

The allergen profile of ash (*Fraxinus excelsior*) pollen: cross-reactivity with allergens from various plant species

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Summary

Background Ash, a wind-pollinated tree belonging to the family *Oleaceae*, is distributed world-wide and has been suggested as a potent allergen source in spring time.

Objective The aim of this study was to determine the profile of allergen components in ash pollen in order to refine diagnosis and therapy for patients with sensitivity to ash pollen

Methods The IgE reactivity profile of 40 ash pollen-allergic patients was determined by immunoblotting. Antibodies raised to purified pollen allergens from tree and grass pollens were used to identify cross-reactive structures in ash pollen extract. IgE immunoblot inhibition studies were performed with recombinant and natural pollen allergens to characterize ash pollen allergens and to determine the degree of cross-reactivity between pollen allergens from ash, olive, birch, grasses and weeds.

Results The allergen profile of ash pollen comprises Fra e 1, a major allergen related to the major olive allergen, Ole e 1, and to group 11 grass pollen allergens, the panallergen profilin, a two EF-hand calcium-binding protein, a pectinesterase-like molecule and an allergen sharing epitopes with group 4 grass pollen allergens. Thus, the relevant allergens of ash are primarily allergens that share epitopes with pollen allergens from other tree, grass and weed species.

Conclusions Allergic symptoms to ash pollen can be the consequence of sensitization to cross-reactive allergens from other sources. The fact that ash pollen-allergic patients can be discriminated on the basis of their specific IgE reactivity profile to highly or moderately cross-reactive allergens has implications for the selection of appropriate forms of treatment.

Keywords allergen profile, antibodies, ash pollen, IgE inhibition, recombinant allergens, spring pollinosis

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Introduction

Ash is a wind-pollinated tree that is widely distributed in Europe (Great Britain, Ireland, Scandinavia, Russia, Central and Southern Europe) and in America, where it is frequently grown as a source for hardwood timber [1–4]. In Europe, where ash blossoms from March to May, heavy loads of ash pollen have been measured and sensitization to ash pollen has been recognized as an important problem [5–9].

The genus *Fraxinus* belongs to the family *Oleaceae*, division *Magnoliophyta*, class *Magnolopsida*, order *Scrophulariales*. It is closely related to jasmine (*Jasminum*), lilac (*Syringa*), privet (*Ligustrum*), forsythia (*Forsythia*) and olive (*Olea*). Due to the close taxonomical relationship between ash and olive, cross-reactivity between these two pollen species has been reported. Studies performed in populations from

Mediterranean areas have shown that almost all patients exhibited concomitant skin sensitivity to both ash and olive pollen [9, 10]. Using ash and olive pollen extracts for IgE inhibition studies, evidence was provided for the presence of cross-reactive allergens/epitopes in both pollen sources [10, 11].

Several clinical observations indicated the possibility of cross-reactivity between ash pollen and birch pollen, the latter being one of the most important allergen sources in spring. These observations were confirmed by RAST inhibition studies revealing the presence of cross-reactive epitopes in ash and birch pollen [12]. A recent immunoblotting study performed with ash pollen extract provided evidence for the occurrence of allergens with cross-reactive potential (e.g. profilin and calcium-binding allergens) in ash pollen as a possible explanation for cross-reactivity between the pollens of ash and other unrelated plants [13].

In order to precisely identify the allergen profile in ash pollen we studied 40 clinically well-documented patients with sensitization to ash pollen. The allergen-profile recognized by the patients' sera was analysed by IgE immunoblotting.

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Ash pollen antigens with cross-reactivity to tree and grass pollen allergens were identified with specific antibodies raised against a panel of purified plant allergens (e.g. birch-, timothy grass pollen allergens). Furthermore, we used a panel of recombinant and natural plant allergens for IgE inhibition studies to identify cross-reactive ash allergens. In order to determine the degree of cross-reactivity between ash pollen allergens and pollen allergens from other sources, IgE inhibition experiments were performed crosswise. Our findings that all identified ash pollen allergens (e.g. profilin, two-EF hand calcium-binding allergens, Fra e 1, a pectinesterase, a grass pollen group 4-related allergen) were cross-reactive components suggests that allergic reactions to ash pollen are often due to sensitization with homologous allergens from other tree, grass and weed species. Our results indicate that the determination of the IgE reactivity profile to ash pollen allergens may discriminate patients with a genuine ash pollen sensitization from patients sensitized to other plant allergens which cross-react with ash pollen allergens. The determination of the precise sensitization profile to ash pollen allergens may thus help to refine diagnosis and treatment of ash pollen allergy.

Material and methods

Characterization of ash pollen-reactive patients

Forty ash pollen-allergic patients were selected from 106 patients with a case history indicative for tree pollen allergy (presence of at least one of the symptoms: rhinitis, conjunctivitis or asthma during spring, the flowering period of ash) (Table 1). The selection was performed according to positive skin prick test results with ash pollen extract (Stallergènes, Antony, France) and/or positive CAP RAST (Pharmacia, Uppsala, Sweden) results to ash pollen. The skin prick tests were performed with commercial extracts from ash, birch, olive tree, grass mix, weed mix, animal dander (cat/dog) and mites (Der p, Der f) (Stallergènes) and by prick to prick, mainly with apple. The IgE reactivity profile to nitrocellulose-blotted ash and birch pollen extracts was determined by IgE immunoblotting [14, 15]. IgE antibodies binding to recombinant allergens (rBet v 1, rBet v 2, rPhl p 7) were measured by ELISA [14, 15].

Natural pollen extracts

Pollens from ash (*Fraxinus excelsior*), birch (*Betula verrucosa*), timothy grass (*Phleum pratense*), ragweed (*Ambrosia elatior*) and olive (*Olea europaea*) were purchased from Allergon (Välinge, Sweden). Aqueous pollen allergen extracts were prepared as previously described [14, 15]. The protein contents and the quality of the extracts were analysed by SDS-PAGE and Coomassie Blue staining (Biorad, Richmond, CA, USA) [16, 17].

Recombinant allergen components

Recombinant birch pollen allergens (rBet v 1; rBet v 2 = birch profilin) were obtained from BIOMAY (Vienna, Austria); rBet v 1 and rBet v 2 represent the isoforms encoded by the cDNA originally isolated by IgE immunoscreening [18, 19]; rPhl p 7 was expressed in *Escherichia coli* and purified, as described previously [20].

Determination of IgE antibodies against rBet v 1, rBet v 2 and rPhl p 7 by ELISA

ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with 5 µg/mL rBet v 1, rBet v 2 or rPhl p 7 and stored at 4 °C overnight. The plates were washed three times with buffer B (TBS, 0.05% vol/vol Tween 20) and blocked with 1% wt/vol BSA in buffer B at room temperature for 4 h. The plates were incubated with 100 µL/well of the sera diluted 1:10 in buffer B at 4 °C overnight and washed five times with buffer B. An alkaline phosphatase-coupled mouse monoclonal anti-human IgE antibody (PharMingen, San Diego, CA, USA) diluted 1:1000 in TBS, 0.05% vol/vol Tween 20 and 0.5% wt/vol BSA was used to detect bound IgE antibodies. After 24 h of incubation at 4 °C, the plates were washed three times with buffer B and the colour reaction was started with alkaline phosphatase substrate (p-nitrophenyl phosphate, disodium, hexahydrate; Sigma Diagnostics, St Louis, MO, USA). The optical density at 405 nm was determined with an ELISA reader (Dynatech, Denkendorf, Germany). All measurements were carried out in duplicate.

IgE immunoblot/IgE immunoblot inhibition experiments

Ash, olive and birch pollen extracts (approximately 200 µg of protein per cm gel) were separated by 12.5% preparative SDS-PAGE and blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) [21]. The sera from ash pollen-allergic patients were diluted 1:10 in buffer A (50 mmol/L Na phosphate [pH 7.5], 0.5% wt/vol BSA, 0.5% vol/vol Tween 20, and 0.05% wt/vol NaN₃) and used to probe nitrocellulose-bound ash and birch pollen extract. Bound IgE antibodies were detected as described [22]. To study the cross-reactivity between ash pollen allergens and rBet v 1, rBet v 2, rPhl p 7 and natural allergens from birch, timothy grass, ragweed and olive pollen, IgE-immunoblot inhibition experiments were performed. The sera were diluted 1:10 in buffer A and pre-incubated overnight at 4 °C either with recombinant allergens (5 µg of each recombinant allergen per 1 mL of serum dilution), with 40 µg of natural ash, birch, timothy grass, olive and ragweed pollen extract, or for control purposes, with BSA (40 µg per 1 mL of serum dilution). The pre-adsorbed sera were then incubated overnight at 4 °C with nitrocellulose-blotted allergen extracts (ash and olive pollen), and bound IgE antibodies were detected as described above.

Detection of cross-reactive allergens in ash pollen extract using specific antisera

Polyclonal rabbit antisera were raised against purified recombinant tree pollen (rBet v 1, rBet v 2, rAln g 4) [18, 19, 23] and timothy grass pollen (rPhl p 1, rPhl p 2, rPhl p 5) [24, 25] allergens by immunizing New Zealand white rabbits with the purified recombinant allergens emulsified in complete Freund's adjuvant (CFA) (Charles River, Kisslegg, Germany). Likewise, a rabbit antiserum was raised against the recombinant C-terminal 70 amino acids of pectinesterase-like birch pollen allergen, Bet v 8 [26, 27] and against purified natural Phl p 4 from timothy grass [28]. Pre-immune sera were obtained from each of the rabbits prior to immunization. Nitrocellulose-blotted ash pollen extract was exposed overnight at 4 °C to the rabbit immune sera or the corresponding pre-immune sera

Table 1. Demographic, clinical and serological characterization of 40 ash pollen-allergic patients

No.	Age	Sex	Allergies				SPT			RAST Ash (kUA/L)	ELISA		
			Positive SPT	Symptoms	Season (months)	Duration (years)	Histamine (MD)	Ash (MD)	Birch (MD)		rBet v 1 OD	rBet v 2 OD	rPhl p 7 OD
1	38	m	a, t	r, c	3–5	2	4.5	8.5	neg.	5.65	–	–	–
2	21	f	t, g, w, a	r, c, a	3–7	15	6.5	10	11.5	6.51	0.257	0.470	0.139
3	36	m	t, o, g, w, mi, a	r, c, a	3–8	10	6	8.5	7.5	8.39	1.385	0.241	–
4	27	m	t, o, w, mi	r, c, a	3–6	6	6.5	13	8.5	26.5	1.900	0.154	–
5	51	m	t	r, c, a	2–5	30	5	5	5.5	0.71	0.255	–	–
6	45	f	t, o, g, w, f	r, c, a	2–7	30	5.5	8.5	8.5	5.93	0.906	0.357	–
7	40	m	t, o, g, a, f	r, c, a	2–7	32	5	10	11	2.70	0.208	–	–
8	42	m	t, o, f	r, c	4–5	10	7	11.5	n.d.	0.68	1.200	–	–
9	24	f	t, o, g, a, f	r, c, a	2–6	10	6	12.5	9	46.0	1.249	0.048	–
10	21	f	t, o, g, w, f	r, c	2–6	15	8	5	7	2.27	0.373	0.146	–
11	31	f	t, g	r	4–6	n.k.	5	4.5	7.5	1.19	0.641	–	–
12	37	f	t, o, g, f, a	r, c, a	1–4	10	5	9	5	9.63	1.317	–	–
13	33	m	t, o	r, c	2–9	4	6.5	7.5	7	0.37	0.244	–	–
14	45	m	t, o	r, c, a	3–4	2	7.5	13.5	6	8.64	–	–	–
15	39	f	t, o, g, f	r, c, a	3–5	9	5	8	14.5	2.77	0.641	–	0.078
16	52	f	t, o, g, w, mi, f	r, c, a	1–5	10	5.5	7.5	6	4.34	0.696	–	–
17	n.k.	m	t, o, a	r, c	2–6	15	5	4	7.5	–	0.533	–	–
18	16	f	t, o, w, f	r, c, a	1–7	6	5.5	5	11	1.32	0.893	–	–
19	15	f	t, o, g, w, a	r, c, a	1–8	10	n.d.	n.d.	n.d.	13.4	–	0.270	0.559
20	58	m	t	r, c	3–5	3	7.25	n.d.	9.75	1.90	0.166	–	–
21	26	f	g, w	r, c	5–6	3	7.25	8	–	3.23	–	–	–
22	18	f	t, o, g, mi, a, f	r, c	2–6	6	8	14	8	5.34	1.199	–	–
23	53	f	w	r, a	3–5	1	8	8.5	–	1.57	–	–	–
24	25	f	t, o, g, w, f	r, c, a	2–6	15	3.5	5	5	0.95	1.235	0.211	–
25	29	m	t, o	r, c, a	2–5	2	n.d.	n.d.	n.d.	7.86	1.246	–	–
26	25	f	t, o	r, c, a	2–7	9	5.5	8	7.5	0.57	0.113	–	–
27	34	f	t, o, mi, a, f	r, c	3–5	16	5.5	7	9	0.67	0.244	–	–
28	23	m	t, o, g, w, mi	r, c	4–7	10	6	5	9	0.40	0.380	0.087	–
29	20	f	t, o, g, w, f	r, c, a	3–9	5	6.5	5.5	7	11.5	1.605	0.101	–
30	42	m	t, o, g, w, mi, f	r, c, a	1–5	20	5	8	6.5	7.87	1.426	0.200	–
31	53	m	t, o	r, c	1–5	4	4.5	4.5	6	13.9	0.165	–	–
32	27	m	t, o, g, f	r, c, a	2–6	10	5.5	n.d.	5.5	34.3	1.458	–	1.448
33	32	m	t, o, w, mi, a, f	r, c, a	3–5	24	5.5	7	6.5	4.95	1.194	0.155	–
34	47	f	t, o, g, w, f	r, c, a	2–8	20	5	10.5	8.5	0.54	0.563	–	–
35	22	m	t, o, g, f	r, c	2–8	n.k.	5.5	10.5	4.5	2.91	1.167	–	–
36	31	f	t, o, g, w, f	r, c, a	1–5	5	7	6	9	3.12	0.919	0.312	–
37	50	m	t, o, a	r, c	1–4	4	6.5	6.5	5.5	0.94	0.091	–	–
38	25	f	t, o, w, f	r, c	1–5	5	5.5	n.d.	6	17.30	1.329	0.104	–
39	52	f	t, w	r, c, a	3–5	8	8	8.5	0	2.05	n.d.	n.d.	n.d.
40	21	m	t	r, a	3–5	n.k.	5.5	5	n.d.	2.62	1.157	–	–

SPT with olive pollen extract was not performed in patients 1, 2, 5, 11, 20, 21, 23, 39, 40. n.k. = not known, n.d. = not done, – = negative. Sex: m = male, f = female. Allergies: SPT = skin prick test, t = trees, o = olive, g = grasses, w = weeds, mi = mites, a = animals, f = foods. Symptoms: r = rhinitis, c = conjunctivitis, a = asthma. MD = mean diameter of the weal reaction. OD = optical density.

diluted 1:1000 in Buffer A. Bound rabbit antibodies were detected with ¹²⁵I-labelled donkey anti-rabbit antiserum (Amersham, Buckinghamshire, UK) and visualized by autoradiography.

Results

Clinical and serological characterization of 40 ash pollen-allergic patients

Ash pollen-allergic patients were selected from 106 patients with spring pollinosis according to positive CAP RAST and/or skin prick test results with ash pollen extract. Thirty-seven

per cent (40/106) of these patients were sensitized to ash pollen. Serological and clinical data of these patients are displayed in Table 1.

The investigated group consisted of 19 men and 21 women. All had rhinitis, 37 complained about conjunctivitis and 25 suffered from asthma. Skin prick testing indicated sensitization to the following allergen sources: pollen of trees belonging to the *Fagales* (birch, alder, hazel, hornbeam) for 38; olive pollen for 31; grass pollen (mixture) for 21; weeds (mugwort, ragweed, plantain) for 18; mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*) for 8, animal allergen extracts (cat and dog) for 12, and apple for 20 of the 40 patients tested in this study (Table 1).

Skin prick tests with ash pollen extract were performed in 35 patients and were positive in all cases. Skin prick tests with birch pollen extract performed in 35 patients were positive in 32 and negative in three cases. CAP RAST determinations with ash pollen extract were positive in 39 out of 40 patients.

All but six patients contained IgE antibodies against recombinant Bet v 1, the major birch pollen allergen. Fourteen of the sera displayed IgE reactivity to recombinant birch profilin (rBet v 2) and four sera reacted with rPhl p 7, a two EF-hand calcium-binding allergen sharing epitopes with homologous allergens from birch (Bet v 4), alder (Aln g 4) and rape (Bra r 1) [20] (Table 1).

IgE reactivity profiles of the patients to nitrocellulose-blotted ash pollen extract

IgE immunoblot experiments with ash pollen extract are displayed in Fig. 1(a and b) and summarized in Table 2. The most frequently recognized allergen was a 21 to 25 kDa band detected by 23/40 patients (57.5%). Twelve to eighteen kDa components were detected by IgE antibodies from 20/40 patients (50%). Twelve patients (30%) displayed IgE reactivity to an antigen cluster consisting of several bands of more than 30 kDa (HMW allergens). A single band of 35 kDa was recognized by IgE antibodies from six patients (15%). Three sera (7.5%) bound to an allergen of 30 kDa and two patients (5%) had IgE antibodies to antigens of 6 to 8 kDa.

Twenty-one out of 40 patients exhibited IgE reactivity to several components of different molecular weights (Table 2). Nineteen sera displayed exclusive IgE reactivity to single bands in blotted ash pollen extract. Six sera reacted exclusively with the 21 to 25 kDa components, eight sera reacted exclusively with the 12 to 18 kDa and three bound exclusively to the 35 kDa band (Figs 1a and b; Table 2).

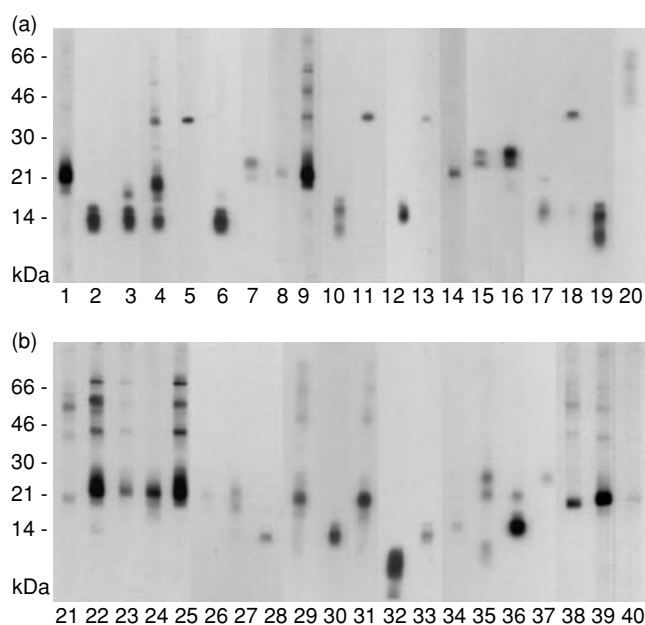


Fig. 1. IgE reactivity profile to nitrocellulose-blotted ash pollen extract. The sera from the 40 patients characterized in Table 1 were exposed to blotted ash pollen extract (a, sera 1–20; b, sera 21–40). Molecular weights are displayed on the left side.

The levels of ash pollen-specific IgE and skin sensitivity to ash pollen extract were low in the group of patients with exclusive reactivity to the 35 kDa band (sera# 5, 11, 13) (Fig. 1, Table 1). No association between skin sensitivity to ash pollen extract (mean weal diameters) and the levels of ash pollen-specific serum IgE (kUA/L) were noted (Fig. 1, Table 1).

Rabbit antisera raised against purified recombinant and natural pollen allergens detect cross-reactive antigens in ash pollen extract

In order to identify cross-reactive ash pollen allergens we used rabbit antisera that were raised against purified recombinant tree and grass pollen allergens and against the natural timothy grass pollen allergen, Phl p 4. Using these antisera we detected profilin and a two-EF hand calcium-binding allergen as major cross-reactive ash pollen allergens (Fig. 2). A rabbit antiserum raised against the C-terminal portion of a cross-reactive birch pollen allergen, Bet v 8, representing a pectinesterase, cross-reacted with several bands in ash pollen extract (Fig. 2). Likewise we found that a rabbit antiserum raised against natural Phl p 4, a high molecular weight cross-reactive timothy grass pollen allergen, reacted specifically with several bands ranging from 20 to 70 kDa in ash pollen extract (Fig. 2). By contrast, rabbit antisera raised against recombinant allergens that occur exclusively in grass pollens (group 1: rαrPhl p 1; group 2: rαrPhl p 2; group 5: rαrPhl p 5) did not react with ash pollen extract (Fig. 2). A rabbit antiserum raised against the major birch pollen allergen, Bet v 1, gave a very weak reactivity with an ash pollen antigen with a molecular weight comparable with Bet v 1 (i.e. 17 kDa).

Table 2. Summary of the IgE recognition profile of ash pollen-sensitized individuals ($n = 40$). (molecular weights of IgE-reactive bands, frequencies of recognition)

Molecular weight	Number of reactive sera	Number of monoreactive sera
6–8 kDa	2	1
12–18 kDa	20	8
21–25 kDa	23	6
30 kDa	3	0
35 kDa	6	3
HMW (> 30 kDa)	12	1

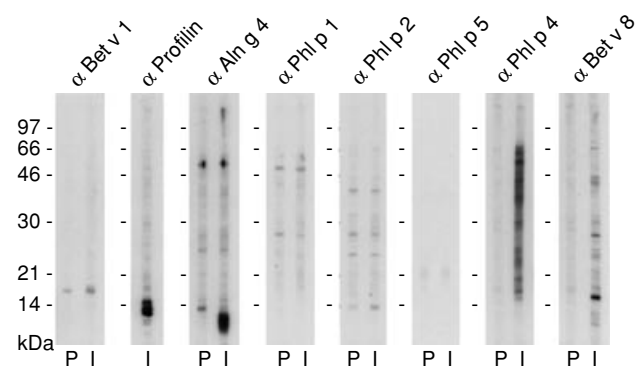


Fig. 2. Reactivity of allergen-specific rabbit antisera with cross-reactive ash pollen antigens. P = pre-immunization rabbit serum, I = rabbit serum after immunization with purified allergen components. Molecular weights are displayed on the left side.

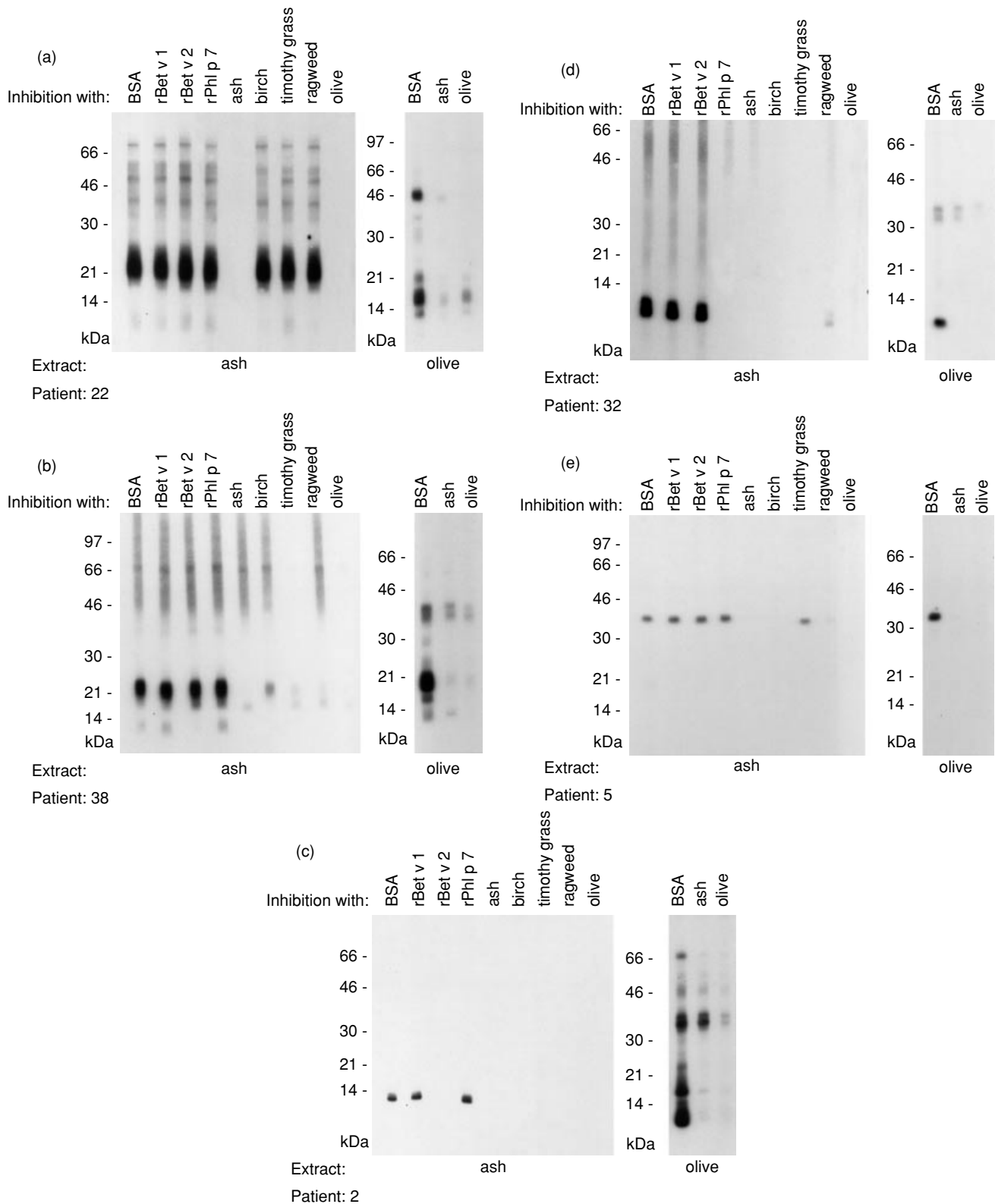


Fig. 3. (a–e). IgE inhibition experiments. The sera from five representative ash pollen-sensitized patients (a = serum 22; b = serum 38; c = serum 2; d = serum 32; e = serum 5) were exposed to nitrocellulose-blotted ash pollen (left panel, ash) or olive pollen extract (right panel, olive). Sera were pre-adsorbed with the antigens described on top of the Figure. Molecular weights are displayed on the left margins.

IgE inhibition experiments with recombinant and natural allergens identify cross-reactive allergens in ash pollen

Five sera (#2, #5, #22, #32, #38) exhibiting a range of typical IgE reactivity profiles to ash pollen extract were used for IgE immunoblot inhibition experiments to identify cross-reactive allergens. The sera were selected to contain high levels of IgE against birch profilin (rBet v 2) (e.g. serum #2), against rPhl p 7 (e.g. serum #32), to Western-blotted Fra e 1 (e.g. sera #22, 38) or from a patient with exclusive reactivity to the 35 kDa ash pollen component (serum #5). The sera were pre-adsorbed with recombinant allergens (rBet v 1, rBet v 2, rPhl p 7) or with natural pollen extracts from ash, birch, timothy grass, ragweed and olive before incubation with nitrocellulose-bound ash pollen extract (Figs 3a–e, left). A cross-inhibition experiment was performed by pre-adsorption of the same sera with ash or olive pollen extract before incubation with nitrocellulose olive pollen extract (Figs 3a–e, right). The following results were obtained.

1. A major 21 to 25 kDa allergen of ash pollen shares IgE epitopes with the major allergen of olive Ole e 1, and cross-reactive grass and weed pollen allergens The immunological relationship of the most frequently recognized 21 to 25 kDa ash pollen protein, tentatively designated Fra e 1, with allergens in other pollen extracts was investigated by IgE immunoblot inhibition experiments. IgE reactivity of serum #22 to the 21 to 25 kDa major ash pollen allergen was abolished after pre-adsorption of the serum with ash and olive pollen extracts (Fig. 3a). IgE recognition of the 21 to 25 kDa ash pollen allergen by serum #38 was strongly reduced after pre-adsorption with birch pollen extract (Fig. 3b). Pre-adsorption with timothy grass, ragweed, and olive pollen extract completely abolished IgE binding to this component. Pre-adsorption of sera 22 and 38 with rBet v 1, rBet v 2 and rPhl p 7 did not affect IgE binding to Fra e 1 (Figs 3a and b). Both sera displayed IgE reactivity with a 21-kDa component in olive pollen extract that was inhibited after pre-adsorption with ash pollen extract (Figs 3a and b, panels on the right).

2. Ash pollen contains a profilin-homologous allergen Serum #2 reacted exclusively with a 10 to 14 kDa component in blotted ash pollen extract and contained high levels of rBet v 2-specific IgE antibodies (Fig. 1a, Table 1). Pre-adsorption of this serum with rBet v 2 abolished IgE binding to the 10 to 14 kDa ash pollen allergen (Fig. 3c). Likewise, pre-adsorption of this serum with pollen extracts containing profilin (e.g. birch, timothy grass, ragweed and olive pollen) inhibited IgE binding to ash pollen profilin (Fig. 3c). Serum #2 also reacted with a 14-kDa band in olive pollen that was inhibited after pre-adsorption with ash pollen extract (Fig. 3c, panel on the right).

3. Ash pollen contains a 2-EF-hand calcium-binding allergen Serum #32 reacted exclusively with a 6 to 8 kDa ash pollen allergen and rPhl p 7, a highly cross-reactive calcium-binding allergen from timothy grass. Pre-adsorption of this serum with rPhl p 7 but not with rBet v 1 or rBet v 2 abolished IgE binding to the 6 to 8 kDa ash pollen allergen (Fig. 3d). All pollen extracts containing Phl p 7 homologous allergens (birch, ragweed and olive pollen) inhibited IgE binding to the two-EF hand calcium-binding allergen from ash pollen. Patient #32 was sensitized exclusively against a 6 to

8 kDa component in olive pollen extracts and this reactivity was completely blocked by pre-adsorption with ash pollen extract (Fig. 3d, panel on the right).

4. Ash pollen contains a 35-kDa allergen with cross-reactivity to birch, ragweed and olive pollen allergens

Three ash pollen-sensitized patients displayed exclusive IgE reactivity to a 35-kDa ash pollen allergen (Fig. 1) and their sera also contained IgE antibodies to components of comparable molecular weight in birch pollen extract (data not shown). Pre-adsorption of serum #5 with birch, ragweed and olive pollen extract inhibited IgE binding of this serum to the 35 kDa ash pollen allergen, whereas pre-adsorption with timothy grass pollen extract or recombinant allergens (rBet v 1, rBet v 2, rPhl p 7) did not affect IgE reactivity to this allergen (Fig. 3e, panel on the left). Serum #5 reacted exclusively with a 35-kDa in olive pollen extract and this IgE binding was inhibited by ash pollen extract (Fig. 3e, panel on the right).

5. Thirty percent of ash pollen-allergic patients react with high molecular weight components > 30 kDa Twelve patients had IgE antibodies to several high molecular (HMW) ash pollen allergens of more than 30 kDa, one of them (#20) reacting exclusively with HMW allergens. IgE reactivity of serum #38 in Fig. 3(b) with the high molecular ash pollen allergens was inhibited after pre-adsorption with timothy grass and olive pollen extracts. The experiments performed with rabbit antisera raised against Phl p 4 from timothy grass and against a recombinant peptide from a birch pollen pectinesterase indicate that certain of the high molecular weight allergens contain cross-reactive polypeptide epitopes (Fig. 2).

Cross-inhibition experiments indicate that ash pollen contains allergens that cross-react with pollen allergens from other plant species

To determine if ash pollen-sensitive patients were primarily sensitized to ash or to the taxonomically closely related olive tree, cross-inhibition experiments with olive pollen extracts were performed in 10 sera (sera #22, #38, #2, #32 and #5, Figs 3a–e, panels on the right; sera #19, #24, #35, #36, #39, data not shown). In all cases pre-adsorption of sera with olive pollen extracts led to a strong reduction or complete inhibition of IgE binding to nitrocellulose-blotted ash pollen allergens, whereas incomplete inhibition of IgE binding to olive pollen extracts was observed when sera were pre-adsorbed with ash pollen extract. These results, together with the data obtained by IgE inhibition experiments, indicate that allergy to ash pollen is in most cases may be caused by sensitization to cross-reactive pollen allergens from other plant species (e.g. grasses, weeds and trees).

Discussion

It has been reported that almost 20% of pollen-allergic patients are sensitized to ash pollen, which has therefore been proposed as a relevant allergen source, particularly in spring [13, 29]. Although some of the ash pollen allergens have been characterized [13], there has been limited information regarding the allergenic components of ash pollen and their immunological

relationship to components present in other allergen sources. In order to study the allergen profile of ash pollen we used sera from a group of 40 ash pollen-sensitive patients living in an area (Strasbourg, France) exposed to high loads of ash pollen in spring. Purified recombinant as well as natural pollen allergens from various plant species were used for IgE inhibition experiments to identify cross-reactive ash pollen allergens. In addition, we used a panel of specific rabbit antisera that were raised against purified recombinant and natural pollen allergens to further characterize ash pollen allergens. The results of our analysis indicate that allergens in the 21 to 25 kDa weight range contain the major ash pollen allergen, Fra e 1, exhibiting immunological similarities to the major olive pollen allergen, Ole e 1 [30, 31]. It is possible that the 21 to 25 kDa complex may contain, in addition to Fra e 1, other allergens. Fra e 1 has previously been described as the most important allergen in ash pollen [13]. We also found that a high percentage of patients reacted with this component. Furthermore many of the Fra e 1-reactive patients contained high levels of ash pollen-specific IgE and exhibited pronounced skin sensitivity to ash pollen extract. Fra e 1-specific IgE antibodies cross-reacted with Ole e 1 and interestingly almost all of the Fra e 1-positive patients showed sensitivity to olive pollen, although olive trees are uncommon in the Strasbourg area.

Ole e 1 belongs to a family of conserved plant proteins with sequence similarities to soybean trypsin inhibitor-related proteins that occur in pollens of many unrelated plant species (e.g. trees, grasses and weeds) and hence may act as cross-reactive allergens [30–35]. Due to a considerable sequence diversity among the allergens of the Ole e 1 family, cross-reactivity of IgE antibodies can be limited [30], a hypothesis that is supported by the results of our experiments indicating a limited cross-reactivity between ash, ragweed and timothy grass, which could be due to the fact that the botanical relationship between ash, ragweed and timothy grass is lower than that between ash and olive.

A considerable percentage of ash pollen-allergic patients reacted with profilin, a cytoskeletal actin-binding protein that represents one of the most cross-reactive allergens [19,22,36,37]. In addition to profilin we identified a 2 EF-hand calcium-binding protein as an allergen in ash pollen, both having previously been described as ash pollen allergens by Hemmer *et al.* [13]. Two EF-hand calcium-binding allergens occur as highly cross-reactive allergens in pollens of botanically unrelated tree, grass and weed species [38–40, reviewed in 41]. IgE-binding to ash pollen profilin and the 2 EF-hand ash allergen were completely inhibited after pre-adsorption of the sera with birch profilin (rBet v 2) and rPhl p 7, the 2 EF-hand allergen from timothy grass pollen, respectively. The latter result indicates cross-reactivity as an explanation for the observation that patients containing IgE antibodies to rBet v 2 and rPhl p 7 appeared to be sensitized to a variety of pollen extracts and plant food.

Ash pollen, as well as pollen extracts from a variety of other plant-species (trees, grasses, weeds), contains IgE-reactive structures of >30 kDa molecular weight (i.e. high molecular weight: HMW allergens). There is increasing evidence that these IgE-reactive structures consist of several immunologically distinct glycoproteins with varying cross-reactive potential [27,28,42,43]. While there is evidence for the presence of IgE-reactive carbohydrate epitopes in this HMW allergen complex [44,45], several allergens have been identified by

molecular cloning as high molecular weight polypeptide allergens. One of these allergens is Bet v 8, a pectinesterase-like birch pollen allergen [27]. Other high molecular weight protein allergens with cross-reactive potential have been characterized also in cypress pollen [46,47]. The presence of a Bet v 8-related allergen in ash pollen is indicated by the reactivity of Bet v 8-specific antibodies with antigens of similar molecular weight in ash pollen. Furthermore, we demonstrate that a rabbit antiserum raised against the cross-reactive HMW grass pollen allergen, Phl p 4, cross-reacts with a related ash pollen allergen. Our observation that the 35 kDa ash pollen allergen has a molecular weight similar to Bet v 5, the birch pollen isoflavone reductase, and the fact that birch pollen extract inhibited binding to the 35 kDa ash pollen allergen suggest the possibility that an isoflavone-reductase-like protein might exist in ash [48].

That IgE reactivity to the HMW ash pollen allergens and to the 35 kDa ash pollen allergens may be of little clinical relevance is suggested by the fact that the sera of patients reacting exclusively with these components contained low amounts of ash pollen-specific IgE and that these patients exhibited weak skin sensitivity to ash pollen extract. This is in accordance with previous reports suggesting poor biological activity of HMW IgE-reactive glycoproteins [44].

In conclusion, our study determines the allergen profile of ash pollen and provides tools (i.e. nitrocellulose-blotted ash pollen extract, recombinant allergen molecules) for component-resolved analysis of ash pollen sensitization. We believe that the determination of the allergen-profile of ash pollen can have clinical relevance because it allows the identification of a group of ash pollen-sensitized patients with a preferential reactivity to Fra e 1. Due to the extensive cross-reactivity of Fra e 1 with Ole e 1 it may be possible to design immunotherapy protocols for such patients that use vaccine formulations based on the Fra e 1/Ole e 1 cross-reactive epitopes. The other group of ash pollen-sensitized patients who reacted preferentially to highly cross-reactive allergens (e.g. profilin, 2 EF-hand allergens) showed allergic symptoms to several other allergen sources and therefore pharmacological treatment would be more appropriate. In the case of monosensitizations to the cross-reactive allergens, immunotherapy treatment with vaccine formulations based on the cross-reactive allergens may be considered.

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