

Podocarpus gracilior and *Callitris verrucosa*—newly identified allergens that crossreact with *Cupressus sempervirens*

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Summary

Thirty-six symptomatic patients, with positive skin reactions to *Cupressus sempervirens* pollen extract were skin-tested with pollen extracts of *Podocarpus gracilior* and *Callitris verrucosa*, of these 17 (47%) had positive responses to *P. gracilior*, nine (25%) to *C. verrucosa*, and six (17%) to both. None of the non-atopic healthy controls had positive reactions to either of the extracts. Radioallergosorbent test (RAST)-inhibition studies were performed with pooled sera from three patients. Fifty per cent inhibition was obtained with 11 µg protein of *C. sempervirens*, 54 µg of *P. gracilior*, and 71 µg of *C. verrucosa*; however, when pollen extract of *Olea europaea*, an unrelated allergen, was tested, 265 µg protein were needed to obtain 50% inhibition. One-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of pollen extracts from the three species revealed that they had several very similar protein bands. Using Western blot analysis, several closely related IgE binding proteins were identified in the three species. It was concluded that the pollen grains of *P. gracilior* and of *C. verrucosa* are potentially allergenic. The presence of common allergenic proteins indicate partial crossreactivity with *C. sempervirens*.

Clinical and Experimental Allergy, Vol. 25, pp. 456–460. Submitted 30 December 1992; revised 24 June 1994; accepted 1 December 1994.

Introduction

Cupressus sempervirens is one of the most important aeroallergens in the Mediterranean area [1,2]. This tree is commonly used for afforestation and ornamental purposes. Increased awareness of its allergenic properties has stimulated a search for substitutes for those that are planted today, especially in populated areas.

The two conifers *Podocarpus gracilior* and *Callitris verrucosa* have been considered as potential substitutes for *Cupressus sempervirens* because of their similar horticultural qualities and because neither has been reported to be allergenic [3,4]. *Cupressus sempervirens* and *C. verrucosa* belong to the family Cupressaceae, *P. gracilior* to the closely related family Podocarpaceae [5].

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All three species have similar patterns of pollination and pollen dispersal, with similar pollen size and weight. Since crossreactivity is known to occur among various Cupressaceae [3,4,6–8] and among other coniferae [7,9], we examined the allergenicity of *P. gracilior*, *C. verrucosa*, and *C. sempervirens*, and their possible crossreactivity.

Materials and methods

Extract preparation for skin testing

Pollen extracts of *P. gracilior*, *C. verrucosa*, and *C. sempervirens* were prepared as previously described [10,11] by sonication of the pollen in Coca's solution and 50% w/v glycerol. Pollen extract of *C. sempervirens* was also obtained from Dome/Hollister-Stier (Spokane, Washington, USA).

Protein determination

Protein concentrations of the extracts were measured by the Lowry modification of the Folin method, using bovine serum albumin (BSA) as a standard [12]. The protein content of the extracts was 2.7 µg/µl for *C. sempervirens*, 1.6 µg/µl for *P. gracilior* and 1.5 µg/µl for *C. verrucosa*.

Patients

Thirty-six patients were previously tested for common inhalant allergens: house dust mite (*Dermatophagoides farinae*); grasses (perennial ryegrass, bermuda grass and orchard grass); trees (European olive, white oak and Arizona cypress); compositae (sagebrush, rough pigweed, English platan). They all had shown positive skin-prick test responses to *C. sempervirens*. The patients suffered from allergic rhinitis, conjunctivitis, asthma or from a combination of these symptoms. Nineteen of these patients had seasonal symptoms and the remainder had symptoms throughout the year with exacerbation in the early spring, i.e. the period of cypress flowering.

Skin testing

Patient sensitivity to pollen extracts of *P. gracilior*, *C. verrucosa* and *C. sempervirens* was tested using skin-prick tests. A weal area of 9 mm² or more was considered positive. Antihistamines were withheld for at least 48 h prior to skin testing [13,14]. A histamine solution of 1 mg/ml and a saline-glycerol solution was used as controls [15,16]. Six healthy non-atopic control subjects were tested concomitantly. The tests were done during the winter and spring of 1989–1990.

Radioallergosorbent test (RAST) inhibition

Sera from three patients (Nos. 2, 4 and 13; Table 1) with high RAST scores to cypress allergens were pooled and used for the RAST inhibition tests. None of the patients had ever undergone immunotherapy before.

RAST inhibition testing was performed as follows: 50 µl of the pooled sera were mixed with 50 µl of a series of dilutions of pollen extracts of *P. gracilior* or of *C. verrucosa*. The mixtures were allowed to incubate at 30°C for 30 min in a shaker and were then kept overnight at 4°C. The next day an antigen-coupled paper disc of *C. sempervirens* was added, and the experiments were completed using RAST testing [17].

A pollen extract of *Olea europaea* (an unrelated antigen) was used as a control. A commercially available pollen extract of *Cupressus sempervirens* was also

tested as an inhibitor. The results of the RAST inhibition tests were expressed as [17]:

$$\% \text{ inhibition} = \frac{\text{CPM(RAST)} - \text{CPM(inhibitor)}}{\text{CPM(RAST)}} \times 100$$

where c.p.m. = counts per minute, c.p.m.(RAST) = c.p.m. of non-inhibited serum control.

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

Protein extracts were prepared in a sample buffer (e.g. Laemmli [18] with 2 mM phenylmethylsulfonyl fluoride [19]). The protein content of each extract was estimated by the spot technique [20] using bovine serum albumin [BSA] as a standard. Electrophoresis was done according to Laemmli in a small, cooled, vertical slab unit (SE-250 — Hoeffer Scientific Instruments, San Francisco, CA, USA).

Western blot

Proteins were blotted onto nitrocellulose membranes according to Towbin *et al.* [21]. The nitrocellulose sheets were blocked by 3% w/v BSA in phosphate buffered saline (PBS) with 0.5% w/v Tween 20 and probed by sera containing antibodies to *C. sempervirens*. Consequently, rabbit anti-human IgE (Dakopats, Denmark) diluted 1:200 and ¹²⁵I-labeled donkey anti-rabbit IgE (0.5 µCi of specific activity 520 kBq/µg protein) (Amersham International, Buckinghamshire, UK) were used to detect the human IgE binding proteins.

Results

Of the 36 patients who responded to *C. sempervirens*, 17 (47%) also responded to *P. gracilior*, nine (25%) to *C. verrucosa*, and six (17%) to both (Table 1). None of the healthy, non-atopic individuals had positive reactions to any of the extracts.

The RAST inhibition curves are presented in Fig. 1. The amount of protein needed to produce 50% inhibition were 11 µg of *C. sempervirens*, 54 µg *P. gracilior*, and 71 µg of *C. verrucosa* pollen. However, 265 µg protein of an *O. europaea* pollen extract, were needed to reach the same level of inhibition.

The slopes of the inhibition curves (Fig. 1) showed the allergenic relatedness (already known) between the three related species. *C. sempervirens* and *C. verrucosa* (of the family Cupressaceae) had similar slopes, whereas *P. gracilior* (of the closely related family Podocarpaceae) had a slightly different one. *O. europaea*, unrelated to the other three species had a distinctly different slope, supporting the difference in its allergenic properties.

The results of SDS-PAGE showed that although each species had a characteristic protein pattern, some of the protein bands of the three investigated species had a similar molecular weight (Fig. 2). For example, while the 66 kDa band appeared in all three species, the 33 kDa band, a more specific protein, appeared mainly in *C. verrucosa*.

Results of the Western blot analysis comparing the IgE binding proteins of *C. sempervirens*, *P. gracilior* and *C. verrucosa*, revealed several allergenic bands such as the 36 kDa and the 60 kDa, that are common to the three species; however, each plant also contains some unique bands. For example, the 25 kDa band appeared only in the *C. sempervirens* immunoblot (Fig. 3).

Table 1. Results of skin tests of 36 patients to pollen extracts of *C. sempervirens*, *P. gracilior* and *C. verrucosa*

Allergic symptoms of patient	Occurrence (seasonal/ perennial)	Pollen allergens			Other allergens**
		<i>C. sempervirens</i>	<i>P. gracilior</i>	<i>C. verrucosa</i>	
1. Rhinitis, conjunctivitis	S	35	24	40	M,G,T,C
2. *Rhinitis, asthma, conjunctivitis	S	35	9	40	M,G,T,C
3. Rhinitis, asthma, conjunctivitis	P & S	9	99	9	M,G,T,C
4. *Rhinitis	P & S	40	16	25	M,G,T,C
5. Rhinitis, asthma	S	20	16	16	M,G,T,C
6. Rhinitis, asthma	S	20	9	20	G,T
7. Rhinitis, asthma	S	48	30	0	M,G,T,C
8. Rhinitis	P & S	72	30	0	M,G
9. Rhinitis, asthma	P & S	35	16	0	M,G,T,C
10. Rhinitis, conjunctivitis	S	36	16	0	G,T,C
11. Rhinitis	S	42	16	0	—
12. Rhinitis	P & S	28	20	0	M,G,T,C
13. *Rhinitis, conjunctivitis	S	32	9	0	M,G,T,C
14. Rhinitis	S	35	16	0	M,G,C
15. Rhinitis, asthma	P & S	16	56	0	—
16. Rhinitis, asthma	S	16	16	0	M,G,T,C
17. Rhinitis	P & S	100	25	0	M,G
18. Rhinitis	S	90	0	16	M,G,T,C
19. Rhinitis, asthma, conjunctivitis	S	24	0	80	M,G,T
20. Rhinitis, conjunctivitis	S	30	0	16	G,T
21. Rhinitis, conjunctivitis	P & S	36	0	0	M,G,T,C
22. Rhinitis, asthma, conjunctivitis	S	16	0	0	G,T
23. Rhinitis, conjunctivitis	S	35	0	0	0
24. Rhinitis, conjunctivitis	S	25	0	0	M,G,T,C
25. Rhinitis	P & S	30	0	0	M,G,T,C
26. Rhinitis, dermatitis	P & S	40	0	0	M
27. Rhinitis, conjunctivitis	S	25	0	0	G,T,C
28. Rhinitis, conjunctivitis, dermatitis	P & S	42	0	0	M,T,C
29. Rhinitis	P & S	63	0	0	G
30. Rhinitis	P & S	9	0	0	G,T,C
31. Rhinitis, asthma	P & S	16	0	0	M,G,T,C
32. Rhinitis, conjunctivitis	S	16	0	0	G,T,C
33. Rhinitis, asthma, conjunctivitis	P & S	16	0	0	M,G,T,C
34. Rhinitis	P & S	9	0	0	M,G,T
35. Rhinitis, conjunctivitis	P & S	12	0	0	M,G,T,C
36. Rhinitis, conjunctivitis	S	40	0	0	M,G,T,C

—, Were not tested. 0, No response. Mean weal size is based on positive reaction.

**: M — house dust mite; G — grasses; T — trees; C — composita, see text for details.

* Patients 2, 4, 13: sera from these patients were pooled for RAST inhibition. Patients 2, 13: sera from these patients were examined by Western blotting.

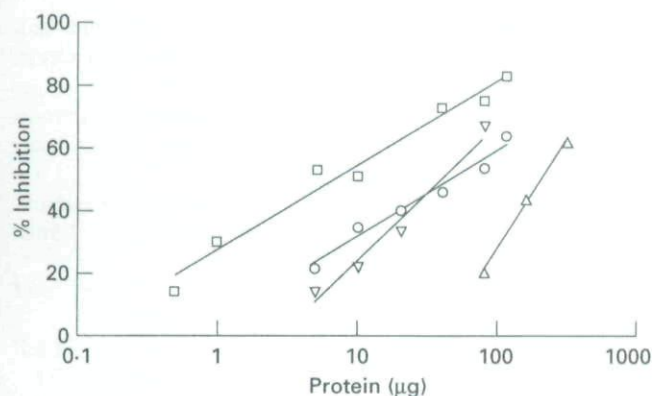


Fig. 1. RAST inhibition curves of *Cupressus* pollen extracts, by the pollen of the four species tested. □. *C. sempervirens*. ○. *C. verrucosa*. ▽. *P. gracilior*. △. *O. europaea*.

Discussion

A similar allergenicity among *C. sempervirens*, *C. verrucosa*, and *P. gracilior* is reported here for the first time. The results demonstrate that atopic individuals who are allergic to *C. sempervirens* pollen, develop positive skin reactions also to pollen extracts of *P. gracilior* and *C. verrucosa*. This finding is interesting for two reasons. Firstly, *P. gracilior* has not been previously reported as an allergenic species, even in areas where it is commonly planted [3]. Secondly, *P. gracilior* does not grow in Israel, and *C. verrucosa* is quite rare there. Although aerobiological studies conducted in Israel revealed the frequent presence of air-borne pollen of Cupressaceae (but not of the Podocarpaceae [22]), Israeli patients who were sensitized to *C. sempervirens* also demonstrated

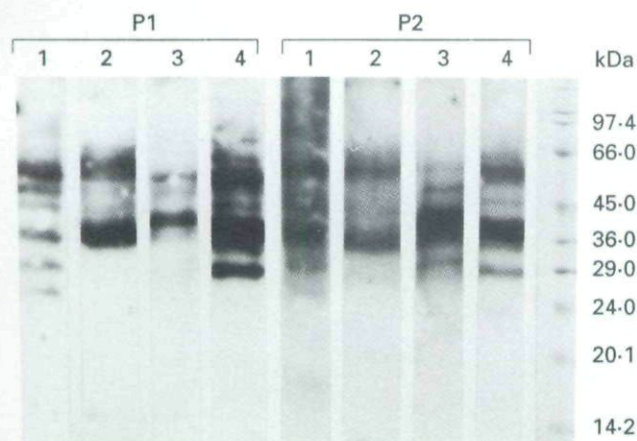


Fig. 2. SDS-PAGE analysis of the pollen extracts of three species. Lane 1. *C. sempervirens*. Lane 2. *C. sempervirens* (Dome/Hollister-Stier). Lane 3. *P. gracilior*. Lane 4. *C. verrucosa*. kDa. Molecular weight of markers.

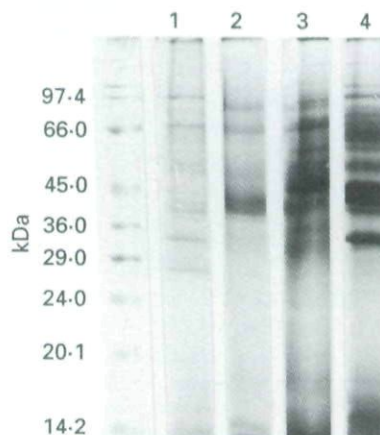


Fig. 3. Autoradiography of an IgE immunoblot analysis, using sera of two patients (P1 and P2) allergic to *C. sempervirens*. Lane 1. *C. sempervirens*. Lane 2. *C. sempervirens* (Dome/Hollister-Stier). Lane 3. *P. gracilior*. Lane 4. *C. verrucosa*. kDa. Molecular weight markers. P1. Patient no. 13 (Table 1). P2. Patient no. 2 (Table 1).

skin-test reactivity to pollen extracts of those two species. Moreover, since *C. verrucosa* is relatively new to Israel, the close relatedness with *C. sempervirens* may cause it to develop into a noxious new allergen.

The frequent skin responses to pollen extracts of all three species, in patients who had no previous exposure to two of them, indicate that we were dealing with a case of crossreactivity. This assumption is substantiated by the results of the RAST inhibition test and the Western blot analysis. In addition, it should be emphasized that according to the slopes of the RAST inhibition curves, the relationships among the three species are not equal; that is, *C. verrucosa* seems to be a closer kin to *C. sempervirens* than to *P. gracilior*. Indeed, these findings suit the botanical taxonomy.

Several investigators have reported crossreactivity between various species of the family Cupressaceae (e.g. among *Cupressus*, *Thuja* and *Juniperus*) [6,7] and among a member of a closely related family of conifers (i.e. *Cryptomeria japonica* of the Taxodiaceae) [7,9].

Thus, our findings support the concept that botanical relatedness of plants is closely associated with crossreactivity between the corresponding pollen allergens.

The data presented herein reveal that the three species investigated have several common IgE binding proteins. Most probably, such antigens are responsible for the crossallergenicity that was indicated by the skin tests and by the RAST inhibition analysis. However, the precise contribution of each of the allergenic bands to the development of the positive skin reactions and to the

allergic responses of the patients remains unclear. The potential crossreactivity demonstrated between the pollen extracts of *C. sempervirens*, *C. verrucosa* and *P. gracilior* precludes the later two to be used as substitutes for cypress trees [22].

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