on immunoblotting, particularly among the junipers.

IgE immunoblotting was sensitive enough to detect minor cross-reactivity of MC with proteins of both the Pinaceae and the angiosperm (not seen in the monoclonal immunoblot). This is not an unexpected result, given reports of limited immunologic similarities between divergent species.^{29,30} Given the negative skin test results to these two trees, it is unclear whether the autoradiography results would translate to clinical allergy.

The cross-reactivity results of this study are in concert with previous reports.⁹⁻¹⁴ The 42 to 43 kD proteins of *C. semipervens* and *C. arizonica* have been shown to be both major and cross-reactive allergens.¹⁴ Cross-reactivity between *C. japonica* and *C. semipervens* has been demonstrated and confirmed using monoclonal antibodies specific for *C. japonica* major

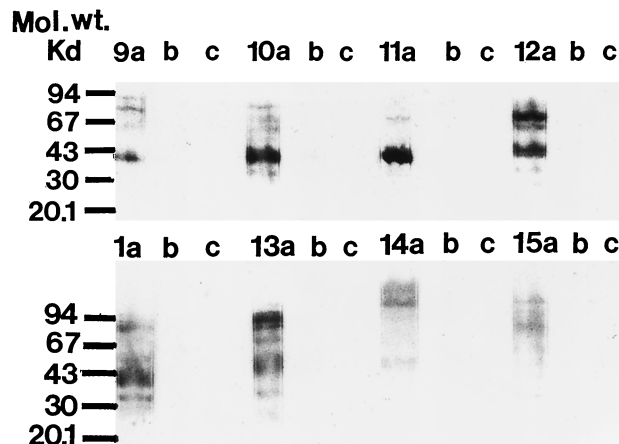


Figure 4. Human IgE immunoblots. Autoradiographic detection of IgE bound to tree pollen extract proteins following SDS-PAGE. Trees are numbered as in Table I (1. *Juniperus ashei* or mountain cedar, 9. *Juniperus virginiana*, 10. *Libocedrus decurrens*, 11. *Cupressus arizonica*, 12. *Chamaecyparis lawsonia*, 13. *Cryptomeria japonica*, 14. *Cedrus deodora*, and 15. *Tamarix gallica*). Lane "a" is uninhibited human MC-hypersensitive sera; lane "b" is sera inhibited by mountain cedar extract; and lane "c" is human cord sera control.

allergens.^{12,31} The current study strengthens the conclusion that the major Japanese cedar allergens Cry j I and Cry j II may represent proteins homologous to major Cupressaceae allergens of molecular weights 40 to 42 kD and 36 kD. Furthermore, both Cry j I and the MC major allergen contain identical N-terminal 5 amino acid residues.^{16,31} It is interesting to speculate that an identical N-terminal sequence would be found for other Cupressaceae 40 to 42 kD allergens.

The use of taxonomy to judge the probability of cross-reactivity depends on two assumptions. First, that closely related plants have more shared antigens. Second, that standard botanical classification actually reflects phylogeny, ie, two plants in the same genus had a common ancestor, two in the same family a more distant ancestor and so on. Two plants in the same genus should, therefore, share most antigens while two plants in the same family should share a lesser number and two in the same order fewer still.³² Our results support the suggestion of others,¹² that a close relationship exists between the Taxodiaceae and Cupressaceae families, with the Pinaceae family being more distantly related.

Study of the cross-reactivity of Cupressaceae is useful for the following reasons. First, the significance of Cupressaceae pollinosis is growing. Since 1986 a number of articles regarding Cupressaceae allergy have appeared.^{1,2,12-14,19} Increased exposure is due to reforestation efforts and widespread landscaping using nonindigenous species. A notable increase in Japanese cedar pollinosis may be partly due to increases in air pollution.⁴ Epidemiologic data shows a higher incidence of symptomatic patients living near major highways, even though they are not exposed to higher pollen counts. Muranaka et al demonstrated an adjuvant effect of diesel exhaust fumes on Japanese cedar pollen hypersensitivity in mice.³³ Second, knowledge of cross-reactive structures in different allergen sources may contribute to the understanding of clinically relevant cross-reactivity phenomena. Lastly, knowledge of cross-reactivity allows for the development of specific molecules for a component-based type of immunotherapy. Among Juniper and *C. japonica* allergic patients it may be possible to standardize one extract for testing and immunotherapy purposes.

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