

Standardization of an Ash (*Fraxinus excelsior*) Pollen Allergen Extract

Maud Hrabina^a Ashok Purohit^b Jean-Philippe Oster^c Ioanna Papanikolaou^c
Karine Jain^a Poncet Pascal^d Hubert Sicard^a Brigitte Gouyon^a
Philippe Moingeon^a Gabrielle Pauli^b Claude André^a

^aDépartement Scientifique et Médical, Stallergènes SA, Antony, ^bHôpitaux Universitaires de Strasbourg, Strasbourg, ^cHôpital de Colmar, Colmar, and ^dInstitut Pasteur, Immunité Anti-Virale, Biothérapie et Vaccins, Paris, France

Key Words

Allergens • Ash pollen • *Fraxinus excelsior* • Immunotherapy

Abstract

Background: Ash tree (*Fraxinus excelsior*) is the main representative of the Oleaceae family in temperate zones. Diagnosis of ash pollen allergy is made difficult due to (1) an overlapping pollinization period with Betulaceae, (2) non-inclusion in current diagnostic assays, and (3) some cross-reactivity with minor allergens from Betulaceae. The aim of this study was to calibrate an ash pollen in-house reference preparation (IHRP) in allergic patients in order to produce standardized products for diagnosis and immunotherapy purposes. **Methods:** Ash pollen IHRP was extracted, ultrafiltered and freeze dried. Allergens in the extract were detected after 2-dimensional PAGE using specific sera and a monoclonal antibody. The Fra e 1 content of IHRP was evaluated by quantitative immunoprint. Forty-eight subjects from the North-East of France exhibiting clinical symptoms, a positive skin test and specific IgE levels $\geq$ class 2 to ash pollen were recruited. IgE immunoprints were performed to select patients sensitized to the ash Fra e 1 allergen as opposed to cross-reacting allergens. Serial 10-fold dilutions of the IHRP were tested by skin prick tests in order to determine the concentration inducing a geometrical mean wheal diameter of 7 mm, said to correspond to an index of reactivity (IR) of

100 per millilitre. **Results:** IgE-reactive molecules in IHRP comprise Fra e 1, Fra e 2, a 9-kDa molecule (presumably Fra e 3), as well as a doublet at 15 kDa and high molecular weight allergens. The 100 IR concentration of IHRP inducing a geometrical mean wheal diameter of 7 mm in 22 patients sensitized to Fra e 1 corresponds to the 1/126 (w/v) extraction ratio (i.e. 259 μ g/ml of protein by Bradford) and contains 17 μ g/ml of Fra e 1. The variability in total activity of 5 batches of standardized extracts was found to be significantly reduced when compared with 7 non-standardized extracts. **Conclusion:** An ash pollen IHRP was defined and molecularly characterized. Its successful standardization at 100 IR/ml in patients specifically sensitized to Fra e 1 allowed a skin reactivity-based calibration in properly diagnosed patients. Such a standardized ash pollen extract is a reliable tool to support immunotherapy of ash pollen allergy.

Copyright © 2007 S. Karger AG, Basel

Introduction

The ash tree (*Fraxinus excelsior*) is a wind-pollinated member of the Oleaceae plant family including Olea, Ligustrum (privet), Syringa (lilac) and Forsythia. *Fraxinus* is widely distributed [1, 2]. In middle European temperate zones (France, North of Spain, Great Britain, Central Europe and South of Scandinavia), it grows naturally in

woods and is planted in gardens, parks and streets where it cohabits with members of the Betulaceae family. In areas without any olive trees, ash is the main representative of the Oleaceae family. In Strasbourg, atmospheric levels of ash pollen can reach a concentration of 1,000 grains/m³ [3, 4].

Ash pollen has been recognized in recent years as a frequent cause of spring pollinosis [3, 5–8]. Ash sensitization occurs more frequently in polysensitized patients [9, 10]. In Switzerland, ash pollen is responsible for spring pollinosis just as frequently as birch pollen [11].

The impact of ash pollen allergy in upper respiratory tract diseases has often been underestimated (1) due to the overlapping of its pollinization period (March to April) with other trees from the Betulaceae family [3, 4] and (2) because ash pollen extracts are not included in routine screening assays for inhalant allergies [12, 13]. The differential diagnosis of ash and birch pollen allergies based on total extracts is difficult because of immune cross-reactivity between these two pollens. Previous studies have described the existence of immunologic cross-reactivity among Oleaceae members due to their phylogenetic proximity [7, 14–18]. Currently available in vivo or in vitro diagnostic reagents cannot distinguish cosensitization to the two pollens from cross-sensitization due to minor allergens.

Based on previous studies [9, 10], we reasoned that diagnosis of ash pollen allergy should be complemented by a direct confirmation of IgE reactivity against Fra e 1. In this regard, an IgE assay using a recombinant Fra e 1 molecule [3] would be useful for routine in vitro screening, in complement to an ash skin prick test. Such an assay would help selecting the most appropriate extract to be used for specific immunotherapy in patients with positive skin tests to birch and ash.

The ash pollen major allergen Fra e 1 is a 21- to 25-kDa molecule exhibiting a high amino acid sequence identity with Ole e 1 [3, 19]. Following SDS-PAGE, it appears as a 17- to 21-kDa band [5]. Fra e 1 is recognized by 57–70% of ash pollen-allergic patients [5, 6]. In addition, minor allergens include: (1) the profilin Fra e 2 (13–16 kDa) recognized by 50% of ash-allergic patients, for which recognition by IgE is totally inhibited by olive, birch and timothy grass extracts [5, 20]; (2) a 35-kDa allergen homologous to Bet v 5, recognized by 15–33% of ash-allergic patients, for which recognition by IgE is totally inhibited by olive and birch extracts [5]; (3) Fra e 3, a 6- to 10-kDa minor allergen homologous to Bet v 4 and Ole e 3, for which recognition by IgE is totally inhibited by timothy grass/birch extracts [5, 6]; (4) glycoproteins with a mo-

lecular weight higher than 35 kDa recognized by 50–80% of ash-allergic patients, for which recognition by IgE is totally blocked by olive and timothy grass extracts [5, 6], and (5) a 45-kDa 1,3-b-glucanase [21].

Ash pollen extracts currently available for diagnosis and immunotherapy have not been standardized. The need for standardization of allergenic extracts used for diagnosis or treatment has been recognized since the early days of allergen immunotherapy. The purpose of allergen standardization is to keep the potency of allergen extracts constant as well as to minimize batch to batch variation in composition. However, due to the complexity of allergen extracts, it is not possible to measure all allergen molecules. Consequently, the Note for Guidance on Allergen Products CPMP/BWP/243/96 establishes that in Europe, commercial batches must be compared with an in-house reference preparation (IHRP) using a number of biological and analytical methods to ensure batch to batch consistency [22, 23].

The objective of this study was to define an ash pollen IHRP and to characterize it in terms of its allergen composition. We also calibrated the biological potency of the IHRP in vivo by skin prick tests on more than 20 patients allergic to ash pollen. We describe herein an IHRP which can be used as a standard to assess the biological activity of commercial batches.

Material and Methods

Patients and Sera

For our biological standardization procedure, 48 subjects aged 18–56 years, with seasonal symptoms (rhinoconjunctivitis with or without asthma) occurring in March to April, a positive skin test to a commercially available ash extract and specific IgE serum levels $\geq$ class 2 to ash pollen, were recruited in 2 centres in the North-East of France (Strasbourg and Colmar) for skin prick testing. Informed consent was obtained from each patient.

The serum pool used in the study is composed of five sera from patients clinically allergic to Oleaceae pollen with specific IgE levels superior or equal to 2.6 kIU/ml and specific IgE reactivity against Oleaceae group 1 major allergen documented by immunoblotting.

An individual serum sensitized to ash (class 4), olive (class 5), oak (class 5), beech (class 5) and hornbeam pollen (class 5) was also tested for comparison.

Preparation of an Ash Pollen IHRP Standard

Ash pollen *F. excelsior* (Pharmallerga, Czech Republic) was extracted at a 1/20 (w/v) ratio in a 4-g/l ammonium bicarbonate solution for 24 h at 4°C with stirring. The extract was centrifuged and the supernatant was submitted to clarifying filtration and ultrafiltered with a cut-off of 1,000 Da. After addition of 2% mannitol and filtration through a 0.22- μ m membrane (Millipore,

Molsheim, France), the extract was divided into 1-ml aliquots and freeze dried. IHRP vials were stored at -20°C for 5 years. The stability of IHRP was confirmed by assessing skin prick test reactivity over 5 years (data not shown). One vial of IHRP will be used as standard for the determination of allergenic activity by ELISA inhibition of each commercial ash pollen extract batch.

In vitro Characterization of Ash Pollen Allergen Extracts and Patient IgE Profiles

Measurement of Total Protein

The total protein content of ash pollen IHRP was measured by the Bradford method (Bio-Rad Protein Assay Kit, Richmond, Calif., USA) according to the manufacturer's instructions using bovine serum albumin as the reference.

One-Dimensional SDS-PAGE (Laemmli Method)

Ash pollen IHRP proteins (15 μg) boiled in sample buffer containing 1% SDS and 5% β -mercaptoethanol were applied on a 12.5% Excel-gel (GE Healthcare, Orsay, France). Electrophoresis was carried out in parallel for samples and low molecular weight protein markers (GE Healthcare) for 1.2 h at 600 V using a Multiphor apparatus (GE Healthcare).

Two-Dimensional Electrophoresis

Two-dimensional (2-D) electrophoresis was performed as previously described [24]. Briefly, Immobiline DryStrip pH 3–10, 11 cm (GE Healthcare), was rehydrated with a rehydration solution (thiourea, urea, CHAPS 4%, glycerol 10%, dithiotreitol and IPG buffer) and ash pollen IHRP was applied. Focusing was performed on a Multiphor II horizontal 2-D electrophoresis unit. The second dimension run was performed on ExcelGel 2-D homogeneous 12.5% (GE Healthcare) for 0.7 h at 20 mA and for 1.2 h at a maximum of 600 V. Low molecular weight protein markers were run in parallel on the second dimension of the 2-D gel. Protein detection was performed using a silver staining kit (GE Healthcare).

Immunoblotting

Immunoblotting was performed as described elsewhere [24]. Briefly, proteins from 1-D or 2-D gels were transferred onto 0.45 μm pore size nitrocellulose membranes and stained with Ponceau-S red to demonstrate successful transfer of proteins. After three washes and a blocking step, membranes were cut in strips and incubated overnight at room temperature with each of the 48 individual sera from patients recruited for the clinical study diluted 1:10. After washing, the membrane was incubated for 1.5 h at room temperature with a 1:2,500 dilution of a goat IgG anti-human IgE antibody labelled with peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, Md., USA), washed again and then revealed using a West Pico substrate (Perbio sciences, Brebières, France), prior to contact with an X-OMAT film (Kodak). The first blot from 2-D gels was incubated with an anti-Ole 1 monoclonal antibody at 0.25 $\mu\text{g}/\text{ml}$ followed by sheep IgG anti-mouse IgG peroxidase conjugate diluted 1:1,000 (Sigma, Saint Quentin Fallavier, France). The second blot made from 2-D gels was incubated with an individual human serum diluted 1:10 followed by phosphatase alkaline-coupled goat anti-human IgE antibody (Sigma Biochemicals, St. Louis, Mo., USA) revealed by the substrate 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma Biochemicals). The third blot was incubated

with a pool of sera containing Oleaceae-specific IgE diluted 1:7, followed by rabbit IgG anti-human IgE (Dakocytomation, Trappes, France), followed by peroxidase-labelled goat IgG anti-rabbit IgG (Calbiochem Merck KGaA, Darmstadt, Germany), and was then revealed using a West Pico substrate. The luminescent signal was detected using a highly sensitive camera, Chemismart 2000 (Vilbert Lourmat, Marne la Vallée, France).

Biological Standardization of the Extract

Skin prick tests were performed to calibrate skin reactivity to the ash pollen IHRP. An arbitrary index of reactivity (IR) of 100 was assigned to a dilution of allergenic extracts eliciting a geometric mean wheal diameter of 7 mm when evaluated by skin prick testing using a Stallerpoint® device. As previously described [24, 25], three 10-fold dilutions of IHRP in glycerol-saline diluent were tested (1:20, 1:200, 1:2,000; w/v). Skin reactivity was simultaneously assessed with codeine phosphate (9%) and histamine hydrochloride (10 mg/ml) as positive controls. Forty-eight patients were included for skin prick testing. Patients in whom the negative control was less than 3 mm and the positive control was greater than 4 mm were selected. IHRP concentration corresponding to 7 mm was obtained from the dose-response curve using the following formula: $\log(\text{wheal diameter}) = a + b \log(\text{concentration})$, where the wheal diameter = the geometric mean diameter of the wheals obtained, a = intercept, b = slope, concentration = concentration (w/v) of the extract tested [26]. The protocol was approved by the French Ethical committee (Strasbourg) and the study performed as per Good Clinical Practices.

Fra e 1 Quantification

Fra e 1 was quantified by immunoprint using a specific Ole e 1 monoclonal antibody in parallel with an Olive pollen extract with a known quantity of Ole e 1. Briefly, several dilutions of olive pollen extract and ash pollen IHRP were run on 12.5% SDS-PAGE gel following immunoprint. Luminescent signals corresponding to Fra e 1 and Ole e 1 bands were quantified using a Chemismart 2000 camera (Vilbert Lourmat).

Batch to Batch Consistency of Non-Standardized versus Standardized Ash Pollen Extracts

Total biological activity of seven non-standardized batches and five standardized batches of ash pollen extracts were compared by ELISA inhibition. Briefly, extract dilutions were incubated with the serum pool in ELISA plates coated with ash pollen IHRP extract. After washing, anti-IgE antibodies labelled with peroxidase were added. After further washing, the substrate was added and the intensity of the reaction was measured. The relative biological activity of the various extracts was measured as an inhibitory 50% concentration.

Results

Molecular Characterization of an Ash Pollen IHRP

Following 2-D electrophoresis of ash pollen IHRP, silver staining (fig. 1a) revealed at least 200 spots. Immunodetection with a pool of sera from patients sensitized to Oleaceae revealed at least 50 IgE-reactive spots (fig. 1b).

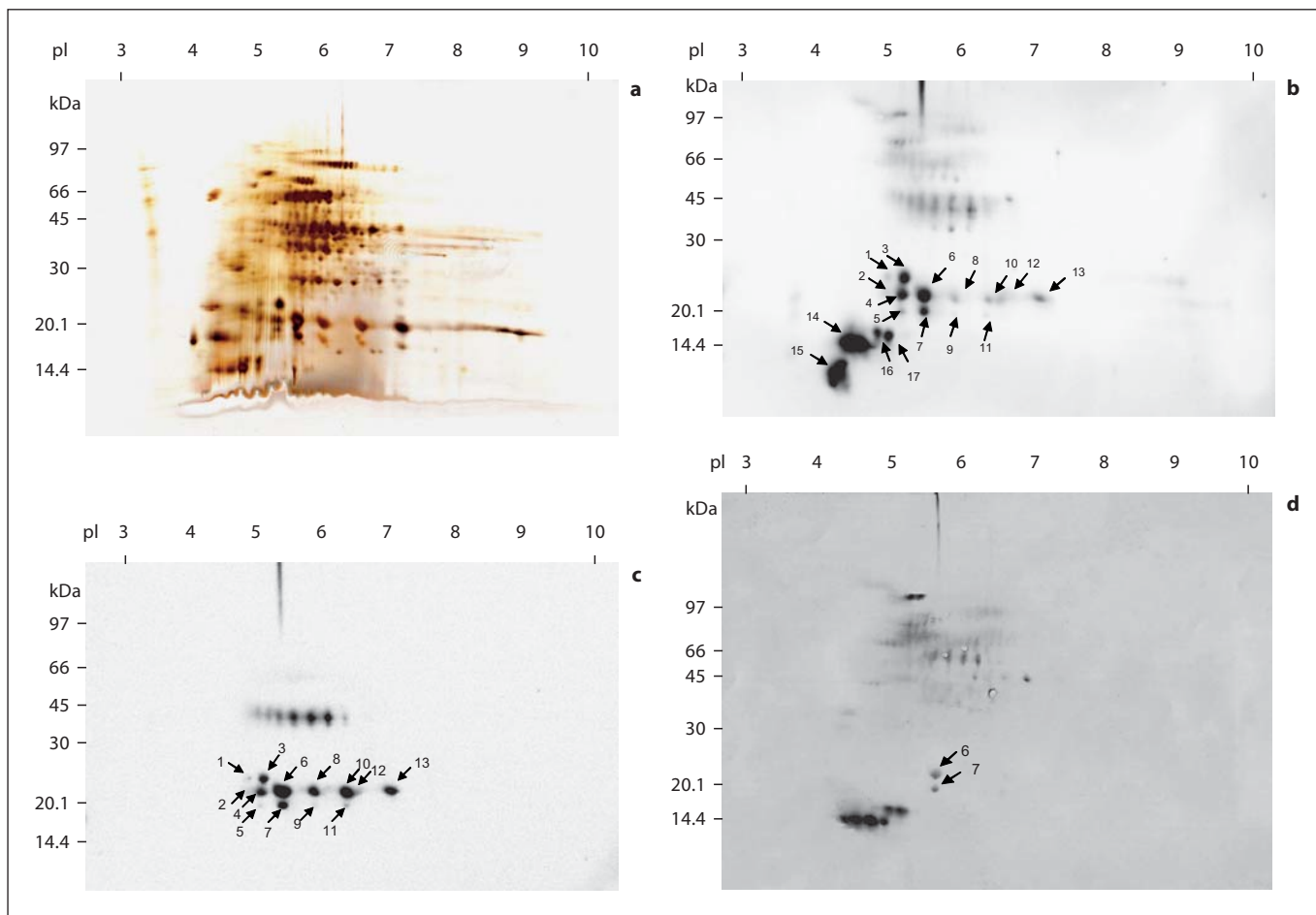


Fig. 1. Ash pollen IHRP 2-D electrophoresis and immunoprints. **a** Silver staining for protein profile (quantity of protein loaded: 15 μ g). IgE immunoblotting after 2-D electrophoresis of IHRP (quantity of protein applied: 60 μ g) using a semidry transfer onto nitrocellulose. Incubation with a mixture of sera containing Oleaceae-specific IgE (1/7 dilution) (**b**), anti-Ole e 1 monoclonal antibody (0.25 μ g/ml) (**c**) and individual sera (1/10 dilution) from an ash-allergic patient (**d**).

Six groups of proteins were observed: (1) proteins with a molecular weight between 18 and 25 kDa and a pI ranging from 5 to 7.5, comprising 13 distinct spots (numbered 1–13). These spots were shown to be identical to those revealed with an anti-Ole e 1 monoclonal antibody (fig. 1c) and, for 2 of them, with an individual serum (spots 6 and 7, fig. 1d). We conclude that these spots correspond to group 1 major allergen Fra e 1 isoforms. (2) A 14-kDa allergen with a pI ranging from 4.5 to 5, also revealed by the individual serum. This allergen is likely to be the already described ash profilin Fra e 2 (see Discussion). (3) A 9-kDa spot with a pI value of 4.5 which might correspond to the known Fra e 3, although no formal identification was conducted. (4) Two spots with an ap-

parent molecular weight of 15 kDa and a pI 5–5.2 not previously described in the literature. (5) A group of about 10 spots including a main 45-kDa protein, and (6) spots with a molecular weight superior to 50 kDa. Altogether, the ash IHRP contains all previously known allergens reported in the literature. These results also show that specific IgE directed against the various allergens are found in the serum pool used to assess the activity of commercial extracts by ELISA inhibition.

We performed a quantification of Fra e 1 in the IHRP by immunodetection with a cross-reactive anti-Ole e 1 antibody, in parallel with a standard containing a known concentration of Ole e 1. The Fra e 1 content was estimated to 17 μ g/ml for 100 IR/ml.

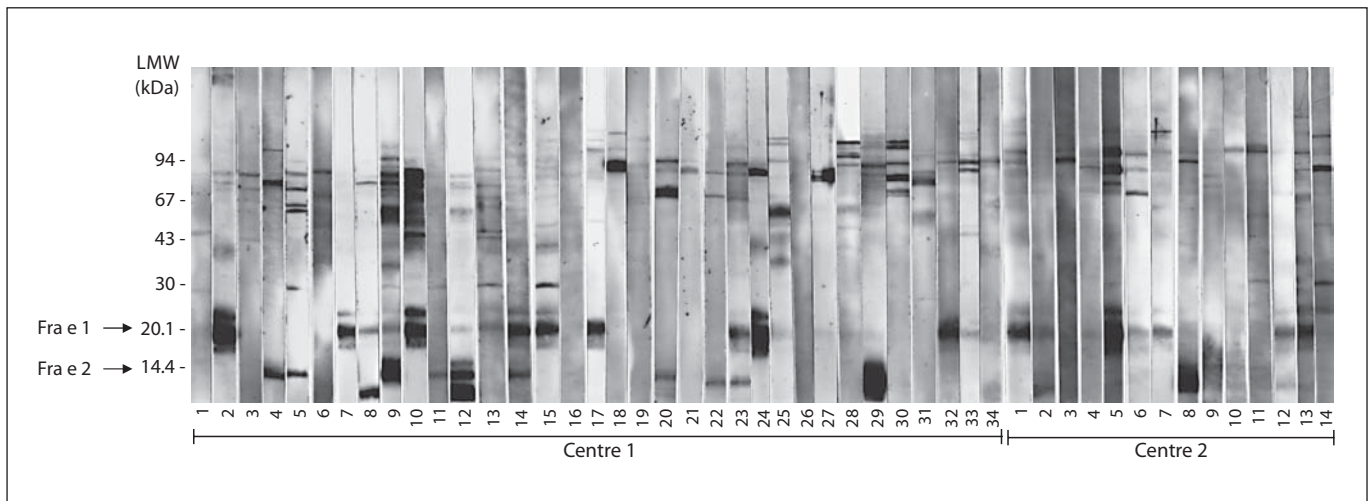


Fig. 2. IgE immunoblotting after SDS-PAGE of 48 individual sera (quantity of ash protein applied: 10 µg; semidry transfer onto nitrocellulose, incubation with sera 1/7 dilution, and revelation by chemiluminescence). LMW = Low molecular weight.

Immunodetection with Patient Sera IgE

IgE profiles obtained by 1-D immunoprint with 48 patient sera are presented in figure 2. Out of 48 patients with a documented clinical history, a positive skin test and specific IgE to ash pollen, 26 patients (54%) exhibited IgE reactivity to the major allergen Fra e 1. This frequency is similar to the one previously reported in the literature (e.g., 57–70%) [5, 6].

Biological Standardization of Ash Pollen IHRP

Skin prick tests were performed in quadruplicate in 48 patients with three serial dilutions (1:20, 1:200, 1:2,000; w/v) of IHRP. Out of the 26 patients sensitized to Fra e 1, as demonstrated by immunoprint, 4 were excluded because of a wheal diameter less than 4 mm for the positive control. Clinical data, laboratory results and geometric mean wheal diameters obtained in the 22 remaining Fra e 1-positive patients are shown in table 1.

The IHRP concentration inducing a mean wheal diameter of 7 mm (i.e. 100 IR) in these patients sensitized to Fra e 1 was equivalent to the 1/126 (w/v) extraction ratio corresponding to 259 µg/ml of protein. The regression line and the equation obtained are shown in figure 3. For comparison, the concentration inducing a mean wheal diameter of 7 mm in patients with no detectable sensitization to Fra e 1 was equivalent to the 1/78 (w/v) extraction ratio corresponding to 417 µg/ml of protein (data not shown).

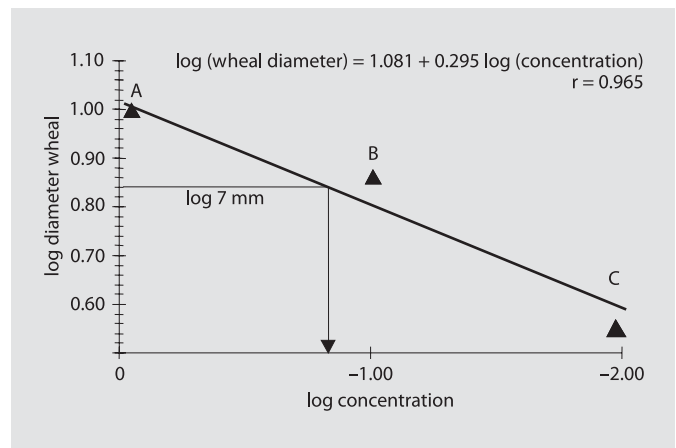


Fig. 3. Dose-response curve: $\log \text{ wheal diameter} = a + b \log \text{ concentration}$.

Batch to Batch Consistency of Non-Standardized versus Standardized Ash Pollen Extracts

The biological activity of 7 non-standardized and 5 standardized batches was compared. Those activities varied from 77 to 265 and from 55 to 99 IR/ml, respectively, with a mean of 201 and 71 IR/ml. Standard deviations were 63% for non-standardized and 16% for standardized batches. The standard deviation of biological activity of standardized extracts is reduced 4-fold in com-

Table 1. Clinical data, laboratory results and skin prick test results with three 10-fold dilutions of ash pollen IHRP

Centre	Patient No.	<i>Fraxinus americana</i> (CAP Pharmacia)		<i>Fraxinus excelsior</i> (RAST)		IgE immuno-print		Mean wheal diameter, mm				
		IgE kIU/ml	class	IgE kIU/ml	class	Fra e 1	Fra e 2	PC	NC	ash extract 1/20 (w/v)	ash extract 1/200 (w/v)	ash extract 1/2,000 (w/v)
1	2	13.9	3			+		4.56	0	7.05	5.40	3.05
	7	2.27	2	106.40	>5	+		4.55	0	10.26	8.10	3.27
	8	17.8	4	54.16	>5	+		5.17	0	12.03	7.00	3.99
	9		3	17.84	4	+	+	6.97	0	9.20	7.35	1.79
	10			32.06	4	+		7.60	0	15.81	8.87	2.16
	12	100	5	121.3	>5	+	+	4.80	0	10.38	8.22	3.67
	13			4.22	3	+		4.43	0	13.43	8.28	3.35
	14			13.28	3	+	+	5.40	0	12.12	8.37	2.92
	15	100	5	13.12	3	+		5.40	0	7.51	6.10	3.83
	17	16.5	3	34.74	4	+		4.95	0	8.01	6.49	2.78
	23			21.63	4	+	+	5.80	0	12.38	9.88	3.24
	24			96.47	>5	+		4.60	0	8.84	4.77	1.00
	25			>154.97	5	+		5.99	0	14.95	8.51	2.69
	27			>153.19	5	+		6.49	0	10.39	7.56	2.34
	28	2.34	2	39.33	4	+		5.48	0	11.52	6.72	2.68
	29			>66.56	5	+	+	6.50	0	10.50	7.89	2.56
	32	0.83	2	19.76	4	+		5.08	0	9.42	6.88	1.81
	33			133.82	>5	+		6.59	0	8.57	5.78	2.44
2	4			4.75	3	+		5.90	2.30	10.34	8.14	3.00
	5		4	5.21	3	+		4.77	0	9.84	7.77	2.76
	12			15.04	3	+	+	6.68	0	14.13	9.29	3.77
	13			25.98	4	+	+	8.42	0	18.87	12.19	5.18
								Geometric mean		10.83	7.55	2.78

PC = Positive control; NC = negative on 22 ash-allergic patients included in biological standardization of ash pollen extract.

parison with non-standardized extracts. These results illustrate a better batch to batch consistency for standardized versus non-standardized batches.

Discussion

Biological extracts currently available for prick test diagnosis and immunotherapy of ash pollen allergy have not been previously standardized. Consequently, ash pollen immunotherapy is not widely performed. Some patients sensitized to ash pollen are diagnosed and treated with a standardized olive extract on the basis of the known homologies between members of the Oleaceae family [14]. However, it is not yet routine clinical practice to include pollen extracts from trees absent in the local flora in diagnostic assays, and thus, olive extracts are not commonly used in the North-East of France.

In Europe, in the absence of any international harmonization, each allergenic extract producer defines its own IHRP tested in a limited number of allergic volunteers. In vivo calibration studies are performed in patients allergic to the allergen source on the basis of clinical criteria, positive skin prick test with the allergen extract and serological testing for specific IgE antibodies. In the case of ash pollen allergy, due to the cross-reactivity between minor allergens from ash and Betulaceae pollens, it is impossible to discriminate co-sensitization and cross-sensitization to birch and ash pollen by skin tests using allergen extracts. Several studies have also demonstrated that 20 [27] to 50% [5] of positive skin tests to ash pollen are due to cross-sensitization to pollen panallergens. Thus, clinical history and skin tests alone are not sufficient to diagnose ash pollen allergy.

Diagnostic testing with marker allergens should allow to discriminate between sensitized patients and those

who appear falsely positive in extract-based tests because of reactivity with highly cross-reactive allergens from other sources [28, 29]. As Fra e 1 is the major ash pollen allergen and Fra e 1 sensitization is constantly associated with mono- and oligosensitization (while its frequency is below 50% in subjects with multiple pollen allergies) [6], we decided to select patients for the calibration study on the basis of this inclusion criteria. At present, no commercial Fra e 1 IgE assay is currently available. Thus, we performed IgE immunoprint using the ash pollen IHRP with individual sera to select patients sensitized to the major allergen Fra e 1.

On the basis of skin reactivity, potencies are usually calculated according to internal methods drawn from Nordic Guidelines or Food and Drug Administration regulations and expressed in potency units defined by each single producer. Production batches are subsequently standardized by *in vitro* methods such as ELISA inhibition with serum pools, using the IHRP as reference.

In the present study, an ash pollen IHRP was defined. Characterization by immunoprint after 2-D gel electrophoresis demonstrated the presence in the IHRP of ash allergens previously described in the literature [3, 5, 6, 20, 21]. Following 2-D gel electrophoresis, at least 200 protein spots were visualized in the ash pollen IHRP, of which 50 are reacting with IgE. Fra e 1, the major allergen, was clearly identified in our IHRP using our pool of sera because (1) IgE-reactive proteins are found in the 20-kDa region corresponding to the expected molecular weight for this allergen [3, 5, 6], (2) the immunoprinting pattern obtained with a cross-reactive anti-Ole e 1 antibody overlaps with these spots, and (3) the 20-kDa proteins recognized by IgE from the individual serum (fig. 1d) were shown to be Fra e 1 by mass spectrometry [Poncet et al., manuscript in preparation]. Analysis with mass spectrometry also reveals that the 3 spots at 14 kDa with an acidic pI, recognized by the individual serum (fig. 1d) as well as by the pool of Oleaceae-specific sera, corresponds to Fra e 2 profilin. The protein detected at 9 kDa with an acidic pI is likely to be Fra e 3 [6]. High molecular weight allergens (>45 kDa) are also detected by our pool sera in agreement with previously reported data [21], and 2 unknown proteins with a molecular weight around 15 kDa, with acidic pI, are visualized. Altogether, our extract contains all the representative allergens from ash pollen.

We could demonstrate that the mean wheal diameter is greater in patients selected for skin test calibration than in patients with no detectable Fra e 1-specific IgE. The concentration of the IHRP inducing a 7-mm wheal diameter would have been 1.6-fold higher if the calibration had

been performed in this second group of patients, although the prevalence of asthma is higher. The Fra e 1-reactive population selected for this study has higher skin reactivity to ash pollen (data not shown).

The 4-fold reduction in standard deviation of activity in standardized commercial ash pollen batches compared with non-standardized extracts showed that standardization allows a better batch to batch consistency in terms of total activity. The mean of total activities of standardized batches is arbitrarily defined at 100 IR/ml corresponding to a 7-mm mean wheal diameter. On the one hand, this level of potency guarantees the efficiency of standardized diagnosis and, on the other hand, provides a basis for determining the efficient dose for treatment.

Altogether, successful standardization of ash pollen extracts has been achieved and an IHRP has been characterized. Such a standardization guarantees batch to batch consistency in terms of biological activity.

Acknowledgements

We thank Air Allergy and the 'Allergy and Environment' group (directed by Gabriel Peltre) for kindly providing specific sera. The study was supported by Stallerg nes SA, France.

References

- D'Amato G, Mullins J, Noland N, Spieksma FT, Wachter R: City spore concentrations in the European Economic Community (EEC) VII. Allergy 1988;18:541-547.
- Liccardi G, D'Amato M, D'Amato G: Oleaceae pollinosis: a review. Int Arch Allergy Immunol 1996;111:210-217.
- Barderas R, Purohit A, Papanikolaou I, Rodriguez R, Pauli G, Villalba M: Cloning, expression and clinical significance of the major allergen from ash pollen Fra e 1. J Allergy Clin Immunol 2005;115:351-357.
- Papanikolaou I, Barderas R, Thibaudon M, Pauli G: Ash tree pollinosis: botanical aspects, description of allergens and cross-reactivities. Rev Fr Allergol Immunol Clin 2005;45:395-405.
- Niederberger V, Purohit A, Oster JP, Spitzauer S, Valenta R, Pauli G: The allergen profile of Ash (*Fraxinus excelsior*) pollen: cross-reactivity with allergens from various plant species. Clin Exp Allergy 2002;32:933-941.
- Hemmer W, Focke M, Wantke F, Gotz M, Jarisch R, Jager S, Gotz M: Ash (*Fraxinus excelsior*) pollen allergy in central Europe: specific role of pollen panallergens and the major allergen of ash pollen, Fra e 1. Allergy 2000;55:923-930.
- Bousquet J, Guerin B, Hewitt B, Lim S, Michel FB: Allergy in the Mediterranean area. 3. Cross reactivity among Oleaceae pollens. Clin Allergy 1985;15:439-448.
- De Blay F, Bessot JC, Pauli G: New aero-allergens. Rev Pneumol Clin 1996;52:79-87.
- Pauli G, Papanikolaou I, Niederberger V: Sensitization to ash: specific and/or cross-reacting allergens? Rev Fr Allergol Immunol Clin 2003;43:120-124.
- Wahl R, Schmid-Grendelmeier P, Cromwell O, Wüthrich B: In vitro investigation of cross-reactivity between birch and ash pollen allergen extracts. J Allergy Clin Immunol 1996;98:99-106.
- Schmid-Grendelmeier O, Peeters AG, Wahl R, Wüthrich B: Zur Bedeutung der Eschenpollenallergie. Allergologie 1994;11:535-542.
- Helbling A, Leuschner RM, Wüthrich B: Pollinosis. 4. Welche Pollen sollen in der Allergie-Praxis getestet werden? Schweiz Med Wochenschr 1985;115:1150-1159.
- Lelong M, Catelain MC, Sulmont G: Existe-t-il en France, une pollinose au frêne? Rev Fr Allergol Immunol Clin 1992;32:41-42.
- Pajaron MJ, Vila L, Prieto I, Resano A, Sanz ML, Oehling AK: Cross-reactivity of *Olea europaea* with other Oleaceae species in allergic rhinitis and bronchial asthma. Allergy 1997;52:829-835.
- Obispo TM, Melero JA, Carpizo JA, Carreira J, Lombardero M: The main allergen of *Olea europaea* (Ole e I) is also present in other species of the Oleaceae family. Clin Exp Allergy 1993;23:311-316.
- Liccardi G, Russo M, Saggese M, D'Amato M, D'Amato G: Evaluation of serum specific IgE and skin responsiveness to allergenic extracts of Oleaceae pollens (*Olea europaea*, *Fraxinus excelsior* and *Ligustrum vulgare*) in patients with respiratory allergy. Allergol Immunopathol 1995;23:41-46.
- Lombardero M, Obispo T, Calabozo B, Lezaun A, Polo F, Barber D: Cross-reactivity between olive and other species. Role of Ole e 1-related proteins. Allergy 2002;57(suppl 71):29-34.
- Martin-Orozco E, Cardaba B, del Pozo V, de Andres B, Villalba M, Gallardo S, Rodriguez-Garcia MI, Fernandez MC, Alche JD, Rodriguez R: Ole e 1: epitope mapping, cross-reactivity with other Oleaceae pollens and ultrastructural localization. Int Arch Allergy Immunol 1994;104:160-170.
- Villalba M, Batanero E, Lopez-Otin C, Sanchez LM, Monsalve RI, Gonzalez de la Pena MA, Lahoz C, Rodriguez R: The amino acid sequence of Ole e I, the major allergen from olive tree (*Olea europaea*) pollen. Eur J Biochem 1993;216:863-869.
- Ledesma A, Rodriguez R, Villalba M: Olive-pollen profilin. Molecular and immunologic properties. Allergy 1998;53:520-526.
- Palomares O, Villalba M, Quirarte J, Polo F, Rodriguez R: 1,3-b-glucanases as candidates in latex-vegetable food cross-reactivity. Clin Exp Allergy 2005;35:345-351.
- Lowenstein H, Larsen JN: Recombinant allergens/allergen standardization. Curr Allergy Asthma Rep 2001;1:474-479.
- Lombardero Vega M: Biological standardization systems for allergenic extracts. J Invest Allergol Clin Immunol 1997;7:354-355.
- Hrabina M, Dumur JP, Sicard H, Viatte A, Andre C: Diagnosis of cypress pollen allergy: in vivo and in vitro standardization of a *Juniperus ashei* pollen extract. Allergy 2003;58:808-813.
- Turjanmaa K, Palosuo T, Alenius H, Leynander F, Autegarden JE, Andre C, Sicard H, Hrabina M, Tran TX: Latex allergy diagnosis: in vivo and in vitro standardization of a natural rubber latex extract. Allergy 1997;52:41-50.
- Brighton WD, Topping MD, Henocq E: Activity units for allergen extracts. Clin Allergy 1979;9:591-596.
- Papanikolaou I, Barderas R, Purohit A, Villalba M, Rodriguez R, de Blay F, Pauli G: Confrontation des résultats d'IgE spécifiques par les méthodes RAST et ELISA chez des sujets sensibilisés au pollen de frêne. Rev Fr Allergol Immunol Clin 2004;44:356.
- Kazemi-Shirazi L, Niederberger V, Linhart B, Lidholm J, Kraft D, Valenta R: Recombinant marker allergens: diagnostic gatekeepers for the treatment of allergy. Int Arch Allergy Immunol 2002;127:259-268.
- Mothes N, Horak F, Valenta R: Transition from a botanical to a molecular classification in tree pollen allergy: implications for diagnosis and therapy. Int Arch Allergy Immunol 2004;135:357-373.