

Cypress pollen allergy. Identification of allergens and crossreactivity between divergent species

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Summary

Studies employing sera from 34 subjects allergic to white cypress pine (*Callitris glaucophylla*) pollen identified 18 IgE antibody-binding components in the pollen of this species, five of which (MWs ~94, 68, 64, 43 and 34 kDa) were recognized by all of the sera. Protein blotting and quantitative inhibition studies revealed clear cross-reactivity between *C. glaucophylla* and *Cupressus sempervirens* pollen proteins and striking similarities in the IgE recognition band patterns of the two pollens. Inhibition experiments with other pollen extracts revealed that sera from *C. glaucophylla* pollen-allergic subjects can be divided into two groups — those inhibited only by extracts from the two Cupressaceae pollens and those inhibited both by these pollen proteins and by pollen extracts from other species. Most of the crossreactions in the latter group cannot be explained on the basis of taxonomic relationships or separate sensitizations. As with previous studies on birch and olive pollens, we conclude that pollen allergenic crossreactivity is much more wide-ranging than generally believed.

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Introduction

Despite early studies which implicated cypress pollens in some allergic respiratory reactions [1–3], the allergenicity of pollens from the Cupressaceae family and their role in inhalant allergies have only recently been intensively examined. In studies on two species of pollens, *Callitris glaucophylla* and *Cupressus sempervirens* from two different genera of the Cupressaceae, we demonstrated clear clinical involvement of the pollens and their IgE antibody-binding properties and blotting studies enabled us to define the allergenic spectrum of the latter species [4,5].

C. glaucophylla, commonly called white cypress, or Murray, pine [6,7] (Fig. 1a) is indigenous to Australia. The species has evolved in geographical isolation from other Cupressaceae and is well adapted to the harsher conditions of some of the inland areas. White cypress pine is a small to medium sized tree with a characteristic

cone (Fig. 1b). Its pollen, which is produced in abundance, may be distinguished from *C. sempervirens* pollen by its small size (Fig. 1c).

After identifying *C. glaucophylla* pollen as an important seasonal allergen source [4], it became important for management of patients to determine their responses to other Cupressaceae pollens (Fig. 2), particularly the widely distributed species *C. sempervirens* or Italian cypress. In undertaking this study we set out to define the allergenic spectrum of *C. glaucophylla* pollen and investigate its allergenic crossreactivity with *C. sempervirens* pollen. Results of these investigations are reported here.

Materials and methods

Pollen extracts

Dry *C. glaucophylla* pollen was collected at Dunedoo, western New South Wales, Australia (lot 3C06SE). Pollen (5 g) were extracted in distilled water (50 ml) at 4°C overnight, centrifuged (17 300 $g \times 60$ min) at 4°C and the supernatant dialysed using Spectrapor Membrane

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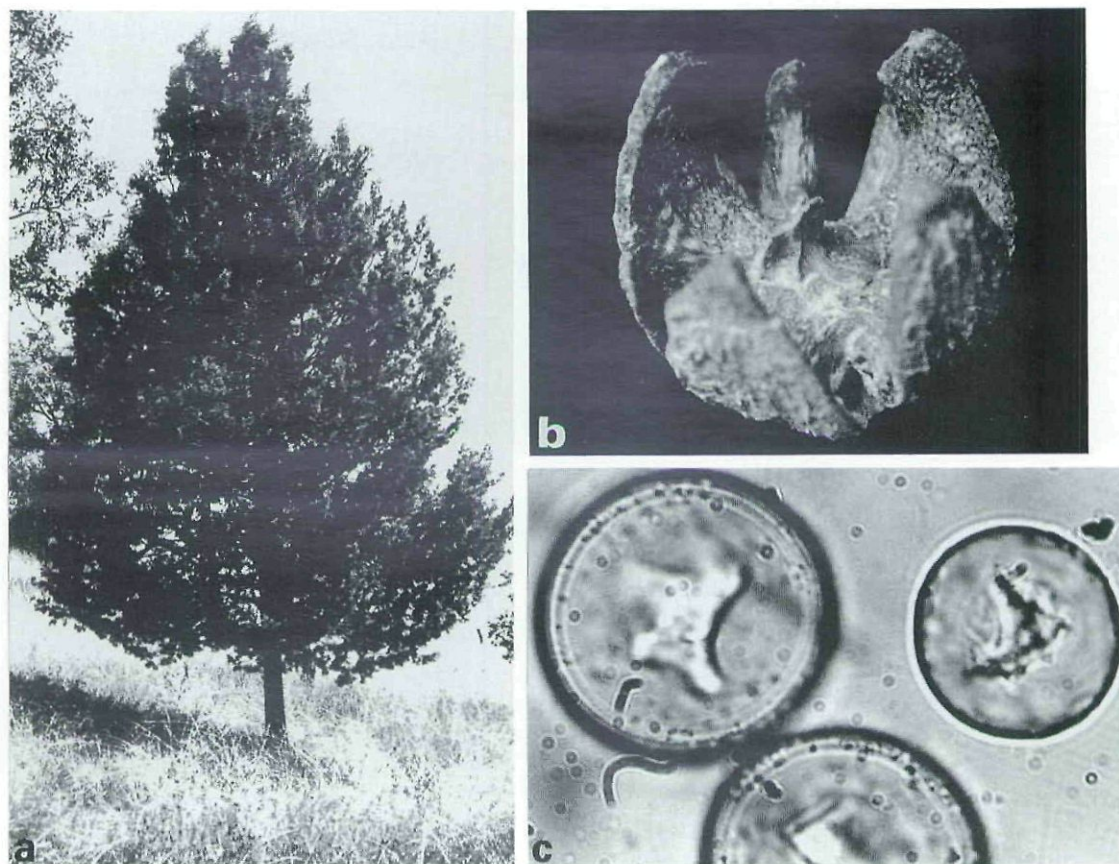


Fig. 1. (a) White cypress pine, *Callitris glaucophylla*. (b) Cone of *C. glaucophylla* with its characteristic central columella about 5–7 mm long. (c) photomicrograph of *C. glaucophylla* pollen grain (right), diameter $\sim 20\ \mu\text{m}$ and *Cupressus sempervirens* pollen grains (left), diameter $\sim 32\ \mu\text{m}$. Magnification $\times 2000$.

Tubing (Spectrum Medical Industry Inc., Los Angeles, USA, mol. wt. cut off 2000 Da) at 4°C overnight against distilled water and freeze-dried. The protein content of the dialysed extract was determined with the Pierce BCA* (Bicinchoninic acid) Protein Assay Reagent [8], and found to be 6.2% w/w. The lyophilized material was stored at 4°C . *C. sempervirens* pollen was obtained and extracted as previously described [5].

Subjects, duration of pollen season and symptoms

For Australian subjects from western NSW with hay fever, symptom scores correlated with counts of *C. glaucophylla* pollen. These subjects generally resided on farm properties where, apart from *C. glaucophylla*, no other species of *Cupressaceae* were growing. Pollination of *C. glaucophylla* commenced in late July, reached over 50 grains/ m^3 in early August and peaked at approximately 8000 grains/ m^3 during the third week of August. Levels then continued at over 3000 grains/ m^3 until mid September when only occasional grains were seen following heavy rain.

Patients sensitive to *C. sempervirens* also tended to be grass pollen-sensitive and with this group symptom scores reflecting rhinitis were more prolonged.

Sera

Serum samples were collected in the Dubbo and Dunedoo districts of western NSW from patients who had a case history of allergy to white cypress pine pollen allergens and a positive skin-prick test to *C. glaucophylla* allergen extract. Sera from subjects allergic to *C. sempervirens* pollen were from Provence, France and Canberra, Australia [5]. Sera were also obtained at Royal North Shore Hospital (RNSH), Sydney, Australia, from subjects allergic to pollens but not white cypress pine pollen and from subjects allergic to foods or house dust mites (*Dermatophagoides pteronyssinus*). Other control sera were obtained from non-allergic subjects with high total serum IgE levels and normal non-allergic laboratory workers. Cord serum was provided by the Department of Obstetrics, RNSH, and used as an IgE-‘free’ control.

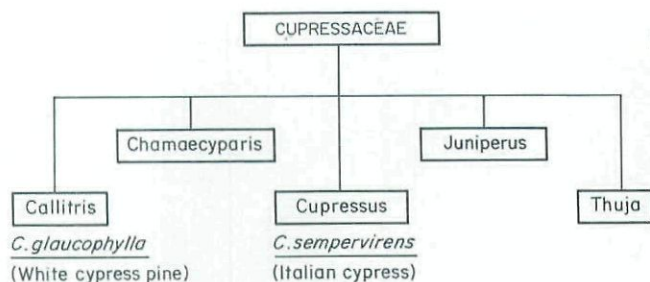


Fig. 2. Scheme showing botanical relationship between white cypress pine *C. glaucophylla* and Italian cypress *C. sempervirens*.

Tests for pollen-reactive IgE antibodies

Sera were tested for *C. glaucophylla* and *C. sempervirens* pollen-reactive IgE-antibodies in assays using 5 mm activated CNBr-discs prepared by reaction with 20 µg pollen proteins per disc [9]. Since previous studies showed that IgE-binding of *C. sempervirens* pollen proteins was increased following reduction of the proteins [5] prior to coupling, *C. sempervirens* pollen extract was reduced with mercaptoethanol. Bound IgE antibodies were detected by the addition of ^{125} I-anti-human IgE obtained from Bioclone Australian Pty Limited, Marrickville, NSW, Australia.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE [10] was carried out as previously described [11]. Pollen proteins in polyacrylamide gels were stained with Coomassie Brilliant Blue (CBB) and silver nitrate [12]. For electroblotting experiments, *C. glaucophylla* pollen extract (1 mg protein/gel) and *C. sempervirens* pollen extract (1.2 mg protein/gel) previously treated with 2-mercaptoethanol were subjected to electrophoresis at 170 V for about 18 h. Low molecular weight (mol. wt.) standards (Pharmacia, Uppsala, Sweden) were used to determine the approximate mol. wt. of the pollen components.

Electrophoretic transfer of proteins to nitrocellulose membrane

Materials and methods for electrotransfer of separated proteins to nitrocellulose were as previously described [13,14] except that transfer times were extended to 2 h and the voltage employed was 60 V. After transfer, membranes were washed for 5 min in 10% methanol in phosphate-buffered saline (PBS) for 1 1/2 h at room temperature to block unbound sites of the membranes.

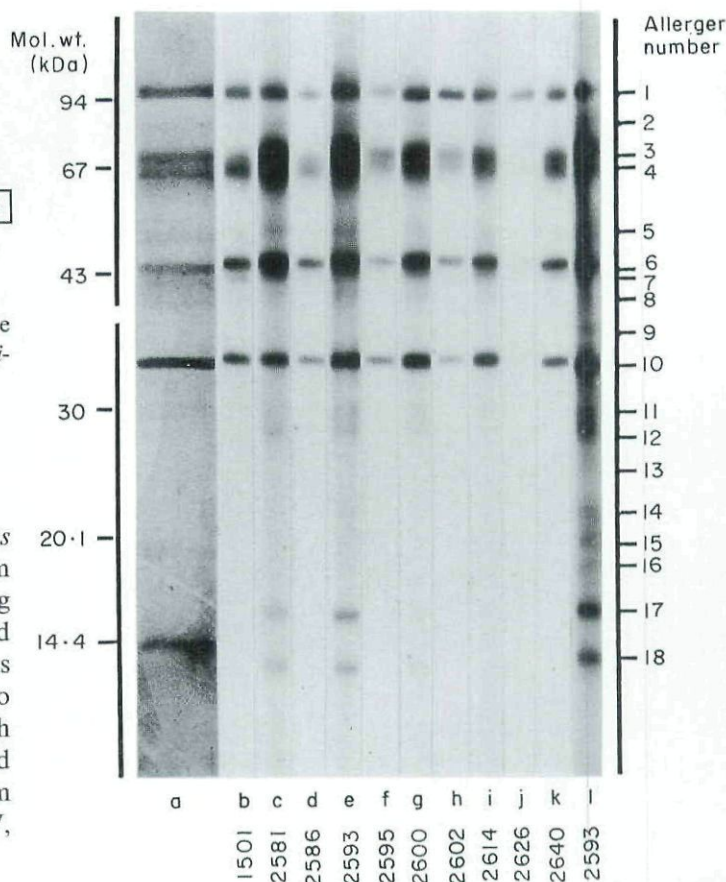


Fig. 3. Protein staining and IgE antibody-binding patterns of blotted *C. glaucophylla* pollen proteins separated by SDS-PAGE. Strip a: *C. glaucophylla* dialysed and reduced pollen extract separated by SDS-PAGE and silver stained. Strips b–k: autoradiographs of blotted pollen proteins on nitrocellulose strips incubated with 10 different sera from subjects allergic to *C. glaucophylla* pollen followed by ^{125} I-anti-human IgE. Strip l: autoradiograph of blotted strip incubated with same serum used with strip e but subjected to a longer period (14 days compared with 7 days for strips b–k) of autoradiography. Sera used for blotting (see Fig. 4): strip b, 1501; c, 2581; d, 2586; e, 2593; f, 2595; g, 2600; h, 2602; i, 2614; j, 2626; k, 2640; l, 2593.

Transferred proteins were detected with Amido black (Sigma Chemical Co., Missouri, USA).

Probing of nitrocellulose strips and autoradiography

Materials and methods for identification, by autoradiography, of IgE-binding proteins were as previously described [15]. Prior to probing with sera, strips were fixed with 0.5% glutaraldehyde (1:50 of 25% solution) in PBS for 15 min, followed by a 30 s wash with PBS, and then incubation in 1% ethanolamine in PBS for 1 h. Detection of IgE-binding proteins was achieved by the addition of ^{125} I-anti-human-IgE (Bioclone).

Patient no.	<i>C. glaucophylla</i> allergen fractions and estimated mol.wt (kDa)																	
	1 94	2 82	3 68	4 64	5 50	6 43	7 42	8 39	9 36.5	10 34	11 30	12 28	13 26	14 22	15 20	16 18.5	17 16	18 14
1501	•	•	•	•	•	•	•	•		•	•							
1942	•		•	•		•				•								
1951	•		•	•	•	•				•								
2174	•		•	•		•				•							•	
2579	•		•	•		•				•								
2581	•		•	•	•	•	•	•	•	•	•	•					•	•
2582	•		•	•		•				•								
2586	•	•	•	•	•	•	•			•								
2587	•		•	•		•				•								
2588	•		•	•	•	•				•								
2589	•		•	•	•	•				•	•	•					•	•
2591	•		•	•		•				•								
2593	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
2595	•		•	•		•	•			•								
2596	•	•	•	•	•	•				•	•	•	•				•	•
2599	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•
2600	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•
2602	•		•	•	•	•	•			•								
2603	•		•	•		•				•								
2606	•		•	•		•				•								
2607	•		•	•	•	•	•	•		•	•							
2608	•		•	•		•				•								
2610	•		•	•		•				•								
2613	•		•	•		•				•								
2614	•	•	•	•	•	•	•	•		•	•	•					•	•
2616	•		•	•		•				•								
2620	•		•	•		•				•								
2626	•		•	•		•				•								
2637	•		•	•	•	•				•								
2640	•	•	•	•	•	•	•	•		•	•						•	•
2643	•		•	•		•				•								
2644	•		•	•		•				•								
2659	•		•	•		•				•								
2714	•		•	•		•				•		•					•	•

Fig. 4. Patients' sera ($n=34$) showing reactions of each with the identified IgE antibody-binding components obtained by transfer of SDS-PAGE-separated *C. glaucophylla* pollen extract.

Results

Qualitative electrophoretic definition of *C. glaucophylla* pollen proteins and identification of IgE-binding components

Silver staining following SDS-PAGE of a post-dialysis,

reduced extract of *C. glaucophylla* pollen, revealed 17 bands, many of them lightly stained, in the mol. wt. range 14–94 kDa. The band pattern was distinguished by six prominently stained bands at approximate mol. wt. regions of 94, 68, 64, 43, 34 and 14 kDa (Fig. 3, strip a).

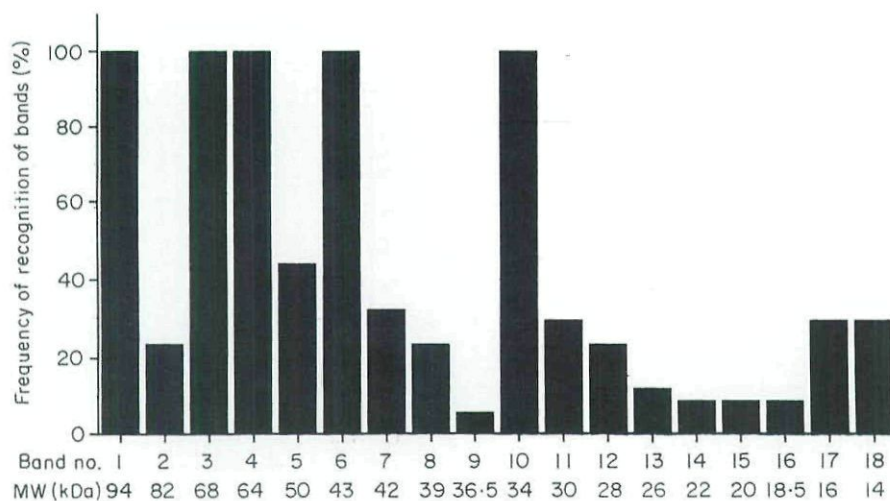


Fig. 5. Histogram showing frequencies of recognition of the IgE antibody-binding components detected in *C. glaucophylla* pollen extract. Frequencies were calculated from results obtained with 34 IgE-binding sera from subjects allergic to *C. glaucophylla* pollen (see Fig. 4).

Employment of sera from 34 patients diagnosed as clinically allergic to white cypress pine pollen with nitrocellulose blots of the pollen proteins revealed 18 IgE-binding bands (Fig. 3b-l; Fig. 4). IgE-binding bands 9 (mol. wt. 36.5 kDa) and 13 (26 kDa), were not detected

on protein-stained gels or blots while a silver-stained band of ~62 kDa did not bind IgE.

All of the sera that proved positive in the blotting study reacted with a least five of the *C. glaucophylla* pollen protein bands viz. numbers 1, 3, 4, 6 and 10 of

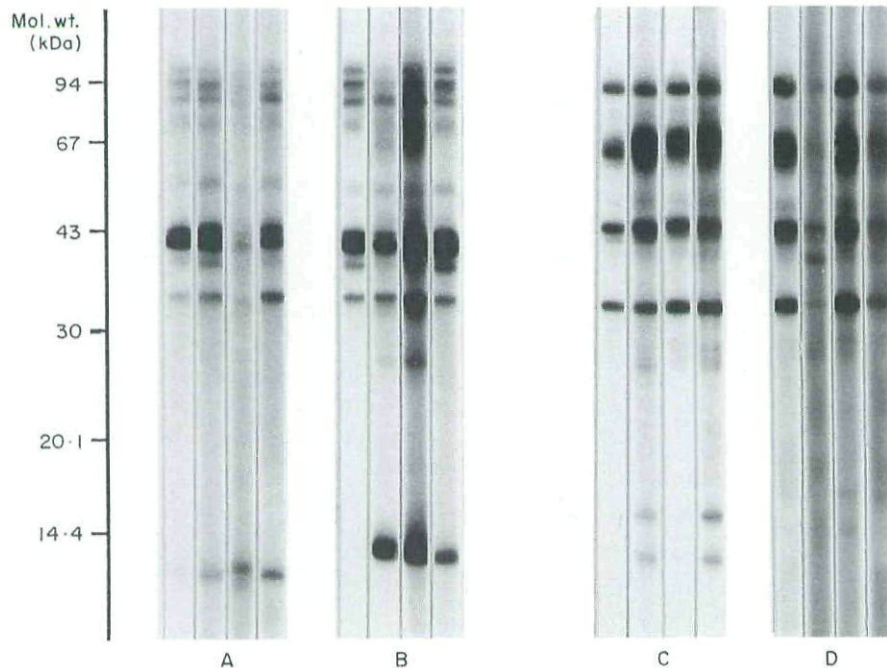


Fig. 6. Protein blots of *C. glaucophylla* and *C. sempervirens* pollen extracts showing similar IgE-binding patterns. A. Strips electroblotted with *C. sempervirens* pollen proteins and probed with sera from four different subjects allergic to this pollen. B. Strips with *C. sempervirens* pollen proteins and probed with sera 2586, 2589, 2596 and 2602 respectively from subjects allergic to *C. glaucophylla* pollen. C. Strips with *C. glaucophylla* pollen proteins and probed with sera from subjects 1501, 2581, 2600 and 2593. D. Strips with *C. glaucophylla* pollen proteins and probed with the same four sera shown in A.

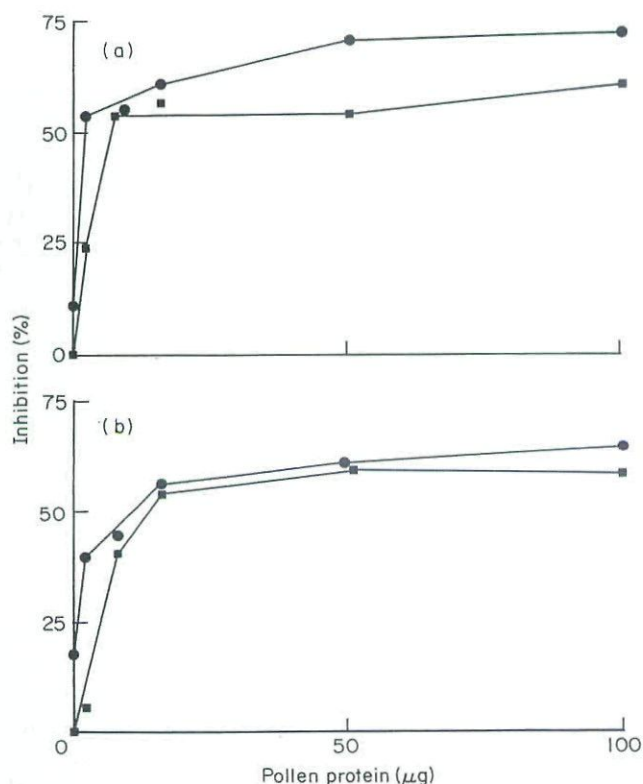


Fig. 7. RAST inhibition results with serum from subject 2593 allergic to *C. glaucophylla* pollen. *C. glaucophylla* (a) and *C. sempervirens* (b) pollen proteins were adsorbed to nitrocellulose discs before addition to diluted serum preincubated with *C. glaucophylla* (●) or *C. sempervirens* (■) pollen proteins.

approximate mol. wt. 94, 68, 64, 43 and 34 kDa respectively (Fig. 3b–l, Fig. 4). These components generally also bound the greatest quantity of IgE as evidenced by the depth of staining on the autoradiographs, although some of the bands were only faintly visible with a few of the sera (see for example Fig. 3, strip j). The 100% recognition frequencies of bands 1, 3, 4, 6 and 10 by the 34 different sera reinforced the conclusion that these components are the dominant allergenic proteins in *C. glaucophylla* pollen (Figs 4 and 5). This finding also highlights the marked homogeneous response to the spectrum of *C. glaucophylla* pollen allergens by the patient group. Bands 17 and 18 of estimated mol. wt., 16 and 14 kDa respectively was another combination of components always recognized together. Along with bands 5 (mol. wt. ~50 kDa), 7 (~42 kDa) and 11 (~30 kDa) these were the only pollen proteins recognized by 30% or more of the sera (Fig. 5). Binding of IgE antibodies by components 2, 5, 7, 8, 9, 11, 12, 13, 14, 15 and 16 were not only moderate to low in frequency but generally, also weak in intensity (Fig. 3).

Table 1. RAST inhibition studies demonstrating different allergen reactivity patterns of sera from *C. glaucophylla* pollen-allergic subjects

Pollen protein extract from	Inhibition (%)* of IgE binding to <i>C. glaucophylla</i> pollen protein-solid phase by 50 μg of pollen proteins with serum:	
	2603†	2593‡
<i>C. glaucophylla</i>	84	70
<i>C. sempervirens</i>	66	54
<i>Lolium perenne</i>	0	56
<i>Cynodon dactylon</i>	5	59
<i>Plantago lanceolata</i>	5	48
<i>Olea europea</i>	0	60

* Numbers in the columns are the percentage inhibition values obtained in experiments measuring uptakes by the allergen discs of ^{125} I-anti-human IgE, and hence IgE, in the subject's serum.

† Serum showing 'simple' recognition pattern of only five IgE-binding bands on *C. glaucophylla* pollen protein blots (see Fig. 4). Serum used at a dilution of 1–12.

‡ Serum showing more complex recognition pattern (18 IgE-binding bands) on blots (Fig. 4). Serum used at a dilution of 1–6.

No binding of anti-IgE (and hence of IgE antibodies) was observed when normal or cord sera or sera from subjects allergic to house dust mites were employed in the protein blotting studies.

Crossreactivity between *C. glaucophylla* and *C. sempervirens* pollen proteins

Protein blotting studies Probing of blots of pollen proteins from the two different cypress species with sera from subjects diagnosed as allergic to *C. glaucophylla* or *C. sempervirens* pollen, revealed striking similarities in the IgE reaction patterns. Fig. 6A and B shows that the reactive band patterns on the electrophoretograms of *C. sempervirens* pollen were almost identical regardless of the sera used for probing. Likewise, the *C. glaucophylla* blots probed with sera from *C. glaucophylla* or *C. sempervirens*-allergic subjects produced autoradiographs that looked strikingly similar (Fig. 6C and D).

Quantitative inhibition studies RAST inhibition experiments with a number of the sera from subjects allergic to *C. glaucophylla* pollen clearly showed that extracts of this pollen, and *C. sempervirens* pollen, inhibited IgE antibody-binding to solid phases prepared from both

pollens. Figure 7 shows results obtained with one of the sera, number 2593 which recognized all 18 IgE-binding *C. glaucophylla* bands (Figs 3 and 4).

Inclusion of other pollens as controls in the inhibition experiments revealed that the sera from *C. glaucophylla* pollen-allergic subjects could be divided into two groups. IgE antibodies in some of the sera, for example serum from subject 2603, were found to be inhibited only by extracts from the two Cupressaceae pollens studied and not by extracts from other tree pollens or pollens from grasses or weeds (Table 1). Sera in this group showed the simplest *C. glaucophylla* allergen reaction profiles with only allergen numbers 1, 3, 4, 6 and 10 being recognized on blots (Fig. 4). Other sera, however, for example serum 2593 showed inhibition profiles in which all of the 'control' pollens tested significantly inhibited IgE antibody binding to the *C. glaucophylla* (or *C. sempervirens*, data not shown) pollen protein solid phase (Table 1). The allergen recognition spectra of these sera proved to be more complex with up to 18 allergens (as with serum 2593) detected on blots (Fig. 4).

Discussion

Results of studies designed to investigate the IgE antibody-binding properties of white cypress pine pollen have confirmed the allergenicity of this species of pollen which was recently implicated as an important seasonal allergen source in parts of rural Australia [4]. In an effort to study and compare reactions to this pollen with reactions to the much more widely distributed Cupressaceae species *C. sempervirens*, direct antibody binding and inhibition experiments were undertaken with sera from allergic subjects sensitized to *C. glaucophylla* or *C. sempervirens* pollen. These experiments revealed striking similarities in the allergenic profiles of the two different pollens and in the IgE antibody recognition spectra of some of the patients' sera. Of 18 different IgE-binding components in *C. glaucophylla* pollen, five bands of mol. wt. approximately 94, 68, 64, 43 and 34 kDa dominate the profile. Two of these, components of mol. wt. ~34 and ~43 kDa appear to have counterparts in *C. sempervirens* pollen. In a recent electroblotting study of *C. sempervirens* pollen, we identified major IgE-binding components with mol. wt. ~42 and ~36 kDa and drew attention to the possibility of a further band, or bands, with mol. wt. ~42 kDa [5]. In the present study, heavy banding on the autoradiographs suggests the existence in both pollen species of at least two IgE-binding components in the mol. wt. region ~42–43 kDa. Inhibition of IgE antibody binding demonstrated clear crossreactivity between the two different Cupressaceae pollen species, a finding relevant to any

allergen avoidance strategy especially for *C. glaucophylla* pollen-allergic subjects in rural areas.

Of particular interest were results with a battery of allergenic extracts from grass, weed and tree pollens all of which clearly inhibited the binding of *C. glaucophylla*-reactive IgE antibodies in some sera to *C. glaucophylla* and *C. sempervirens* solid phases. This finding supports our previous conclusion formed from analyses of IgE reactivity patterns of sera from birch (*Betula pendula*) and olive (*Olea europea*) pollen-allergic subjects that pollen allergenic crossreactivity is much more wide-ranging than is generally believed [16,17]. In addition to the inhibition results presented here, direct binding, protein blotting and adsorption and elution studies (Pham & Baldo, in preparation) all demonstrated that the *C. glaucophylla* pollen-reactive IgE antibodies in some patients' sera (that is patients in the group represented by subject 2593 described in Table 1) reacted with pollens from *C. sempervirens*, birch and olive trees, ragweed, plantain, parietaria, lamb's quarter, couch grass, cocksfoot and ryegrass. Most of these crossreactions cannot be explained on the basis of taxonomic relatedness and separate sensitization to each of the different species was either not involved or, at most, extremely unlikely. From detailed analyses of IgE-reactivity patterns of birch pollen-allergic and olive pollen-allergic subjects, we concluded that pollen-sensitivities in some subjects resulted from exposure to allergens, probably from grass pollens, that crossreact with birch or olive pollen proteins [16,17]. Crossreactions observed with some sera in this study (for example 2593) appear to have a similar basis. The molecular basis of such broad pollen cross-reactivity needs to be elucidated.

Acknowledgements

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