

Short communication

Diagnosis of cypress pollen allergy: *in vivo* and *in vitro* standardization of a *Juniperus ashei* pollen extract

Background: Cypress pollen allergy is a major cause of rhinoconjunctivitis and asthma in the Mediterranean area. The nonstandardized cypress allergen extracts currently available for the diagnosis of cypress allergy have a low level of activity. The search for an active material has led to the selection of *Juniperus ashei* (Ja) pollen because of its very high cross-reactivity with cypress extracts and its superior allergenic activity. The aim of this study was to characterize *in vitro* and calibrate *in vivo* an in-house reference extract (IHRS) of *J. ashei* pollen and determine the specificity and sensitivity of a standardized Ja extract for the prick test diagnosis of cypress allergy.

Methods: *Juniperus ashei* pollen extract was analysed by 2-D electrophoresis. The IHRS Ja extract was calibrated by skin prick testing in 28 cypress-allergic patients. The sensitivity and specificity of cypress allergy diagnosis using a standardized Ja extract was studied by skin prick test in 42 cypress-allergic patients and 53 nonallergic patients. Jun a 1 content of the IHRS was determined by a monoclonal antibody-based electrophoretic technique.

Results: The Jun a 1 content of the 100 IR/ml Ja IHRS extract was 180 µg/ml. For *in vivo* diagnosis of cypress allergy, Ja pollen extract demonstrated a sensitivity of 95%, a specificity of 100%, a negative predictive value of 96%, and a positive predictive value of 100%.

Conclusion: Standardized Ja pollen extract is therefore a very appropriate tool for the *in vivo* diagnosis of cypress pollen allergy and good candidate for specific immunotherapy.

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Cypress pollen allergy is a major cause of rhinoconjunctivitis and asthma in the Mediterranean area. Increased planting of cypress trees for ornamental purposes has probably contributed to the increased prevalence of sensitization to cypress pollen, which is the third most sensitizing allergen source after mites and grass pollens in South-East France (1).

The role of cypress pollen allergy in upper respiratory tract diseases has often been underestimated because of the winter pollination of cypress trees. The lack of efficacy of the available nonstandardized extracts for the diagnosis of cypress pollen allergy is also partly responsible for this situation (1, 2). A biologically active and reproducible, i.e. standardized test, is therefore necessary for *in vivo* diagnosis of cypress pollen allergy.

The cypress family is composed of six genera: *Cupressus*, *Juniperus*, *Thuja*, *Chamaecyparis*, *Callitris* and *Libocedrus* (3). The main species of this family present in the Mediterranean region are the Italian cypress (*Cupressus sempervirens*) (Cs) and the Arizona cypress (*Cupressus arizonica*) (Ca). In the USA, Mountain cedar (*Juniperus ashei* = *J. sabinoide*s) (Ja), which belongs to

the *Juniperus* genus, is a major cause of seasonal allergy in the South-West and Texas (4–6). It is mainly found at high altitudes, and is also cultivated as an ornamental tree. In Europe, this species is present only in the Balkan peninsula and Crimean mountains.

Comparing extracts intended for diagnostic skin prick tests from five different companies, Mari et al. (2) showed that Cs pollen extracts were not sufficiently potent and induced a large number of false-negative reactions. The mean wheal area did not exceed 18 mm² for any of these extracts. The physicochemical properties of cypress pollen grains could be responsible for the difficulty of extracting Cs pollen allergens (7). Mari et al. also demonstrated the marked superiority of a Ja extract compared with Ca and Cs extracts for the diagnosis of cypress allergy by skin prick tests. The Ja extract induced a mean wheal area of 44 mm² (2). Albeit more potent than Cs extracts, the Ca pollen extract was unable to induce an equivalent wheal area without a prior concentration step. These data were confirmed in a previous study (8) by our group that showing that Ja extract was much more potent than Cs and Ca extracts both *in vitro*

and *in vivo*. The *in vitro* allergenic activity of Ja was 20-fold greater than that of Cs and 11-fold greater than that of Ca, despite similar protein contents. The mean wheal diameter induced *in vivo* by 1/20 (m/v) extracts was significantly greater for Ja (8.3 mm) than for Cs (6.3 mm) and Ca (6.7 mm) extracts ($P \leq 0.001$). This higher activity might be explained, at least in part, by a greater concentration of a 42 kDa allergen identified as Jun a 1. Serum specific immunoglobulin E (IgE) levels measured with a solid phase coated with Ja extract were higher than those measured with Cs (8). Mari et al. (2) reported a similar finding with five different assay methods.

A high cross-reactivity has been reported between the *Cupressus* and *Juniperus* genera, which actually have very similar allergenic compositions (3, 8, 9–12). Using immunoblotting-inhibition, Iacovacci (11) showed that the Ja extract totally inhibited IgE binding to a Ca extract, but the opposite was not observed, possibly because of an insufficient concentration of the Ca extract. Immunoblotting experiments indicated that allergens were common to all three extracts. The inhibition curves obtained with the three extracts were parallel. Skin prick test responses to Cs and Ja and to Cs and Ca correlated significantly ($P \leq 0.002$). Serum anti-Ja and anti-Cs IgE levels in patients also correlated significantly ($r = 0.916$, $P < 0.001$, $n = 23$). Altogether of those data confirm the high cross-reactivity, both *in vitro* and *in vivo*, between Cs, Ca and Ja, and the higher allergenic activity of the later. On this basis, Ja was considered the best candidate for the manufacturing a standardized diagnostic extract. Di Felice et al. (3), on the basis of the high IgE cross-reactivity within Cupressaceae species, already considered selecting a representative extract from one species that could be carefully standardized and used for diagnostic and therapeutic purposes.

The objective of this study was to characterize *in vitro* and calibrate *in vivo* an in-house reference extract (IHRS) pollen of *Juniperus ashei* and to determine the specificity and sensitivity of this standardized Ja extract for the prick test diagnosis of cypress allergy.

Material and methods

Patients and sera

Patients and controls undergoing skin prick testing in the biological standardization procedure Twenty-eight cypress-allergic patients, 14 females and 14 males, aged 13–58 years, with a clinical history and typical symptoms of cypress pollen allergy, were recruited in Aix en Provence, France. Relevant clinical data and laboratory results for these patients are presented in Table 1. Informed consent was obtained from each patient or the patient's parents. Fifty-three healthy subjects tested for suspicion of atopy, with or without a history of living in an area planted with cypresses, were used as controls for the skin prick test standardization procedure. Approximately 70% of control subjects had positive skin prick tests to one or several common (non-cypress) allergens.

Preparation of extracts and IHRS

The Ja pollen (Allergon, Angelholm, Sweden) was extracted at a 1/20 (m/v) ratio in 4 g/l ammonium bicarbonate buffer for 24 h at 4°C with stirring. The extract was then centrifuged and the supernatant was submitted to clarifying filtration and ultrafiltration to 1000 Da. After addition of 2% mannitol, and filtration through a 0.22 µm membrane (Millipore, Molsheim, France), the extract was divided into 1 ml aliquots and freeze-dried.

2-D Electrophoresis

A Multiphor II horizontal 2-D electrophoresis unit was used. Immobiline DryStrip pH 3–10, 11 cm (Amersham Biosciences Europe GmbH, Orsay, France) was rehydrated overnight with rehydration solution containing 10 µg of Ja IHRS, according to the manufacturer's instructions. Focusing was performed for a total of 18 000 volt-hours. Strips were then equilibrated with sodium dodecyl sulphate (SDS) equilibration buffer containing 3% SDS (according to the manufacturer's instructions). The second dimension was performed with ExcelGel 2-D homogeneous 12.5% (Amersham Biosciences Europe GmbH). Electrophoresis conditions were 600 V, 20 mA, 30 W for 30 min and then 600 V, 50 mA, 30 W for 70 min. Protein detection was performed with a silver staining kit (Amersham Biosciences Europe GmbH).

Immunoblotting

After electrophoresis, proteins were transferred from the gel onto a 0.45 µm pore size nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by electroblotting at 0.8 mA/cm² for 1 h with a semi-dry transfer kit (Novablot; Amersham Biosciences Europe GmbH). After blotting, the membrane was stained with Ponceau-S red to demonstrate successful transfer of proteins. The membrane was washed three times for 10 min in Tris-buffered saline (TBS), incubated for 15 min in 0.5% glutaraldehyde in phosphate buffer saline (PBS) followed by a 30-s wash with PBS, incubated in 1% ethanolamine in PBS for 1 h and then washed three times for 10 min in TBS (13). The membrane was incubated for 1 h in blocking buffer containing 1% nonfat milk powder in TBS and then washed three times for 10 min in TBS containing 1% Tween-20 (TTBS) with shaking. The serum pool and control serum (diluted 1 : 7) in antibody buffer containing 0.2% nonfat milk powder in TTBS were added, and the membrane was incubated overnight at room temperature with continuous shaking. The membrane was then washed three times for 10 min in TTBS. Rabbit IgG antihuman IgE antibody (Dako, Trappes, France) diluted to 1 : 500 in antibody buffer was then added and the membrane was incubated for 1.5 h at room temperature with shaking. The membrane was washed again and then treated by Immuno-Star Chemiluminescent detection kit (Bio-Rad, Munchen, Germany) according to the method described by the supplier. The allergens were revealed after 3-min contact with X-OMAT film (Kodak, Rochester, NY).

Jun a 1 content determination

High performance liquid chromatography purified Jun a 1 standard (14) and three serial dilutions of Ja IHRS were mixed with 2× SDS-sample buffer, boiled at 100°C for 1.5 min. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed using 4–20% gradient acrylamide gels (Bio-Rad) in 1 l of Tris glycine/SDS buffer for 45 min. Proteins were electrotransferred to nitrocellulose in 1 l of Tris glycine buffer, on ice, for 1.5 h. After blocking

Table 1. Clinical data, laboratory results, and skin prick test results for 28 cypress-allergic patients included in biological standardization of *Juniperus ashei* pollen extract

Patient No.	Age	Sex	Symptoms	Mean wheal diameter (mm)								
				Cs RAST		Ja RAST		PC	NC	<i>Juniperus ashei</i>		
				U/ml	class	U/ml	class			A	B	C
1	58	F	C,R	23.9	4	51.6	5	9.5	1.0	10.6	7.7	4.2
2	35	F	C,R,A	<0.4	0	<0.4	0	7.7	2.4	5.9	3.5	4.2
3	46	F	C,R	12.7	3	28.5	4	6.5	2.0	10.9	7.7	7.0
4	42	M	C,R,A	ND	ND	ND	ND	8.0	3.5	8.3	6.0	4.7
5	58	M	C,R	9.2	3	17.8	3	11.8	2.6	13.6	8.0	5.2
6	42	F	C,R	0.6	1	2.2	2	7.2	2.4	7.5	5.4	4.3
7	49	F	C,R	1.8	2	17.0	3	6.2	2.2	7.0	4.3	3.5
8	36	M	C,R	8.1	3	27.3	4	7.7	1.0	9.9	7.7	5.9
9	44	F	C,R	2.6	2	18.8	4	7.0	1.2	6.8	4.6	3.2
10	40	F	R	0.6	1	3.6	3	7.5	2.2	7.7	3.0	3.4
11	41	M	C,R	0.6	1	3.7	3	7.7	3.3	7.2	6.1	4.4
12	33	F	C,R,A	9.9	3	59.0	5	6.6	2.6	6.8	4.1	4.3
13	56	F	C,R,A	<0.4	0	<0.4	0	7.7	1.7	6.5	6.7	4.8
14	42	F	C,R	1.1	2	4.4	3	7.0	2.0	10.3	9.5	6.9
15	49	M	C,R	1.6	2	7.6	3	7.0	1.4	7.6	5.8	3.5
16	41	F	C,R	1.6	2	22.4	4	7.7	1.4	8.4	6.2	4.9
17	13	M	C,R	ND	ND	ND	ND	6.9	2.0	11.1	7.3	4.0
18	58	M	C,R	ND	ND	ND	ND	7.9	2.4	3.5	3.7	3.1
19	45	M	C,R	1.3	2	10.5	3	4.2	2.4	6.8	5.6	4.1
20	30	F	C,R	9.3	3	23.8	4	7.4	2.0	10.4	9.7	6.2
21	50	M	C,R	<0.4	0	1.0	2	6.5	1.0	7.0	5.6	4.1
22	46	F	C,R	9.4	3	17.1	3	4.2	3.0	9.3	6.1	6.5
23	40	M	C,R,A	5.5	3	13.3	3	5.8	1.4	9.2	7.0	5.6
24	55	M	C,R	9.5	3	16.1	4	6.8	1.4	8.7	5.7	5.8
25	14	F	C,R	7.2	3	25.0	4	6.2	1.4	12.2	7.5	6.1
26	56	M	C,R	8.3	3	13.5	3	4.4	2.0	11.7	7.0	7.2
27	43	M	C,R,A	<0.4	0	0.4	1	8.0	1.0	5.9	5.4	3.6
28	39	M	C,R,A	1.3	2	6.0	3	4.2	3.7	9.3	5.8	5.1
Geometric mean										8.27	5.94	4.70

M, male; F, female; R, rhinitis; C, conjunctivitis; A, asthma; PC, positive control; NC, negative control; A, Ja m/V 1/20; B, Ja m/V 1/200; C, Ja m/V 1/2000; ND, not determined.

with 10% dry fat-free milk in 0.05% TTBS for 2 h and after washing three times, nitrocellulose was incubated with 1 µg/ml of Anti-Jun a 1 monoclonal antibody KW-S91 (15). After washing three times with 0.05% TTBS for 5 min each, nitrocellulose was incubated with 0.1 µg/ml horseradish peroxidase (HRP)-Streptavidin (Zymed, San Francisco, CA), washed three times, incubated with ECL substrate (Pharmacia, Uppsala, Sweden) for 1 min and finally exposed to the films. Protein bands were analysed by Alpha Imager 2200 (Alpha Innotech Corporation, San Leandro, CA). Jun a 1 concentration was calculated with reference to the standard.

Biological standardization of the extract

Skin prick tests were performed to determine the *in vivo* reactivity of the allergen extract. An arbitrary index of reactivity (IR) of 100 was assigned to a dilution of allergenic extracts which, when evaluated by skin prick testing using a STALLERPOINT® (Stallergenes, Antony, France) device in 28 subjects sensitized to the allergen, gave a geometric mean wheal diameter of 7 mm. Three 10-fold dilutions of IHRS in glycerol-saline diluent (1 : 20, 1 : 200, 1 : 2000; m/v) were used. The skin reactivity of these subjects was simultaneously checked with 9 g/l codeine phosphate as positive control. Patients in whom the negative control was <3 mm and the positive control was >4 mm were included. Skin prick tests were performed in quadruplicate with the STALLERPOINT® device on the anterior surface of the forearm

in a predetermined order. The negative (glycerol-saline solvent) and positive (9 g/l codeine phosphate) controls were performed in duplicate. Wheals and flares were outlined after 20 min and the figures were transferred with adhesive tape to test record sheets. The mean diameter of each wheal was measured. The geometric mean wheal diameter was calculated for each dilution. The IHRS dilution inducing a geometric mean wheal diameter of 7 mm was interpolated from the dose-response curve using the formula: $\log(\text{wheal diameter}) = a + b \log(\text{concentration})$ (where wheal diameter = geometric mean diameter of the wheals obtained, a = intercept, b = slope, concentration = concentration (m/v) of extract tested).

Results

2-D electrophoresis and immunodetection

Figure 1 shows the protein and allergen 2-D electrophoresis patterns. Silver staining (Fig. 1A) showed 30 spots located between pH 4.5–7.5 and 14–90 kDa. After immunodetection (Fig. 1B), four different areas showed IgE binding. The most intense binding was observed in the 40–43 kDa and pI 5–6.5 area, which comprised seven distinct spots, corresponding to the isoforms of Jun a 1.

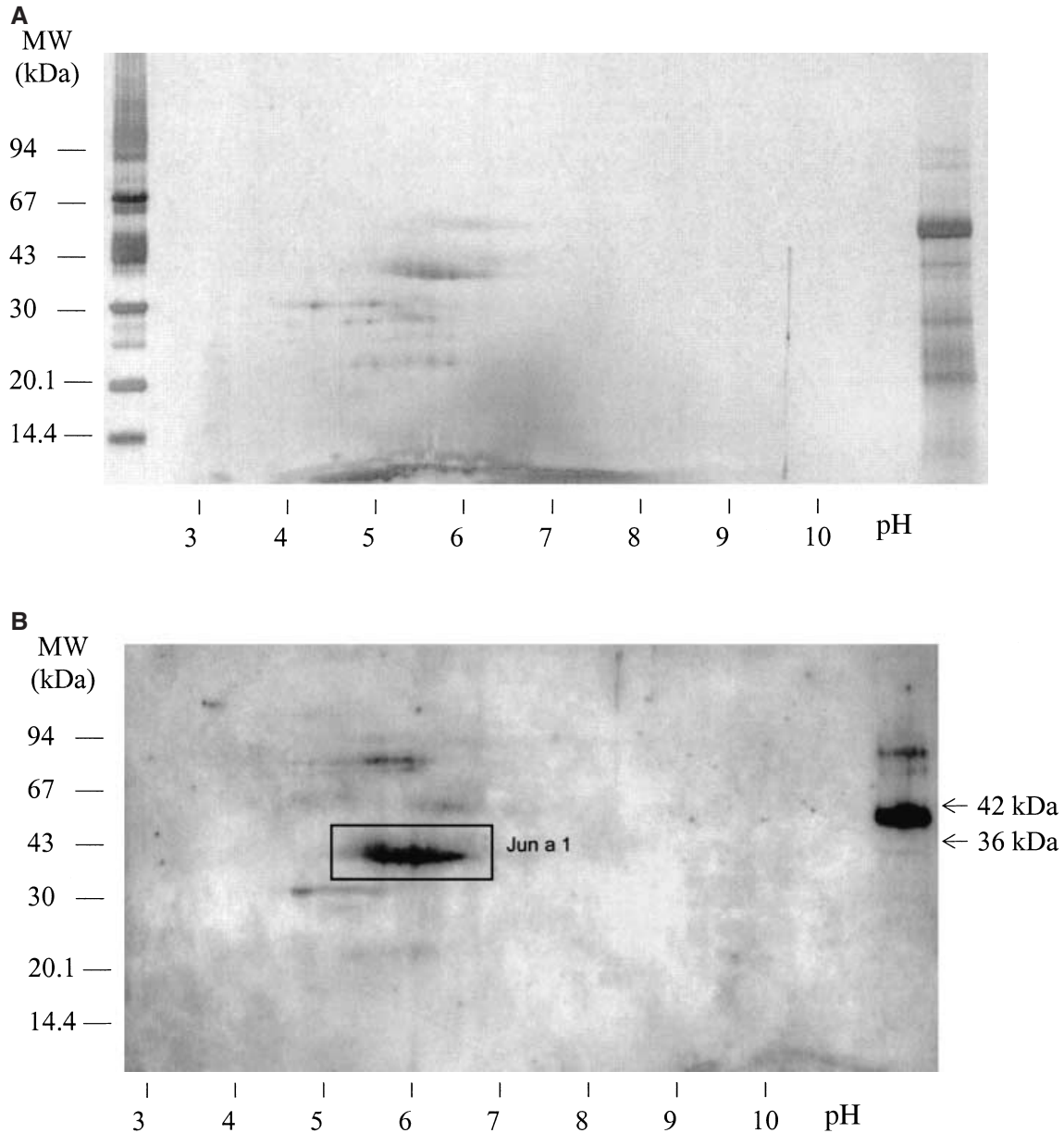


Figure 1. 2-D electrophoresis of Ja IHRS (A) total protein staining (B) IgE immunoblotting (quantity of protein applied: 10 µg; semidry transfer onto nitrocellulose; incubation with a mixture of four sera containing Cs-specific IgE (1/7 dilution) and revelation by chemiluminescence).

The other three areas were a spot at 36 kDa ranging from pI 4.5 to 5.5, a zone at 67 kDa ranging from pI 6 to 7.5 and a zone > 70 kDa ranging from pI 5 to 6.

Biological standardization of Ja extract

Skin prick tests with three successive dilutions of IHRS (1 : 20, 1 : 200, 1 : 2000 dilutions; m/v) were performed in quadruplicate on 28 cypress-allergic patients. Results are presented in Table 1. The IHRS concentration inducing a geometric mean wheal diameter of 7 mm was calculated as 1 : 68 (m/v) and was arbitrarily defined

as 100 IR/ml. The regression line and the equation obtained are shown in Fig. 2. Forty of the 42 cypress-allergic patients had a positive response to 100 IR/ml (wheal diameter $\geq$ 5 mm and/or $\geq$ 2/3 of the wheal diameter because of the positive control). The sensitivity of skin prick testing was therefore 95%. None of the 53 control subjects, tested with the 100 IR Ja skin prick test showed a positive response, corresponding to a specificity of 100%, with a negative predictive value of 96% and a positive predictive value of 100%.

Jun a 1 content of the 100 IR/ml Ja IHRS extract was 180 µg/ml.

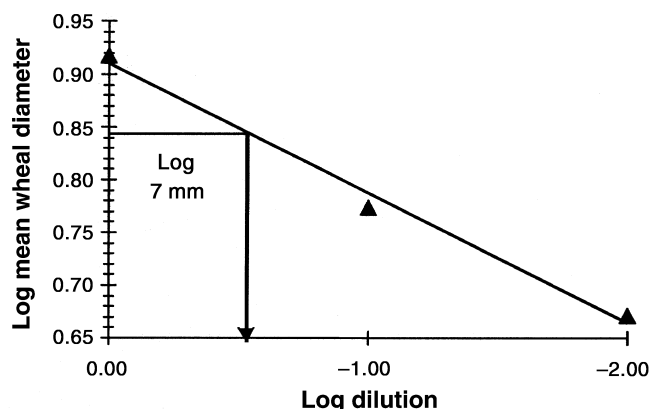


Figure 2. Dose-response curve: $\log \text{ wheal diameter} = a + b \log \text{ dilution}$.

Discussion

The extracts available up until now for the prick test diagnosis of cypress allergy had a low allergenic activity and were not standardized. According to our definition, a 100 IR/ml standardized extract induces a mean wheal diameter of 7 mm, i.e. a mean wheal area of 38 mm². The Cs and Ca cypress pollens are unable to induce this type of skin reactions without a prior concentration step. As several studies (2, 8) have demonstrated the much higher allergenic activity of Ja pollen extracts compared with cypress pollen extracts but also high *in vitro* and *in vivo* cross-reactivities within the Cupressaceae family, we attempt to develop a standardized extract for the diagnosis of cypress allergy using the Ja extract.

The Ja IHRS showed seven 42 kDa isoforms, corresponding to the group 1 allergen Jun a 1. The importance

of the 42 kDa band in cypress pollen allergy has been previously studied. Ford et al. (13) showed that 81% of 91 sera from cypress-allergic patients contained IgE specific for the 42 kDa major band of a Cs pollen extract. Among them, 46.7% only recognized only this single 42 kDa band. Di Felice et al. (16) demonstrated a 100% prevalence of IgE specific for the 42 kDa band. The importance of the 42 kDa band in Ja extracts was confirmed by Iacovacci et al. (11).

Immunodetection after 2D-electrophoresis with cypress-allergic patient serum pool also demonstrated the presence of the minor allergen Jun a 2 (36 kDa) and of a 67 kDa allergen in the Ja IHRS. The frequency of recognition of the 36 and 70 kDa bands did not exceed 10% with a Ca pollen extract (13). Di Felice et al. (16) reported a higher prevalence of recognition of the 70 and 36 kDa bands.

Finally, Ja pollen extract was successfully standardized for the *in vivo* diagnosis of cypress allergy in cypress-allergic patients from France with no history of contact with Ja pollen. This study demonstrates that a *Juniperus ashei* pollen skin prick test reagent therefore represents a documented, standardized, highly sensitive (95%) and specific (100%) solution for *in vivo* diagnosis of cypress pollen allergy. Clinical trials designed to validate the efficacy of cypress pollen specific immunotherapy with the standardized Ja pollen extract are currently underway.

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