

Allergy in the Mediterranean area

III: cross reactivity among Oleaceae pollens

J. BOUSQUET*, B. GUÉRIN†, B. HEWITT†, S. LIM†, and F.-B. MICHEL*

*Clinique des Maladies Respiratoires, CHU, 34059 Montpellier-Cedex, and †Laboratoires des Stallergènes, 160 Quai de Pollengis, Joinville Le Pont, France

(Received in original form 10 December 1984 and in revised form 20 February 1985; accepted for publication 20 May 1985)

Summary

Pollens of closely related species often share common antigens and allergens although this is not always the case. Four species of Oleaceae pollens commonly found in the Mediterranean area were investigated for cross-reactivity: olive (*Olea europaea*), ash (*Fraxinus excelsior*), privet (*Ligustrum vulgare*) and *Phillyrea angustifolia*, a common bush. Twenty individual sera of patients allergic to Oleaceae were investigated for specific IgE antibodies against the four species of Oleaceae pollens. The results indicated a high degree of correlation between RAST titres of Oleaceae pollens but three gave a particular emphasis on one species only. Cross-reactivity among these four pollen species was sought by means of RAST inhibition, iso-electric focusing and tandem cross-immunoelectrophoresis. All tests revealed a high degree of cross reactivity although there is no total identity among these four pollen species.

Introduction

In the northern Mediterranean area, Oleaceae pollens are among the main pollinating plants [1]. Four species predominate: olive (*Olea europaea*), ash (*Fraxinus excelsior*), privet (*Ligustrum vulgare*) and *Phillyrea angustifolia*, a common bush (Fig. 1). Normally, close botanical classification correlates with a high degree of cross-reactivity, whereas more distantly related species have a lower degree of cross-allergenicity [2,3]. However, this is not always the case and it was shown recently that *Urtica* (nettle) and *Parietaria* although belonging to the same family and having very similar morphological patterns did not cross-react [4], so that every example of cross-reactivity requires careful scrutiny.

Many methods make it possible to study patterns of allergic cross-reactivity between pollen allergen extracts but they should include techniques assessing the total allergenic potency such as RAST inhibition [5], complemented with techniques

Correspondence: Dr J. Bousquet, Clinique des Maladies Respiratoires, CHU, 34059 Montpellier-Cedex, France.

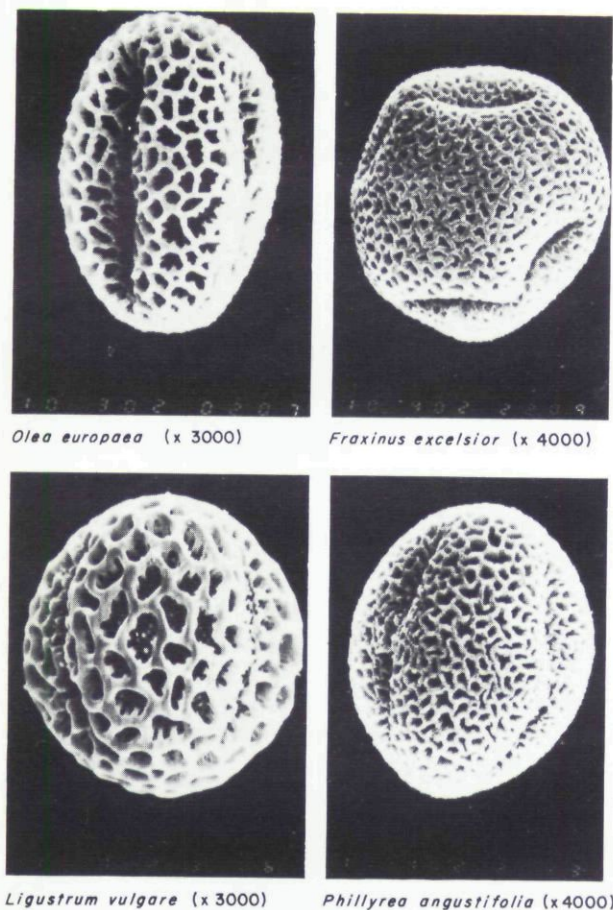


Fig. 1. Microscopic study of the four Oleaceae pollens.

analysing for the constituent proteins or allergens of the extracts. Iso-electric focusing [6] and cross-immunoelectrophoresis [CIE] derived methods [7] are examples of such experimental techniques.

No data exist in the literature concerning cross-reactivity among Oleaceae pollens. The four major Mediterranean members of the Oleaceae family (olive, privet, ash and *Phillyrea*) were studied. Preliminary studies by means of skin testing showed that there might be a possible cross-reactivity among these four pollen species and therefore it was considered important to investigate these observations more fully. The characterization of precise cross-reactivity has been investigated *in vitro* by means of RAST inhibition assays, iso-electric focusing (IEF) and tandem CIE.

Materials and methods

Patients

Twenty patients who presented a seasonal rhinitis during the Oleaceae pollen season were selected on the following criteria:

- (i) a positive skin-prick test with at least one of the extracts of the four Oleaceae

pollen species. The extracts were prepared by the Laboratoires des Stallergènes, Fresnes, France according to the method described below.

(ii) A positive RAST (Pharmacia Diagnostics AB, Uppsala, Sweden) to at least one of the four Oleaceae pollen species. The method for performing the RAST is described below.

(iii) No previous specific immunotherapy.

Materials

Four species of pollens of the Oleaceae family were tested: olive, ash, privet and *Phillyrea* (Fig. 1). All pollens were harvested from cultivated (olive, privet) or wild (ash, *Phillyrea*) population plants after the advice from a botanist. Pollens collected over 3 years were used for each species to cater for possible annual variation in pollens. Pollens were stored at -20°C in vacuum containers before use. All pollen batches were microscopically analysed before use [8]. They were absolutely free of any contamination from other pollen species and they contained less than 2.5% of plant matter.

Ten per-cent (w/v) extracts were made in Coca's solution by stirring overnight at $+4^{\circ}\text{C}$. The extracts were then passed through a sterilizing grade filter and subsequently freeze-dried.

Methods

Serum specific IgE. Individual sera of the twenty patients allergic to Oleaceae pollens were analysed for specific IgE to the four species. Sera were incubated with cyanogen bromide activated paper discs coupled with allergen extracts according to the classical method of Ceska *et al.* [9]. Binding of specific serum IgE to the disc was revealed using ^{125}I anti-IgE (Pharmacia Diagnostics AB, Uppsala, Sweden). RAST was performed according to the usual procedure. All assays were performed in duplicate and results expressed as titre determined from the mean number of counts bound when using 0.03% of human serum albumin. This negative control was used since it was observed that it gave a highly significant correlation with either a pooled cord blood sera or horse sera. In our hands, the intra-assay variation coefficient was less than 10% and the non-specific background using 0.03% of human serum albumin was no greater than 600 counts per min. The cut-off between the absence and presence of allergen-specific IgE was chosen as twice the background. Moreover, ten sera from healthy non-allergic volunteers were tested as a negative control for the RAST discs.

Protein nitrogen units. The PNU content of each extract was determined using the standard Kjeldahl procedure recently standardized [10].

RAST inhibition. RAST inhibition was performed using the method of Gleich *et al.* [5] with a serum pool, and with three individual sera of subjects who had a high titre for one Oleaceae species and two others who had an equal titre (cases 1, 3, 6, 9 and 15). The serum pool was prepared with equal volumes of individual test sera of patients who had both positive prick tests and serum specific IgE.

RAST discs were prepared according to the method of Ceska *et al.* [9] Optimal allergen concentration for the coupling procedure was estimated by direct RAST titration. Serial dilutions of 1/10 (w/v) allergen extracts were tested for their capacity to inhibit binding of the serum pool to the allergen disc. Binding of specific serum IgE antibodies to the disc were revealed using ^{125}I anti-IgE (Pharmacia Diagnostics AB, Uppsala, Sweden). Parallel line bioassay was used to analyse the linearity and the parallelism of the RAST inhibition curves [11].

Iso-electric focusing (IEF). Analytical flat bed IEF in a pH gradient of 3.5–10 was performed to polyacrylamide gel supports (PAG plates, LKB Laboratories, Sweden) as described by Brighton [6].

Tandem cross-immunoelectrophoresis. Tandem CIE as described by Lowenstein [7] was used to compare ash, privet and *Phillyrea* extracts to olive pollen extract. The serum used in the second dimension migration was raised in goats against olive pollens. All experiments were done in duplicate and results were averaged for RAST, RAST inhibition and PNU determinations.

Results

Serum specific IgE of individual patients

RAST titres when testing individual Human sera against the four pollen species are shown in Table 1. Significant correlation was found between all extracts though variation was seen in the capacity of individual sera to bind to the specific allergen.

Table 1. Direct RAST titration of individual sera with the four Oleaceae pollen species

Serum	Allergen			
	Olive	Ash	Privet	<i>Phillyrea</i>
1	3.0	4.2	4.2	4.6
2	1.1	1.0	2.1	1.3
3	2.7	2.1	6.8	1.9
4	2.0	2.1	1.9	1.0
5	2.0	2.3	1.9	2.0
6	12.0	3.9	5.4	5.3
7	4.5	2.0	2.1	2.6
8	4.5	2.0	2.1	2.6
9	8.8	16.7	12.0	13.4
10	7.5	6.0	7.8	7.2
11	2.0	2.0	2.1	2.1
12	2.2	2.1	2.0	2.1
13	2.1	2.1	2.3	2.7
14	7.1	6.2	4.5	7.2
15	13.8	14.0	9.9	11.8
16	1.8	2.0	2.0	2.3
17	2.8	2.8	2.7	3.4
18	2.5	2.0	2.8	3.4
19	5.7	3.5	4.6	5.7
20	11.6	8.5	13.5	8.5
mean	4.98	4.37	4.61	4.5
s.d.	3.86	4.2	3.56	3.5
Statistical analysis:	$r = 0.77$			
	$r = 0.82$			
	$r = 0.87$			
	$r = 0.87$			
Spearman correlation	$r = 0.73$			
	$r = 0.80$			
	$r = 0.81$			

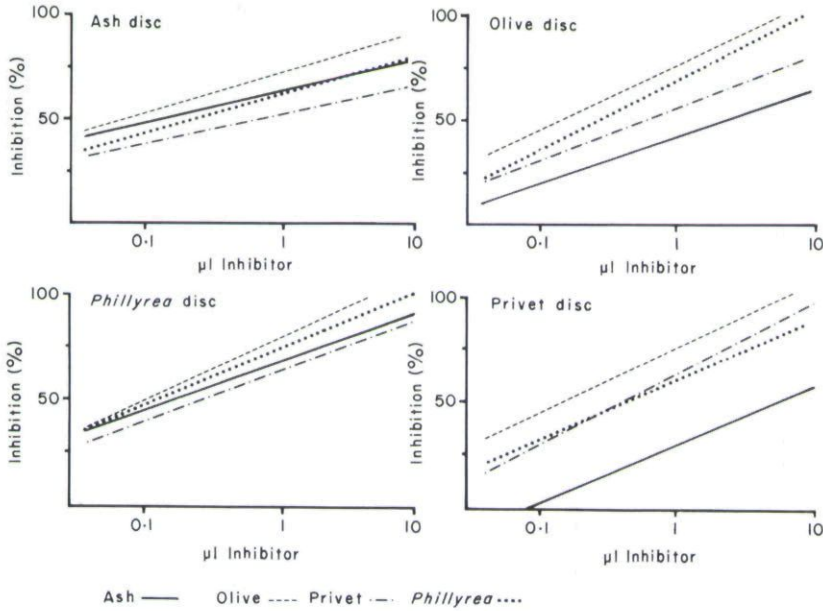


Fig. 2. Comparison of the cross-reactivity of Oleaceae pollen allergens by RAST inhibition.

Four cases expressed different patterns: case 3 had a very high privet IgE titre, case 6 had a very high olive titre and case 9 a much higher titre against olive than against the three other pollen species. On the other hand, case 2 had a low privet titre and a negative titre for the three other species.

PNU content

The PNU content of the four Oleaceae pollen extracts showed that olive had the highest titre (36 250 PNU/ml), ash pollen the lowest (24 000 PNU/ml) and the two others intermediate titres (privet: 34 450 PNU/ml, *Phillyrea*: 27 870 PNU/ml).

RAST inhibition studies

RAST inhibition studies for the serum pool are shown in Figs 2 and 3 and Table 2. It can be seen that whatever the allergen coupled on the RAST disc, the other three pollen allergens were able to inhibit binding of serum pool to the RAST disc indicating cross-allergenicity between these extracts. For all experiments RAST curves were found linear and parallel as show in Table 2. The comparison of the potencies of the extracts was done by means of the 50% RAST inhibition end point titration. Some differences in potency between inhibitors was found. To compare the activity of each pollen in RAST inhibition an arbitrary value of 1 was given when the homologous inhibitor was used in the assay (Fig. 3). It was observed that when the PNU content of the extracts was not taken into consideration (Fig. 3a), olive inhibitor is the most potent whatever the RAST disc. On the other hand, when the results were adjusted to constant PNU (Fig. 3b) results slightly differed since olive inhibitor was still the most potent for olive and privet discs but *Phillyrea* and ash were slightly more effective for their homologous discs. For individual sera, some differences were observed when there was a higher RAST titre for one Oleaceae pollen species (Table 3).

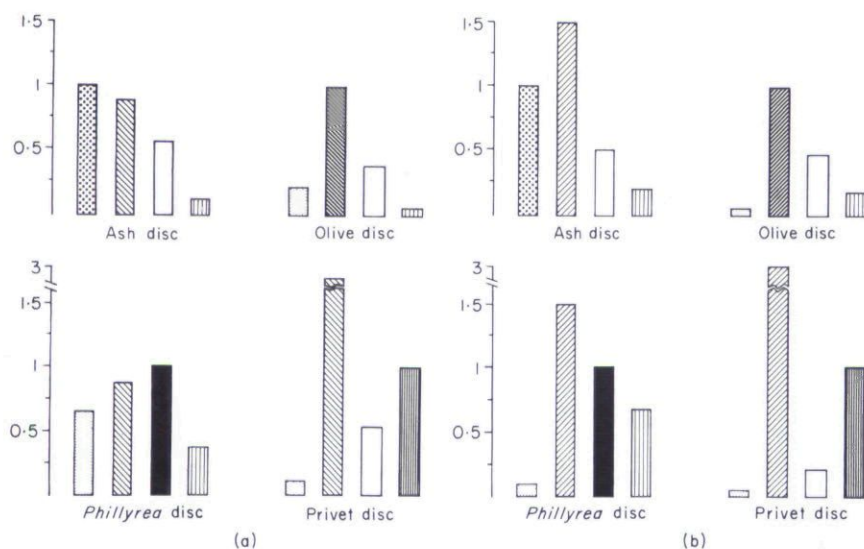


Fig. 3. Inhibitory activities of the various fluid-phase Oleaceae pollen extracts competing for IgE antibodies with solid phase Oleaceae pollen allergens. (a) The extracts were compared without PNU equality; (b) the extracts were compared at constant PNU of the inhibitors. A ratio of 1 was given to the homologous inhibitor: □ ash; ▨ ash inhibitor; ▩ olive; ▧ olive inhibitor; □ Phillyrea; ■ Phillyrea inhibitor; ▨ privet; ▩ privet inhibitor.

Table 2. Results of RAST inhibition experiments using the serum pool

Disc	Inhibiting extract	Linearity*		<i>S</i>	<i>i</i>	50% end-point (μl)	Parallelism†	
		F^0	F_1				<i>F</i>	F_1
Olive	Olive	0.27	6.94	13.34	51.73	1.14	—	—
	Phillyrea	2.58	6.94	15.22	74.78	5.10	1.15	4.28
	Privet	0.81	6.94	11.56	73.22	7.46	2.52	4.28
	Ash	1.72	6.94	12.01	88.73	25.17	1.46	4.28
Phillyrea	Phillyrea	0.47	4.53	11.68	28.87	0.16	—	—
	Privet	0.91	4.53	10.18	37.04	0.30	1.54	2.98
	Ash	1.93	4.53	9.90	35.50	0.23	1.58	2.98
	Olive	1.97	6.94	13.82	20.14	0.12	2.64	3.22
Privet	Privet	2.64	6.94	12.41	37.21	0.36	—	—
	Ash	0.52	6.94	9.64	68.17	6.59	1.34	4.28
	Olive	0.20	6.94	8.80	31.17	0.12	2.00	4.28
	Phillyrea	0.15	6.94	10.42	45.94	0.68	4.79	4.28
Ash	Ash	0.22	6.94	7.33	35.76	0.14	—	—
	Olive	2.69	6.94	8.25	32.46	0.12	1.13	4.28
	Phillyrea	4.21	6.94	9.58	38.90	0.31	1.26	4.28
	Privet	0.15	6.94	9.41	50.70	1.08	1.54	4.28

* RAST curves are linear at a *P* level < 0.05 when $F^0 < F_1$.

† RAST curves are parallel within an experiment at a *P* level < 0.05 when $F < F_1$.

S: slope; *i*: intercept.

Table 3. Results of RAST inhibition experiments using individual sera

Disc	Inhibiting extract	RAST inhibition end-point titre (μ l)				
		pt 1	pt 3	pt 6	pt 9	pt 15
Olive	Olive	1.30	2.52	0.71	1.15	0.72
	<i>Phillyrea</i>	NT	8.12	3.56	2.53	5.31
	Privet	NT	5.23	4.05	2.56	7.45
	Ash	NT	21.56	27.30	15.41	14.65
Privet	Olive	0.76	1.23	0.04	0.16	0.12
	<i>Phillyrea</i>	NT	0.98	0.23	0.34	0.36
	Privet	0.27	0.13	0.57	0.27	0.55
	Ash	NT	9.56	5.67	2.35	3.10

NT: not tested.

All curves were parallel within an experiment.

Iso-electric focusing patterns

Analytical flat bed IEF patterns are presented in Fig. 4. The four Oleaceae pollens share several common proteins in the acidic range of pH (3–4.5). Ash and *Phillyrea* had the same number of proteins in the acidic range of pH but their profiles were less important than those of olive and privet. These two later species had some

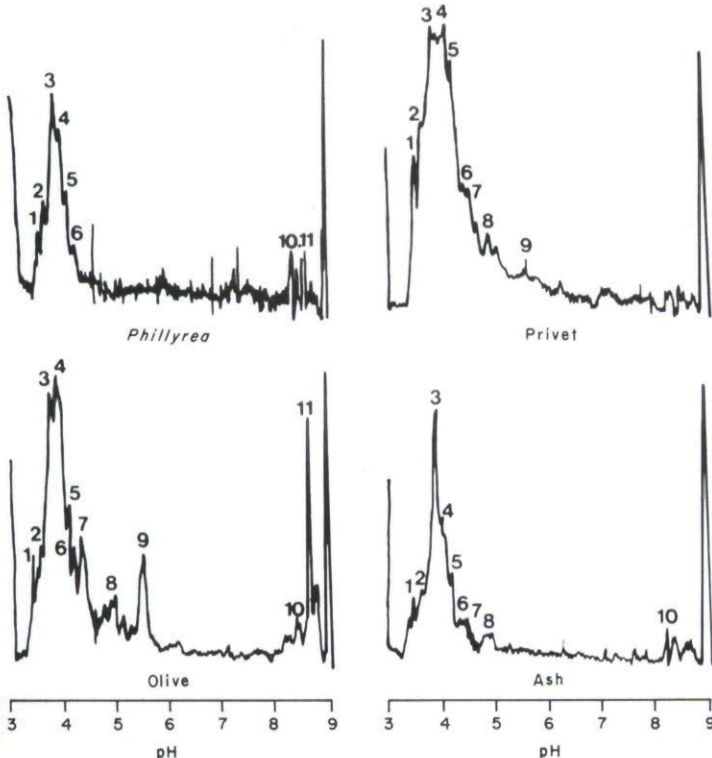


Fig. 4. Iso-electric focusing patterns of the four species of Oleaceae pollens.

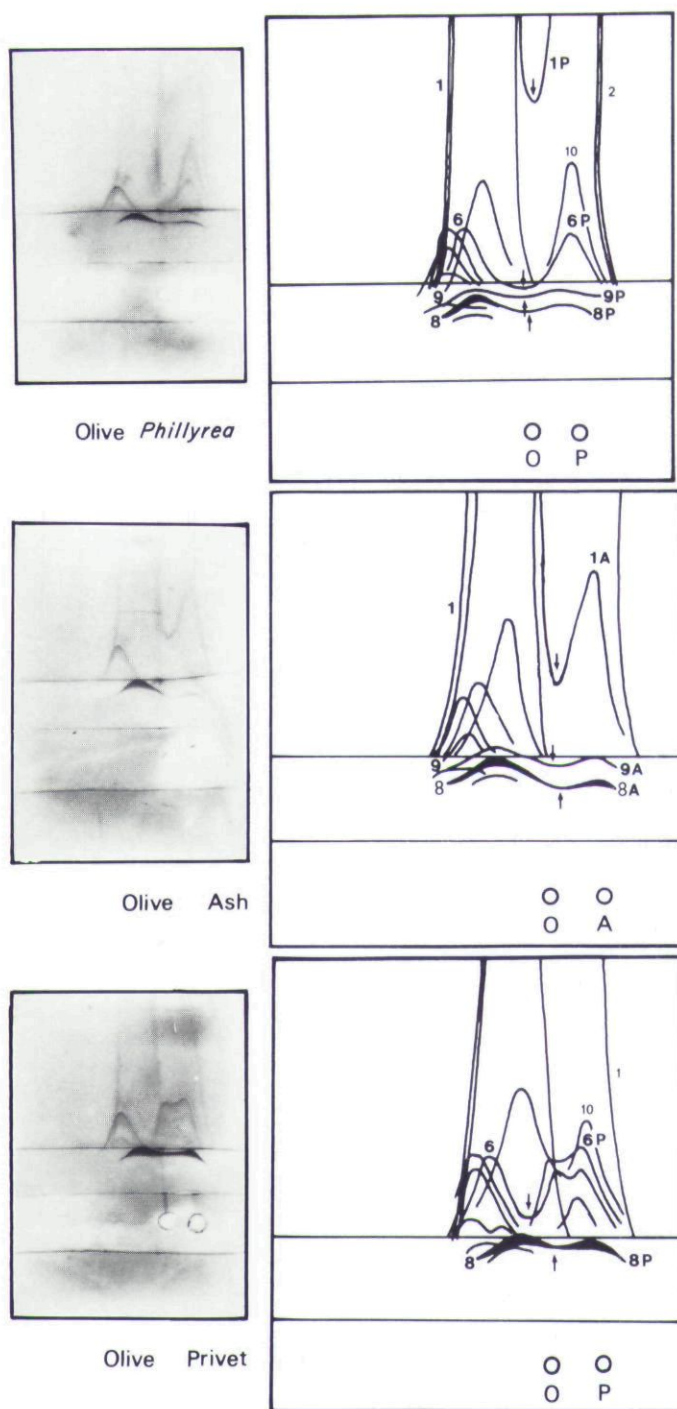


Fig. 5. Cross-antigenicity determined by tandem cross-immunoelectrophoresis.

characteristic proteins in the range of pH 6–7 (proteins nos 8 and 9). Finally, olive pollen had a major peak in the basic range of pH (10) whereas this protein was not very concentrated in the three other pollen species.

Tandem cross-immunoelectrophoresis

Ten antigens were revealed in the olive pollen extract using goat anti-olive antiserum. In tandem CIE (Fig. 5 and Table 4) cross-reacting antigens were clearly demonstrated in the case of a continuous line between the same antigen revealed in the olive and the other extract. Some antigens were not linked by a continuous line but were revealed by the olive antiserum. Antigens 1 and 8 are present in all extracts whereas antigens 2, 6, 9, 10 are only present in three extracts.

Table 4. Cross-reacting antigens between Oleaceae pollens determined by tandem cross-immunoelectrophoresis

Olive antigens	Ash	<i>Phillyrea</i>	Privet
1	CL	CL	NCL
2	NCL	NCL	
3			
4			
5			
6		CL	CL
7			
8	CL	CL	CL
9	CL	CL	
10		NCL	NCL

CL: continuous line; NCL: non-continuous line.

Discussion

All batches of Oleaceae pollens used in these experiments were controlled by a botanist and a palynologist. The batches were species-specific and free from any contamination by other pollen species. This is a necessary prerequisite for any study of cross-reactivity. For all species, batches of pollens harvested over 3 years were used to avoid any possible seasonal difference.

All the techniques used in these experiments have been widely employed and methods for both total allergenicity (RAST inhibition), and protein (IEF) and antigenic (tandem CIE) composition of the extracts were used. Purified antigens of Oleaceae pollens are not yet fully characterized although their fractionation has been attempted [12,13] and therefore it was not possible to use antibodies raised against purified antigens for cross-reactivity studies.

Most patients tested in RAST had a similar titre for the four Oleaceae pollens whereas three had an increased serum IgE level for one species. These results were confirmed by the RAST inhibition studies using individual sera. The results obtained with RAST inhibition using the serum pool show that there exists a strong degree of cross-reactivity among the four species of Oleaceae pollens. All curves of RAST inhibition were parallel suggesting that the most relevant allergens are present in all the extracts, however, RAST can be totally inhibited by major allergens and it is therefore

possible that minor ones are lacking in some species. Individual sera tested in RAST inhibition showed that all curves were parallel suggesting an allergenic identity but greater differences between the 50% end-point titres were observed.

The patterns of IEF were very similar in all four species except for protein no. 11 that is almost exclusively present in olive pollen extract. When grass pollen IEF patterns are examined [2] there are more differences between them than those of the four Oleaceae pollens. The antigenic composition of the Oleaceae pollens was studied by means of tandem CIE. Although a complete identity between two antigens can only be ascertained when there is a continuous line between them it may be postulated that antigens revealed by the anti-olive antiserum are also shared by the two species. Antigens 1 and 8 are shared by the four species whereas antigens 2, 9, 6, 10 are restricted to some species showing that there is no total identity between the four species.

The results of *in vitro* experiments confirm a high degree of cross-reactivity among the four Oleaceae pollens tested (olive, ash, *Phillyrea* and privet). There appear to be closer cross-reactive patterns between these species than between grass pollens, although Oleaceae pollens are microscopically distinct and grass pollens are not. Moreover, in a recent study it was shown that Urticaceae pollens (nettle and *parietaria*) [4] that are morphologically similar do not cross-react. Thus the antigenic and morphological patterns of pollens within a family are not related.

Acknowledgments

The authors thank Drs P. Cour, H. Dhivit, J.P. Marty and C. Seignalet for their help and suggestions.

References

- 1 Bousquet J, Cour P, Guérin B, Michel FB. Allergy in the Mediterranean area. I- Pollen calendar and pollinosis of the Montpellier area. *Clin Allergy* 1984; 14:249-58.
- 2 Bousquet J, Guérin B, Furic R, Michel FB. Standardization of allergens. In: Spector SL, ed. *Provocative challenge*. Boca Raton: CRC Press, 1983:45-95.
- 3 Weber RW. Cross reactivity among pollens. *Ann Allergy* 1981; 46:208-15.
- 4 Bousquet J, Hewitt B, Guérin B, Dhivert H, Michel FB. Allergy in the Mediterranean area. II- Cross allergenicity among Urticaceae pollens. *Clin Allergy* (in press).
- 5 Cleich GJ, Larson JB, Jones RT, Baer H. Measurement of the potency of allergy extracts by their inhibitory capacities in the radioallergosorbent test. *J Allergy Clin Immunol* 1974; 53:158-69.
- 6 Brighton WD. Profiles of allergen extract component by isoelectric focusing and radio immunoassay. *Dev Biol Stand* 1975; 29:362-9.
- 7 Löwesstein H. Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. *Prog Allergy* 1978; 25:1-62.
- 8 Cour P, Loublier Y. Contrôle de l'identité et de la pureté des lots de pollens destinés à la préparation d'extraits allergéniques à usage diagnostique ou thérapeutique. *Rev-franç Allergol* 1980; 20:197-201.
- 9 Ceska M, Eriksson R, Varga JM. Radio-immunosorbent assay for allergens. *J Allergy* 1972; 49:1-12.
- 10 May JC, Sih JTC. Protein nitrogen unit precipitation procedure for allergenic extracts. Collaborative study. *J Assoc Off Anal Chem* 1981; 64:1435-40.
- 11 Rastogi SC. Allergenic extract relative potency calculations by parallel line bioassay method. Bureau of Biologics, Food and Drug Administration, Bethesda, 10 September 1979.
- 12 Vela C, Platas C, Gurbindo C *et al*. Fractionation and biological characterization of *Olea europea* pollen extract. *Int Archs Allergy Appl Immunol* 1982; 68:289-94.
- 13 Blanca M, Boulton P, Brostoff J, Gonzales-Reguera I. Studies of the allergens of *Olea europea* pollen. *Clin Allergy* 1983; 13:473-8.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.