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LOW MOLECULAR WEIGHT ALLERGENS OF THE POLLEN OF *PARIETARIA OFFICINALIS*

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Abstract—The allergenic composition of a low mol. wt fraction of the pollen extract of *Parietaria officinalis* (PO) was investigated. Fraction C, that was eluted after oxytocin (mol. wt 1040) when the pollen extract was gel filtered on Sephadex or on Biogel, was cross-reactive in the RAST with the major allergen PO15 and was capable of eliciting histamine release from leukocytes of sensitive donors. RAST inhibition (RAST I) analysis of the eluate of gel filtration on Sephadex G-10 revealed several peaks of IgE binding activity. Analysis of fine specificity of response of individual patients carried out by skin-prick tests and by RAST I. revealed individual patterns of reactivity, indicating that allergens contained in fraction C were minor allergens.

INTRODUCTION

Pollens of plants causing atopic allergy contain a variety of soluble allergens, identified by tests of sensitivity in atopic subjects and by reaction with serum IgE antibodies. It has been found convenient to subdivide these allergens into "majors" and "minors", according to a double criterion that takes into account both the value of the prevalence of specific responses in the group of sensitive subjects, and the level of specific IgE antibodies in individual sera (Platts-Mills *et al.*, 1981).

Marsh *et al.* (1982a, b) showed that the immune response to Ra5 (mol. wt 4500), a minor allergen of *Ambrosia*, was associated with the haplotype HLA Dw2. The same author had previously advanced the suggestion that the response to minor allergens was a convenient model for the study of genetics of human immune response, arguing that minor allergens had a low mol. wt and possessed few epitopes, so that responsiveness was presumably restricted by single immune response genes and the genetic heterogeneity of the population was exposed (Marsh, 1976).

A preliminary identification of minor allergens is obtained by comparison of individual patterns of IgE antibody response to fractionated pollen extract. Variability of individual patterns of response has been reported for the pollen extracts of *Ambrosia* and *Poa pratensis* fractionated by CRIE (Santilli *et al.*, 1980; Lowenstein and Marsh, 1981; Chakrabarty *et al.*, 1981; Osterballe *et al.*, 1983) and by a combination of gel filtration and skin tests (King and Norman, 1962; King *et al.*, 1967; Adolphson *et al.*, 1978). Minor allergens have been obtained in a purified form from the pollen of *Ambrosia*: Ra3, Ra4 and Ra5 have mol.

wt, respectively, of 15,000, 28,000 and 4500; the prevalence of skin sensitivity in the group of subjects responsive to the major allergen, AgE, was 61.5, 46 and 45% (Santilli *et al.*, 1980).

We have identified and partially purified a major allergen of the pollen of *P. officinalis* (PO) with a mol. wt of approx. 14,500 (PO15) (Oreste *et al.*, 1985a, b). We observed that a fraction of the pollen extract corresponding to mol. wt approximately lower than 1000 (low mol. wt fraction) cross-reacted with PO15 in the RAST. The present study shows that this fraction contains several components that are active in skin tests, able to induce histamine release from leukocytes of sensitive donors and active in RAST inhibition; analysis of individual patterns of response, indicated that these allergens behave as minors.

The response to these minor allergenic components offers an opportunity to investigate genetic restrictions of immune responsiveness. The purification and characterization of these molecules could provide useful tools for the identification of epitopes that are active in the modulation of IgE response, a problem that is crucial to the elaboration of new strategies of vaccination.

MATERIALS AND METHODS

Human sera

The reaginic serum pool consisted of equal vols of sera collected from 45 untreated subjects with atopic allergy to the pollen of *Parietaria*, documented by clinical history. All subjects gave positive reactions to the crude extract of PO pollen by skin-prick tests and by RAST. The control serum pool consisted of equal vols of sera collected from 10 allergic subjects, that gave negative reactions using skin-prick tests and RAST to PO pollen. Sera were stored at -20°C in small aliquots prior to use.

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Parietaria officinalis pollen (PO pollen)

The pollen of PO was purchased from Allergon AB, Engelholm, Sweden (Batch No. 079182101).

Molecular wt indicators

The columns of Sephadex G-25 and G-10 and of Biogel P-2 were calibrated with the following peptides and aminoacids: oxytocin (1040); thyroxine (777); monoiodothyronine (MIT) (398); and thryptophan (204).

Crude extract of PO pollen (CE)

Two grams of pollen were suspended at 4°C with magnetic stirring in 60 ml of 0.125 M NH_4HCO_3 , pH 8.7, containing 0.05% NaN_3 and 5.7×10^{-4} M phenylmethylsulphonylfluoride (Sigma, St Louis, MO) (Fahreny and Gold, 1963). After 12 hr the pollen was separated from the supernatant by centrifugation: 198 mg of protein was recovered in the supernatant as determined by Lowry *et al.* (1951).

Solid-phase antigens

Solid-phase antigens were prepared according to Ceska (1981). Two thousand CNBr-activated discs were reacted for 48 hr at 4°C with 80 ml of a solution of the CE or of pool C (0.6 mg protein/ml) or of PO15 (0.1 mg protein/ml) in 0.1 M NaHCO_3 , pH 8.5. The remaining reactive groups were blocked by reaction with 80 ml of 0.5 M ethanolamine in 0.1 M NaHCO_3 . The discs were stored at 4°C in 0.05 M phosphate buffer, pH 7.4, containing 0.9% NaCl, 0.03% BSA, 0.05% NaN_3 and 0.5% Tween-20 (RAST buffer).

RAST and RAST inhibition (RAST I)

Direct RAST and RAST I were performed according to Ceska (1981). Each serum to be assayed by RAST I was titrated by direct RAST. The amount used in the inhibition assay was such as to bind approx. 60% of the radioactivity bound by undiluted serum (to exclude antibody excess).

For RAST I: 100 μl of inhibitor was incubated overnight at 4°C with 50 μl of the appropriate dilution of the serum and the discs. Inhibition was expressed as $[1 - (b - c/a - c)] \times 100$, where a are cpm bound by discs incubated without inhibitor, b cpm bound by discs incubated with inhibitor and c cpm bound by discs incubated with control serum. Each determination was performed in duplicate, and the values reported are means. c (bkg) represented less than 2% of the cpm added to the incubation mixture.

Skin-prick tests

Skin-prick testing was performed on the forearm of informed volunteers; 20 μl of the solutions to be tested was injected with a prick-test disposable needle (Holljster-Stier). Each test was repeated on the opposite arm. Wheal responses were measured after 5

and 15 min. The responses were graded from 0 to 4 according to the mean diameter of the wheal (d) (Santilli *et al.*, 1980): d: 0–2 mm = 0; d: 2–3 mm = 1; d: 3–4 mm = 2; d: 4–5 mm = 3; d: 5–6 mm = 4. A control test performed with buffer eluted from Sephadex G-10 column yielded negative results.

Histamine release

Histamine release assays of fraction C and PO15 were carried out with leukocytes from five subjects allergic to *Parietaria* and from three subjects non-allergic to *Parietaria*, but allergic to Gramineae, according to Lowenstein *et al.* (1964).

RESULTS

Gel filtration on Sephadex G-25

The CE (0.4 ml), containing 3.5 mg of protein/ml, was gel filtered through a column of Sephadex G-25 (Fig. 1). Alternate fractions of the eluate were assayed as inhibitors of the RAST with reaginic serum pool and discs CE. In the sector of the eluate limited by the elution vols of oxytocin (mol. wt 1040) and thryptophan (mol. wt 204) five major peaks were observed.

Fractions 51–100 of the eluate were pooled. This pool, that accounted for 19% of the total protein content of the eluate and contained proteins with mol. wts approx. lower than 1000, was lyophilized and regelfiltered on Sephadex G-25; fractions 51–100 were again pooled (fraction C).

Fraction C was coupled to paper discs with the procedure described under Materials and Methods. The allergen discs obtained will be referred to as discs C. Serial dilutions of PO15 and fraction C were assayed as inhibitors of the RAST performed with the serum pool, discs C and PO15 (Fig. 2). A partial inhibition of PO15 RAST was obtained with fraction C, while PO15 completely inhibited fraction C RAST.

Fractions of the eluate of Sephadex G-25 gel filtration of the CE were used as inhibitors of the RAST performed with discs C (Fig. 1). The inhibition profile obtained was in good agreement with the profile obtained with discs CE; four additional peaks, comprised between the elution vol of MIT and thryptophan, were clearly delineated. Skin activity of the fraction of the eluate was determined by the skin-prick test on 12 allergic subjects. All subjects responded to fractions containing higher mol. wt allergens; fractions corresponding to mol. wts lower than 1000 were skin active for some patients only. The results are summarized in Table 1; the data of column N are reported in Fig. 1 for comparison of skin activity and RAST inhibition profiles.

The ability of fraction C and PO15 to induce histamine release from leukocytes of allergic donors was investigated. Four typical dose-response curves comparing the activity of fraction C and PO15 are reported (Fig. 3). Fraction C and PO15 did not

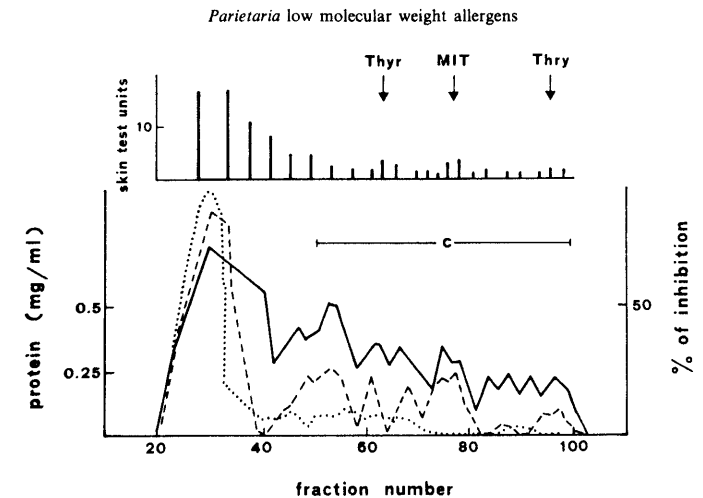


Fig. 1. Gel filtration of CE on Sephadex G-25. The CE (0.4 ml), containing 3.5 mg protein/ml, was applied to a 50×1 cm column. Elution buffer: 0.125 M NH_4HCO_3 , pH 8.7. Fraction vol: 0.38 ml. Molecular wt. markers (left to right): oxytocin (mol. wt 1040); thyroxine (777); monoiodothyronine (MIT) thryptophan (204). (.....): mg protein/ml (Lowry *et al.*, 1951). RAST I: (—) CE-RAST: (bkg: 100 cpm; 100%: 2100 cpm). (---): C-RAST: (bkg: 100 cpm; 100%: 1900 cpm). Serum: pool. Inhibitors: alternate fractions. Bars represent the sum of skin activity grades (column N, Table 1).

induce histamine release from leukocytes of control donors.

The skin activity of fraction C was evaluated by quantitative prick-test titration in 11 allergic subjects. Four serial 5-fold dilutions from 0.8 to 2500 $\mu\text{g}/\text{ml}$ were used to elicit skin reactivity. An increase in size of wheal and flare occurred with increasing concns. The lowest dilution giving a skin reaction of grade 1 (see Materials and Methods) was reported in Fig. 4, and compared with C-RAST values.

Gel filtration on Biogel P2

Fraction C (0.5 ml) containing 6 mg protein/ml, was filtered through a column of Biogel P2. Alternate fractions of the eluate were assayed as inhibitors of the RAST (discs C, serum pool). The inhibition profile showed at least seven peaks eluted after thyroxine (Fig. 5).

Gel filtration on Sephadex G-10

Fraction C (0.5 ml) containing 6 mg protein/ml, was filtered through a column of Sephadex G-10. Alternate fractions of the eluate were assayed as inhibitors of the RAST (discs C, serum pool) (Fig. 6). The inhibition profile obtained showed at least seven peaks in the sector of the eluate delimited by the elution vols of thyroxine and thryptophan.

Thirty subjects allergic to PO were skin-prick tested with 13 fractions eluted after thyroxine and with a solution containing 30 $\mu\text{g}/\text{ml}$ of PO15 (Table 2). All subjects were highly positive to PO15; fractions of the eluate corresponding to higher mol. wts were active for a higher proportion of subjects than

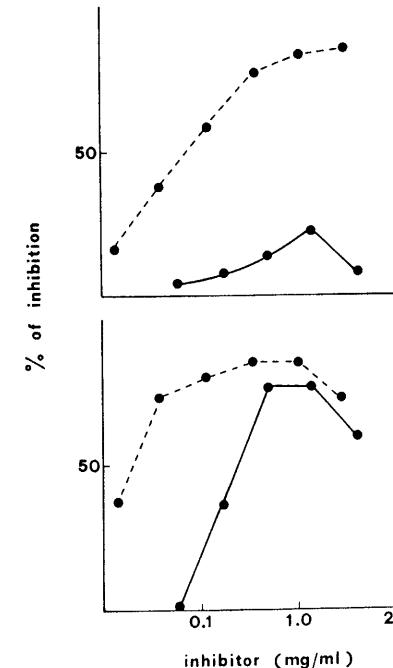


Fig. 2. RAST I: (a) PO15-RAST: (bkg: 100 cpm; 1500 cpm). (b) C-RAST: (bkg: 80 cpm; 100%: 1350 cpm). Serum: pool. Inhibitors: serial dilutions of fraction C and of PO15 (---). Abscissae: mg protein/ml (Lowry, 1951).

Table 1. Subjects

Fractions	1	2	3	4	5	6	7	8	9	10	11	12	N
27	2	2	4	4	2	4	2	0	4	4	2	4	34
33	1	2	3	4	2	4	2	4	3	4	2	3	34
37	1	0	2	3	2	4	0	4	2	1	1	2	22
41	0	0	1	1	2	3	2	3	2	1	1	1	17
45	0	0	1	1	0	2	1	2	1	1	0	1	10
49	0	0	1	1	0	2	1	2	1	1	0	1	10
53	0	0	0	1	0	0	1	0	1	1	0	1	5
57	0	0	0	1	0	0	1	0	0	0	0	1	3
61	0	0	0	1	0	1	1	1	1	0	0	1	6
63	0	0	0	1	0	1	1	1	1	1	0	1	7
65	0	0	0	1	0	0	1	1	1	1	0	1	6
69	0	0	0	0	0	0	1	0	1	1	0	0	3
71	0	0	0	0	0	0	1	0	1	1	0	0	3
73	0	0	0	0	0	0	0	1	1	0	0	0	2
75	0	0	0	0	0	0	0	1	1	2	1	2	7
77	1	0	0	0	0	0	0	0	1	3	0	3	8
79	1	0	0	0	0	0	1	0	1	0	0	0	3
83	1	0	0	0	0	1	1	0	1	0	0	0	4
87	0	0	0	0	0	1	1	0	1	0	0	0	3
88	0	0	0	0	0	0	1	0	1	0	0	1	3
93	0	0	0	0	0	0	1	0	1	0	0	1	3
95	0	0	0	0	0	1	1	0	1	0	0	1	4
97	0	0	0	0	0	1	1	0	1	0	0	1	4

Twelve subjects allergic to PO were skin-prick tested with fractions of the eluate of gel filtration on Sephadex G-25 (Fig. 1). Their reactivity was evaluated as described under Materials and Methods, according to the diameter of wheals.
N = sum of grades of reactivity for all patients.

fractions corresponding to lower mol. wts. The variability of individual patterns of reactivity suggested that these fractions contained different allergens. The grades of skin activity of each fraction were summed (column N) and reported in Fig. 6 for comparison with the RAST I profile.

To further investigate the relationship between skin activity and RAST inhibitory activity, some of the

subjects described in Table 2 were selected according to differences in the skin reactivity pattern: group A was composed of subjects 11, 12, 21, 25 and 27 that were highly sensitive to fraction 6; group B was composed of subjects 10, 17, 18, 22 and 23 that showed no skin reactivity to this fraction. The sum of individual grades of reactivity to fractions 1-8 was calculated for each group from Table 2, and is

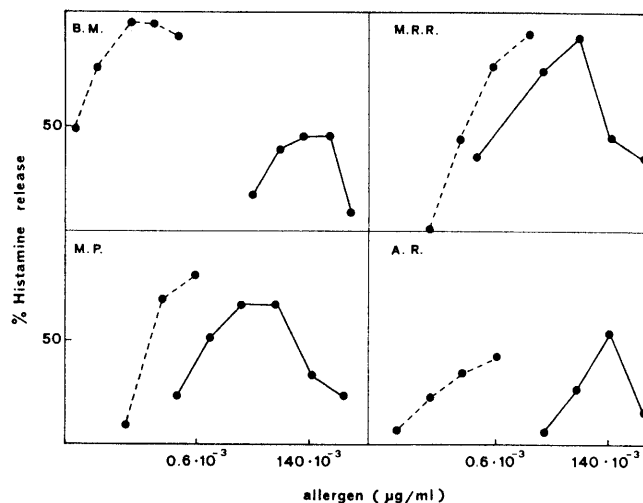


Fig. 3. Dose-response curve with leukocytes of four allergic donors. Comparison of activity of fraction C and PO15 in stimulating the release of histamine. B.M.: w.b.c.: 2.0×10^7 ; histamine available: $0.33 \mu\text{g/ml}$. M.R.R.: w.b.c.: 2.4×10^7 ; histamine available: $0.40 \mu\text{g/ml}$. M.P.: w.b.c.: 1.5×10^7 ; histamine available: $0.12 \mu\text{g/ml}$. A.R.: w.b.c.: 2.4×10^7 ; histamine available: $0.44 \mu\text{g/ml}$.

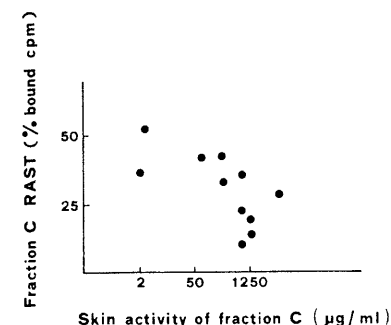


Fig. 4. A comparison of IgE antibody to fraction C and skin activity. Ordinate: C-RAST. Per cent bound c.p.m. Abscissa: skin activity (minimal amount giving a grade 1 response by prick-test—see Materials and Methods).

reported in Fig. 7 for comparison with RAST profiles. Pools of sera drawn from each group (pools a and b), were employed for RAST inhibition (diagrams C), using as inhibitors fractions of the column eluate (Fig. 7, left). Pool a and b identified different allergens, in good agreement with expectations based on skin reactivity patterns.

Rast I was also performed with individual sera obtained from patients that were skin reactive to 12 fractions; three profiles are reported (Fig. 7, right) showing again a good agreement of skin reactivity pattern with RAST inhibition profile.

In summary the data reported in Fig. 7 indicate that the sector of the eluate comprised between the elution vol of thyroxine and MIT contained at least six potential allergens (fractions 1, 2, 4, 5, 6 and 7) active in RAST I and the skin test, that behaved "minor" according to epidemiological criteria.

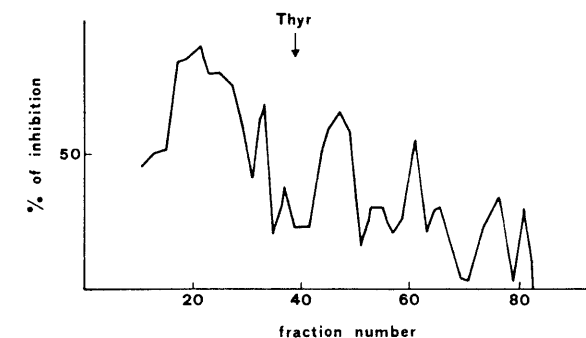


Fig. 5. Gel-filtration of fraction C on Biogel P2. Fraction C (0.25 ml), containing 12 mg protein/ml, was applied to a $0.6 \times 47 \text{ cm}$ column. Elution buffer: $0.125 \text{ M NH}_4\text{HCO}_3$, pH 8.7. Fraction vol: 0.34 ml . (—): RAST I: C-RAST; (bkg 150 cpm; 100%; 1300 cpm). Serum: pool. Inhibitors: alternate fractions. Molecular wt marker: thyroxine.

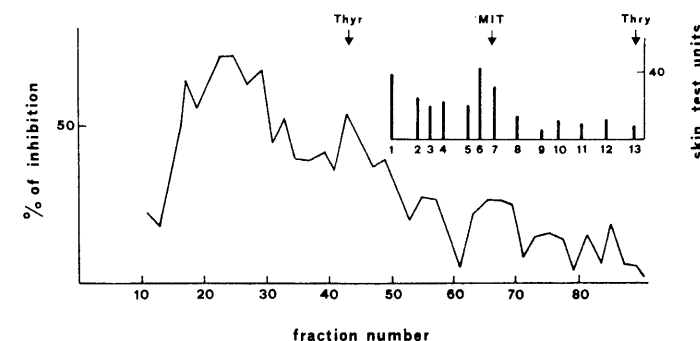


Fig. 6. Gel filtration on Sephadex G-10 of fraction C. Fraction C (0.25 ml), containing 12 mg protein/ml, was applied to a $0.6 \times 47 \text{ cm}$ column. Elution buffer: $0.125 \text{ M NH}_4\text{HCO}_3$, pH 8.7. Fraction vol: 0.34 ml . Molecular wt markers: same as Fig. 1. (—): RAST I: C-RAST. (bkg: 100 cpm; 100%; 1300 cpm). Serum: pool. Inhibitors: alternate fractions. Bars show the sum of skin activity grades (Table 2, column N).

Table 2. Subjects

Fraction	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	2	1	1	3	2	2	2	2	1	1	1	2	1	1	2	2
2	2	2	2	1	2	0	0	1	1	0	0	1	1	0	1	0
3	1	2	2	1	2	0	1	1	1	2	0	1	1	1	1	0
4	1	2	2	2	2	0	1	0	1	1	1	2	1	1	1	0
5	1	1	1	0	3	0	1	0	1	1	0	2	1	1	1	0
6	2	2	2	2	3	1	1	2	2	1	2	4	2	2	2	0
7	0	1	1	1	2	0	1	1	1	1	1	2	1	2	2	2
8	0	0	0	0	3	0	0	0	0	0	0	1	0	1	1	0
9	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
10	0	0	0	0	1	0	0	0	1	0	0	0	1	1	1	0
11	1	0	0	0	2	0	0	1	0	0	0	1	0	0	0	0
12	0	0	1	0	2	0	0	0	0	0	0	1	1	1	1	2
13	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
PO15	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

Fraction	17	18	19	20	21	22	23	24	25	26	27	28	29	30	N
1	1	0	0	2	0	1	2	2	1	2	1	0	1	2	41
2	3	3	0	1	1	1	1	1	0	0	0	0	1	1	27
3	1	1	1	0	0	0	0	0	0	0	1	0	1	1	23
4	0	1	0	0	1	1	1	1	2	1	0	1	2	3	30
5	1	2	1	0	1	1	0	0	1	1	0	0	1	1	24
6	0	0	0	1	2	0	1	0	2	2	2	2	2	3	47
7	0	1	0	0	2	0	2	1	1	1	2	1	2	2	34
8	1	1	1	0	1	0	1	1	1	0	0	0	0	1	15
9	0	0	0	0	1	0	1	0	0	0	0	0	0	0	4
10	0	0	0	0	1	1	1	1	0	0	0	0	0	1	10
11	0	0	0	1	0	0	1	1	0	0	0	0	0	0	8
12	0	0	0	0	1	0	1	1	0	0	0	0	0	0	12
13	0	0	0	0	0	0	1	1	0	1	0	0	0	0	5
PO15	4	4	4	4	4	4	4	4	4	4	4	4	4	4	120

Thirty subjects allergic to PO were skin-prick tested with fractions of the eluate of gel filtration on Sephadex G-10 (Fig. 3) and with a solution of PO15 containing 30 µg/ml. Their reactivity was evaluated as described under Materials and Methods, according to the diameter of wheals. N = sum of grades of reactivity for all patients.

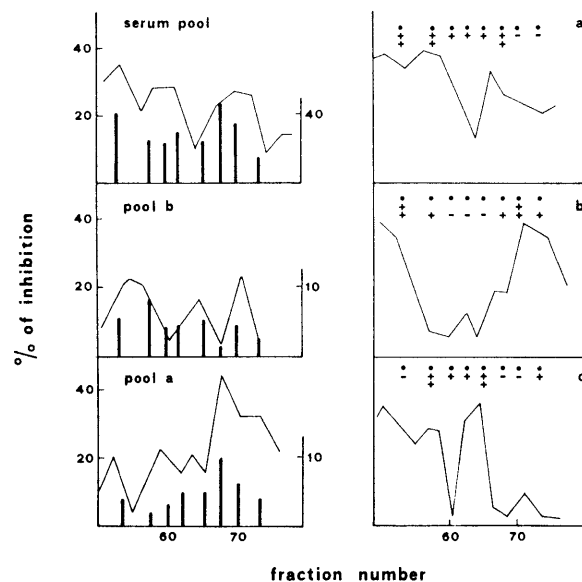


Fig. 7. Comparison of C-RAST inhibition profiles obtained with different sera. Left (from the top): serum pool, subpools a and b. Bars represent the sum of skin activity grades (from Table 2, column N). Right (from the top): individual sera a, b and c. + = 1 skin-test grade. Bkg: 110–130 cpm. 100%: serum pool: 1300 cpm; pool a: 1200 cpm; pool b: 1300 cpm; serum a: 1300 cpm; serum b: 1300 cpm; serum c: 1200 cpm.

DISCUSSION

Pollen allergens with mol. wts lower than 1000 have been described. Feo *et al.* (1984) identified an hapten (mol. wt approx. 1000), obtained by gel filtration from the pollen extract of *P. judaica*, that had ELISA inhibitory activity but was not skin active and disappeared when the extraction was performed in the presence of PMSF. Malley and Harris (1967) and Malley *et al.* (1975) reported that the dialysable fraction of the pollen extract of *Phleum pratense* cross-reacted in the RAST with the major allergen and showed slight skin activity in *Phleum* sensitive patients. Further studies showed that this fraction contained two allergens, D2 and D3, with a mol. wt of approx. 2500 and 1000, that behaved as haptens. Ag D3 was composed of a flavonoid pigment, quercetin, a disaccharide, cellobiose, and the amino acid threonine (Malley *et al.*, 1975, 1979; Malley and Harris, 1967). However, an extensive study on low mol. wt allergenic and/or antigenic components present in pollen extracts has not been carried out.

RAST inhibition in combination with gel filtration and the skin test provided a suitable assay for the identification of low mol. wt allergens of the pollen of *P. officinalis*. Although commonly used for the determination of allergenic potency of pollen extracts and for studies on cross-reactivity (King, 1979; Leiferman *et al.*, 1976; Lowenstein and Marsh, 1981), RAST I has been considered to give results of difficult interpretation with respect to the analysis of allergenic composition of pollen extracts and CRIE has been preferentially used (Adolphson *et al.*, 1978; Akrust *et al.*, 1979; Chakrabarty *et al.*, 1981); the latter assay however is not reliable for identification of low mol. wt allergens (Lowenstein *et al.*, 1981).

Our data suggest that RAST I performed with serum pool and discs bound to the crude extract is biased in favour of the identification of major allergens: minor allergens, that correspond to antibodies scarcely represented in serum pools because of low prevalence of individual responses, require for identification a limitation of the array of allergens bound to the discs and/or the use of individual sera of specifically responsive subjects.

Gel filtration of the low mol. wt fraction of the pollen extract (fraction C) on Sephadex G-10 allowed the separation of at least seven peaks of RAST inhibitory activity eluted after oxytocine (mol. wt 1040) and identified by a serum pool. The variability of individual patterns of response, shown by the skin-prick test and by RAST inhibition with individual sera, indicated that these allergens were not merely different molecular species carrying the same epitopes, but corresponded to molecules with different antigenic specificity in the IgE system.

Even though mol. wts reported are only indicative, it was surprising that low mol. wt allergens had skin activity; formation of aggregates in the eluate cannot be excluded. Skin activity of allergens with low mol.

wt has been reported. A dodecapeptide obtained from tryptic digestion of allergen M from cod, a apamin and mellitin, two allergens of honeybee venom (mol. wts 2840 and 2038) have been found to be skin active (Elsayed *et al.*, 1976; Haberman 1972).

Fraction C cross-reacted with the major allergen PO15 (Fig. 2); further investigations are needed to determine the cross-reactivity of single allergens. Immunological cross-reactivity of allergens derived from the same pollen or from pollens of different species or genera has frequently been reported (Leiferman *et al.*, 1976; Lowenstein and Marsh, 1981; Adolphson *et al.*, 1978). Although the extraction solution contained a proteinase inhibitor (PMSF) the presence of proteolytic activity during the extraction of the pollen cannot be excluded; thus it is possible that allergens of higher mol. wt produce, by enzymatic hydrolysis, low mol. wt fragments carrying few epitopes that reveal the fine specificity of individual responses.

Further investigations will be aimed at the purification and biochemical and immunological characterization of these allergens. This appears promising for the study of the fine mechanisms of genetic control of antigenicity and allergenicity.

Allergy to *P. officinalis* pollen is the major cause of hayfever in the Mediterranean area and the prevalence of this allergy among allergic adults reaches 60%. With respect to the problems related to standardization of commercial reagents for diagnosis and for desensitization therapy, it is important to investigate whether specific patterns of response characterize geographic areas. From a clinical point of view it is also relevant to investigate whether specific patterns of response are predictive of the outcome of therapy and/or correlated with symptomatology.

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