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Identification of Italian cypress (*Cupressus sempervirens*) pollen allergen Cup s 3 using homology and cross-reactivity

Akihisa Togawa, MD^{*,†}, Raphael C. Panzani, MD[‡], Maritza A. Garza, BS^{*}, Reiko Kishikawa, MDPHD^{*}, Randall M. Goldblum, MD^{*}, and Terumi Midoro-Horiuti, MDPHD^{*}

^{*} Department of Pediatrics, Child Health Research Center, University of Texas Medical Branch, Galveston, Texas

[†] Department of ENT, Wakayama Medical University, Wakayama, Japan

[‡] Centre de Recherches en Allergologie, Marseille, France

Abstract

Background—The prevalence of seasonal allergic diseases of the upper airways is increasing in industrialized countries. The Cupressaceae are important causes of pollinosis, particularly in Europe.

Objective—To determine whether the pollen from *Cupressus sempervirens* (Italian cypress) contains a pathogenesis-related group 5 (PR-5) protein, similar to that found in other allergenic Cupressaceae pollens.

Methods—Messenger RNA was purified from Italian cypress pollen, and complementary DNA (cDNA) was synthesized. cDNAs for PR-5 proteins were amplified by polymerase chain reaction and extended by rapid amplification of cDNA ends methods. Recombinant Cup s 3 was expressed in *Escherichia coli* as a fusion protein. Inhibition enzyme-linked immunosorbent assays were used to test the allergenicity of Cup s 3.

Results—Three cDNAs were cloned. These clones had approximately 95% identity to Jun a 3 and Cup a 3. Recombinant Cup s 3.0102 maltose-binding protein inhibited the IgE from most patients from binding to an extract of Italian cypress. The extent of inhibition suggested that antibodies to Cup s 3 were a prominent component of the IgE response to Italian cypress pollen.

Conclusion—Cup s 3, an allergen of Italian cypress pollen, was identified based on cross-reactivity and homology with other pollen PR-5 proteins, despite an apparently low level of protein expression. Variations in the content of Cup s 3 in the pollen from different regions or trees should be considered in the choice of extracts for diagnosis and specific immunotherapy for Italian cypress pollen hypersensitivity.

INTRODUCTION

Pollens from various plants are the major causes of seasonal allergic rhinitis and conjunctivitis and may contribute to asthma. Despite improvements in symptomatic therapy, many patients continue to experience pollinosis. Pollens from plants of the Coniferales order are the major causes of pollen allergy in several regions of the world.^{1–3} An epidemiologic survey in Italy indicated that the prevalence of a positive skin test result for Italian cypress was 17.4% among allergic patients.⁴ Extensive cross-reactivity of the allergens in the extracts of pollen from plants of the Coniferales order has been described by skin testing studies.⁵ Our previous clinical

and immunologic studies of French patients who were allergic to the pollens of Italian cypress and Japanese patients allergic to the pollens of Japanese cedar (*Cryptomeria japonica*) indicated that there is cross-reactivity between these pollen allergens.⁶

The described cross-reactivities between the cedars may have diagnostic and therapeutic value. Hrabina et al⁷ examined the specificity and sensitivity of a standardized prick test, using mountain cedar extracts for diagnosis of cypress allergies. They concluded that mountain cedar pollen extracts can be used for the *in vivo* diagnosis of cypress pollen allergies and are good candidates for specific immunotherapy. However, our understanding of the molecular basis of the cross-reactivities is limited.^{8,9}

Characterizing the allergic responses to plant and animal proteins and the phylogenetic relationships between these organisms can help in predicting cross-sensitization and in selecting therapeutic reagents. The specific allergens that are responsible for the intense allergic sensitization to conifer pollens are beginning to be identified. Recently, a group 1 allergen was identified (Cup s 1, GenBank No. AAF72625). No other allergens from this species have been described.

This study aims to determine whether the pollen from *Cupressus sempervirens* (Italian cypress) contains a pathogenesis-related group 5 (PR-5) protein, similar to that found in other allergenic Cupressaceae pollens. We describe an approach for identifying a new group 3 allergen in Italian cypress pollen based on homology and cross-reactivity with other related allergens. Cup s 3 was identified in the pollen using an antiserum to Jun a 3 of mountain cedar pollen and cloned based on its homology with Jun a 3. By expressing the Cup s 3 complementary DNA (cDNA), we have been able to demonstrate that antibodies to Cup s 3 represent a prominent part of the allergic response to Italian cypress pollen.

MATERIALS AND METHODS

Preparation of Crude Extract From Italian Cypress Pollen and Purification of Jun a 3 From Mountain Cedar Pollen

Pollen of Italian cypress was purchased from Biopol Laboratory Inc (Spokane, WA), and mountain cedar, *Juniperus ashei*, was purchased from Hollister-Stier (Spokane, WA). The crude extract (CE) of Italian cypress pollen was prepared as described previously for mountain cedar.¹⁰ The protein concentration of CE was determined by Coomassie staining of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), using bovine serum albumin (BSA) as a standard. Jun a 3 was isolated from a CE of mountain cedar pollen as described previously.⁹

Human Serum Samples

Serum samples were obtained from 23 patients from Southeast France who were allergic to Italian cypress and maintained freeze-dried until the time of this study. The diagnosis of Italian cypress sensitivity was based on a clinical history of pollinosis, positive skin prick test results, and positive radioallergosorbent test (RAST) results (Pharmacia Diagnostic, Uppsala, Sweden) for IgE antibodies to Italian cypress and mountain cedar CE. Serum samples from 10 control patients were also obtained from allergic patients in France, whose skin test results to Italian cypress were negative. The clinical and laboratory evaluation results of the patients are given in Table 1. Both RAST and skin test results were positive for Italian cypress, except for patient 6, who had a negative RAST result but a positive skin test result, and patient 17, who was not analyzed by RAST.

Detection of Cup s 3 Protein by SDS-PAGE and Western Blotting

A CE of Italian cypress pollen was separated by 4% to 20% gradient (Bio-Rad, Hercules, CA) SDS-PAGE. Proteins on these gels were stained with Coomassie blue or electrotransferred to nitrocellulose membranes for Western blotting analysis.⁹ After blocking, membranes were incubated with patient's sera or an anti-Jun a 3 polyclonal antibody prepared in our laboratory, followed by horseradish peroxidase-conjugated goat anti-human IgE (KPL, Gaithersburg, MD) or horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed, South San Francisco, CA), and were developed with ECL (Amersham Biosciences), according to the manufacturer's recommendations. The intensity of bands of the appropriate migration for Cup s 1 and Cup s 3 was measured by densitometry (AlphaImager, Alpha Innotech Corporation, San Leandro, CA).

Detection of IgE Antibodies to Cup s CE and Native Jun a 3

Polystyrene plates (96 well, Immulon 4HBX, Thermo Lab-systems, Woburn, MA) were coated with 200 μ L of 200 μ g/mL of Italian cypress CE or 5 μ g/mL of purified Jun a 3 in 0.125M borate saline buffer, pH 8.5, and incubated overnight at 4°C. After blocking with 1% BSA in 0.05% Tween 20 in Tris-buffered saline (TBS), pH 7.4, serum from patients with Italian cypress pollinosis, diluted in the Tween-TBS solution, was added. Bound IgE antibodies were detected with an anti-human IgE horseradish peroxidase conjugate and then colorimetric reactions using 3,3',5,5'-tetra methyl benzidine (KPL). Plates were read at 450 nm (Spectra MAX 340 PC, Molecular Devices, Sunnyvale, CA).

cDNA Cloning

Messenger RNA was purified from Italian cypress pollen using oligo-dT cellulose (Amersham Pharmacia Biotech, Pis-cataway, NJ) as described.⁸ First-strand cDNA was synthesized using oligo(dT) and reverse transcriptase (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) primers (forward: 5'-AGAGGCGGGAGTAGTGAAGTTT-3' and reverse: 5'-ATTCGTGGCAGGGCAGTTATC-3') based on Jun a 3 sequences were used.

Rapid amplification of cDNA ends (RACE) was used to sequence the 5' and 3' ends. First-strand cDNA with adapters was synthesized according to the manufacturer's recommended procedure (RLM-RACE, Ambion, Austin, TX). The cDNA was subjected to nested PCR with gene-specific and adapter-specific primers. The gene-specific primers were designed based on the sequence of mountain cedar Jun a 3, 5' outer primer: 5'-GCTGATGGCGATGAATGAACACTG-3'; 5' inner primer: 5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'; 3' outer primer: 5'-GCGAGCACAGAATTAATACGACT-3'; and 3' inner primer: 5'-CGCGGATCCGAATTAATACGACTCACTATAGG-3'. The products of the second PCR were separated by low-melting-point agarose gel electrophoresis, and amplicons of appropriate size were purified using a gel extraction kit (Qiagen, Hilden, Germany). Cup s 3 cDNA was ligated into PCR 2.1 vector (Invitrogen), transformed into *Escherichia coli* DH5 α , cloned, and sequenced using PCR-based techniques and automated detection (Perkin Elmer/Applied Biosystems model 373 A DNA sequencer). Sequences of Cup s 3 were compared against GenBank (GCG, Madison, WI).

Expression of Maltose-Binding Protein (Cup s 3 Fusion Protein)

A cDNA that contained the complete coding sequence of Cup s 3 was amplified with forward (5'-TCTAGAGTTAAGTTTGTATATAAAG-3') and reverse (5'-AAGCTTAAACATAATCCTACG-3') primers that contained restriction sites for *Xba*I and *Hind*III, respectively. The amplicon was inserted into the *Xba*I and *Hind*III sites of pMAL-p2 vector (New England Biolab, Beverly, MA) and transfected into *E coli* HMS 174. Synthesis

of recombinant Cup s 3 (rCup s 3) was induced with 0.5-mmol/L isopropyl β -D-thiogalactoside. Bacteria were harvested by centrifugation, and maltose-binding protein (MBP) Cup s 3 was purified using an amylose resin column (New England BioLabs) and analyzed by SDS-PAGE and Coomassie blue staining. The concentration of MBP Cup s 3 was determined by bicinchoninic acid–based protein assay (Pierce, Rockford, IL) using BSA as a standard.

IgE Inhibition Assay

An inhibition enzyme-linked immunosorbent assay (ELISA) was developed to evaluate the binding of IgE antibodies in the serum of Italian cypress–sensitive patients to recombinant Cup s 3. Maltose (0.58 mmol/L) in TBS-Tween was added to the allergen fusion protein and incubated for 1 hour to saturate the maltose-binding sites. Serum samples were preincubated with MBP Cup s 3 (0.5 mg/mL) or recombinant MBP (0.25 mg/mL) overnight in the wells of an ELISA plate. Next, the mixture was transferred to the high-binding ELISA wells (Immulon 4HBX) previously coated with CE of Italian cypress and blocked as described for the IgE ELISA, except for the addition of the 0.58-mmol/L maltose to the blocking solutions. The IgE that bound to the plate was detected as described herein. The percentage of inhibition of IgE binding to the CE-coated wells was calculated according to the following formula: inhibition (%) = $(OD_B - OD_A)/OD_B \times 100$, where OD_A and OD_B represent the optical density at 450 nm for wells with rMBP Cup s 3 and rMBP only, respectively.

IgE Inhibition Assay With Native Jun a 3

To investigate whether the cross-reactivity of IgE from the serum samples of patients allergic to Italian cypress with Jun a 3 was due to sensitization to Cup s 3, inhibition ELISAs were performed. Patients' serum samples were preincubated overnight with 0.5 mg/mL of native Jun a 3 or an equivalent volume of TBS-Tween. These mixtures were transferred to the ELISA wells coated with MBP Cup s 3, preincubated with maltose. Specific IgE was detected as described herein. The percentage of inhibition was calculated according to the formula above with native Jun a 3 (A) and TBS-Tween (B).

Statistical Analysis

A linear regression analysis was used to evaluate whether the ELISA reactivity to Cup s CE is related to the reactivity to native Jun a 3. The *t* test was used for analysis of inhibition ELISA for the patient group and healthy controls.

RESULTS

Detection of a Jun a 3 Homolog in CE of Italian Cypress Pollen

Based on the finding that a PR-5 protein in mountain cedar is a major allergen for pollinosis patients from Texas, we hypothesized that the highly allergenic Italian cypress pollen would also contain a similar allergen. However, the extracts of Italian cypress pollen we obtained in the United States demonstrated a faint protein band of the appropriate migration on SDS-PAGE stained with Coomassie blue (Fig 1) or on Western blots of these gels that were developed with patient sera and anti-IgE secondary antibody. A rabbit antiserum to Jun a 3 identified a single band of the appropriate migration (approximately 30 kD) for a PR-5 protein (lane 3). Based on the density of Cup s 3, the relative amount of Cup s 1 by the densitometry in the CE from 8 different lots of pollen ranged from 0% to 3.1% (data not shown).

Demonstration of IgE Antibodies That React to Jun a 3 in the Serum Samples of Italian Cypress–Sensitive Patients

To test whether patients allergic to Italian cypress were sensitized to a group 3 allergen, independent ELISAs were performed for IgE binding to CE of Italian cypress and to native

Jun a 3. As shown in Figure 2, most patients had IgE antibodies that bound to Jun a 3. In addition, linear regression analysis indicated the extent of IgE binding to Jun a 3 was highly correlated to the IgE binding to the CE of Italian cypress pollen.

Cloning of Cup s 3

Based on the results reported herein, cloning of cDNAs for homologues of Jun a 3 in Italian cypress was attempted. The initial PCRs, performed using primers based on the nucleotide sequence of Jun a 3, produced a single amplicon of approximately 550 nucleotides. The nucleotide sequence of this product was highly homologous to that of Jun a 3. To obtain the complete coding sequence, 5' and then 3' RACE PCRs were performed using nested primers based on internal sequences and the adaptors. After sequencing each end, a new set of primers was used to produce an amplicon of approximately 900 base pairs (bp). The sequence of this fragment contained a TAG stop codon 675 bp downstream from an ATG start codon.

Three Cup s 3 cDNA variants were obtained repeatedly. We named them Cup s 3.0101, Cup s 3.0102, and Cup s 3.0103. The sequence of Cup s 3.0101 and Cup s 3.0103 differed by only one base, and the inferred amino acid sequences were identical. Cup s 3.0102 differed from Cup s 3.0101 at 5 nucleotide positions, and 2 amino acid residues differed from those of Cup s 3.0101. The consensus amino acid sequences are shown in Figure 3.

Based on the Jun a 3 sequence,⁹ the first 26 N-terminal amino acids of Cup s 3 probably represent a signal peptide. There was also a high degree of amino acid sequence identity between the group 3 allergen of Arizona cypress (Cup a 3, CAC05258) and Jun a 3.^{9,11} We therefore termed the group 3 protein of Italian cypress Cup s 3 (Fig 3).

Demonstration of the Allergenicity of Cup s 3 Using Recombinant Proteins

To confirm Cup s 3's allergenicity, a recombinant fusion protein that contained the microbial MBP and Cup s 3.0102 was synthesized and purified by affinity chromatography. The SDS-PAGE gel shown in Figure 4 shows the purity of the fusion protein and recombinant MBP and native Jun a 3.

The MBP Cup s 3 fusion protein and recombinant MBP alone were used in inhibition ELISAs. Figure 5 shows the percentage of inhibition of serum IgE binding to CE of Italian cypress pollen. Sixteen of 23 patient serum samples demonstrated greater than 20% inhibition, whereas only 1 of 10 control serum samples was inhibited by that CE ($P < .001$) (Fig 5). Interestingly, the binding of IgE from some patient serum samples to extracts of Italian cypress pollen was inhibited by up to 50%. We tested for nonspecific inhibition by the MBP Cup s 3 protein in an assay for IgE anti-*Dermatophagoides farinae* antibodies and did not see significant inhibition (results not shown).

Cross-reactivity of Cup s 3 and Jun a 3

We next questioned whether the association between the IgE reactivity with Cup s CE and Jun a 3 was indeed caused by a cross-reactivity between Cup s 3 and Jun a 3. Inhibition assays were therefore performed in which the binding of IgE to MBP Cup s 3 was inhibited by preincubation with native Jun a 3. As shown in Figure 6, 21 of the 23 serum samples demonstrated inhibition of more than 20%, with a mean inhibition of 48% for the 21 patients with positive inhibitions.

DISCUSSION

Italian cypress pollen is one of the most common causes of seasonal allergic rhinitis in the Mediterranean area.³ However, only one putative allergen, Cup s 1, has been identified

(European Congress Allergology, Paris, France, June 7–11, 2003), and allergenicity of this protein has not been proven yet. We have used the cross-reactivity and sequence homology between Italian cypress and mountain cedar pollen to identify and clone a new allergen (Cup s 3) from the Italian cypress pollen. Recombinant Cup s 3 was then used to evaluate the contribution of this allergen to the IgE response to Italian cypress pollen.

Our first attempts to identify a group 3 allergen in the CE of Italian cypress pollen by SDS-PAGE gel and Western blotting with patient sera did not demonstrate such an allergen. This finding was most likely due to the low concentration of Cup s 3 in the pollen preparations we had available for testing. However, the Western blot using rabbit hyperimmune antiserum to Jun a 3 revealed a protein of the appropriate size. This cross-reactivity suggested the expression of a Jun a 3 homolog in Italian cypress pollen. We were then able to show by ELISA that most serum samples from Italian cypress–allergic patients had IgE antibodies to Jun a 3, the group 3 allergen from mountain cedar. We also found a surprisingly strong correlation between the reactivity of individual serum samples with Jun a 3 and CE of Italian cypress. In fact, approximately 60% of the variability in the reactivity of IgE to CE could be accounted for by reactivity with Jun a 3.

The serologic evidence for the presence of a group 3 allergen in Italian cypress encouraged us to clone that allergen and show that there was a high degree of amino acid sequence identity between Jun a 3 and Cup s 3. We also demonstrated by inhibition ELISA a cross-reactivity between Cup s 3 and Jun a 3 in most of the patient serum samples.

Our study also raises a number of questions about the immune response of the French patients to Cup s 3. Why do these patients respond so vigorously to an allergen that is not abundant in the pollen to which they are exposed? For instance, the proportion of IgE anti-Italian cypress pollen antibody that is directed against Cup s 3 ($47\% \pm 71\%$) seems to be much higher than we have seen for Jun a 3 reactivity in mountain cedar ($12\% \pm 27\%$, data not shown). Others have commented that analysis of Italian cypress pollen allergens was difficult due to the viscous nature and low protein content of aqueous extracts.⁵ However, strong protein bands were seen by Coomassie staining in the region anticipated for the group 1 allergen, Cup s 1.¹²

The PR proteins are produced by plants in response to pathogens or other noxious stimuli,^{9, 13,14} such as drought and flooding; freezing temperature; fungal, bacterial, and viral infections; and ozone and UV light.^{15,16} Another study has shown that PR-5 protein allergen in Japanese cedar pollen, Cry j 3, was induced by exposure to UV light and various chemicals.¹⁷ The low amount of Cup s 3 in our pollen might also be due to the low basal expression of this pathogenesis-induced protein. However, we examined CE from other lots of the pollen from the United States and found Cup s 3 abundance was low, relative to the Jun a 3 in mountain cedar CE.⁹ It is possible that pollen from Italian cypress trees in France contains more Cup s 3 than in our samples. As shown in Figure 3, all known PR-5 homologous allergens have an extremely high degree of homology. It is therefore likely that these proteins may have essential functions for these plants. At least 33 other PR proteins have now been shown to be allergenic in humans.¹⁴ Selection of extracts in which Cup s 3 is well represented should improve both diagnosis and treatment of Italian cypress pollinosis.

In summary, we have cloned and sequenced 3 isotypes of an allergen from Italian cypress that have a high degree of homology with Jun a 3. The IgE of Italian cypress–allergic patients reacted with Cup s 3 and Jun a 3. A more complete identification and structural characterization of Cupressaceae family pollen allergens should allow us to improve the diagnostic and therapeutic application of these allergens and to identify allergenic extracts that can be used in specific immunotherapy in patients with sensitivity to various Cupressaceae species.

Acknowledgements

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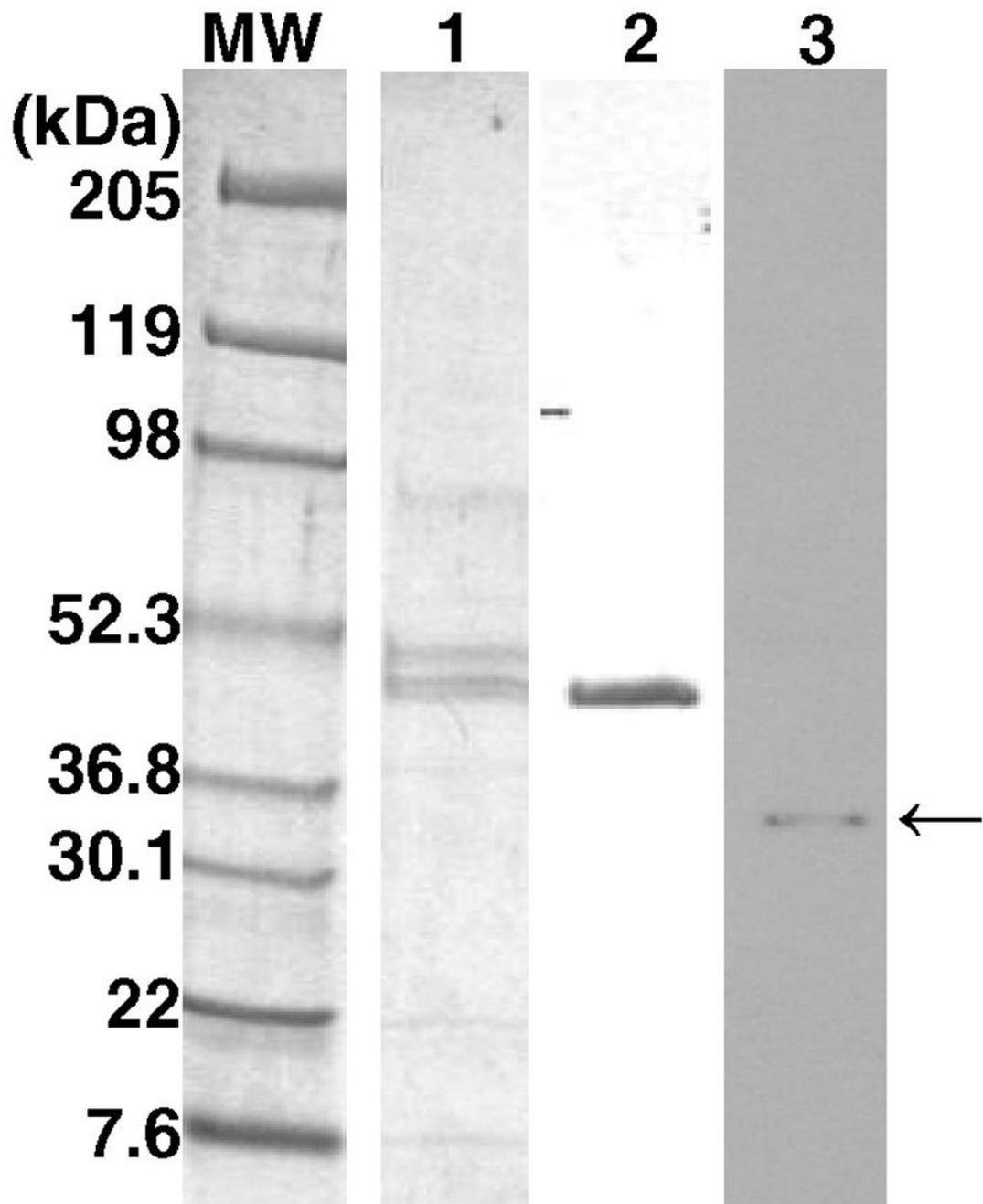


Figure 1.

Cup s 3 identification by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Italian cypress crude extract was separated on the SDS-PAGE and analyzed by Western blot. No band of the appropriate size for Cup s 3 was visible in lane 1 (Coomassie blue) or lane 2 (representative pattern of patients' IgE). Lane 3 (anti-Jun a 3) demonstrates the presence of a protein that cross-reacts with Jun a 3. The bands at 40 to 50 kD are the anticipated size and intensity for Cup s 1. Lane MW contains molecular weight markers.

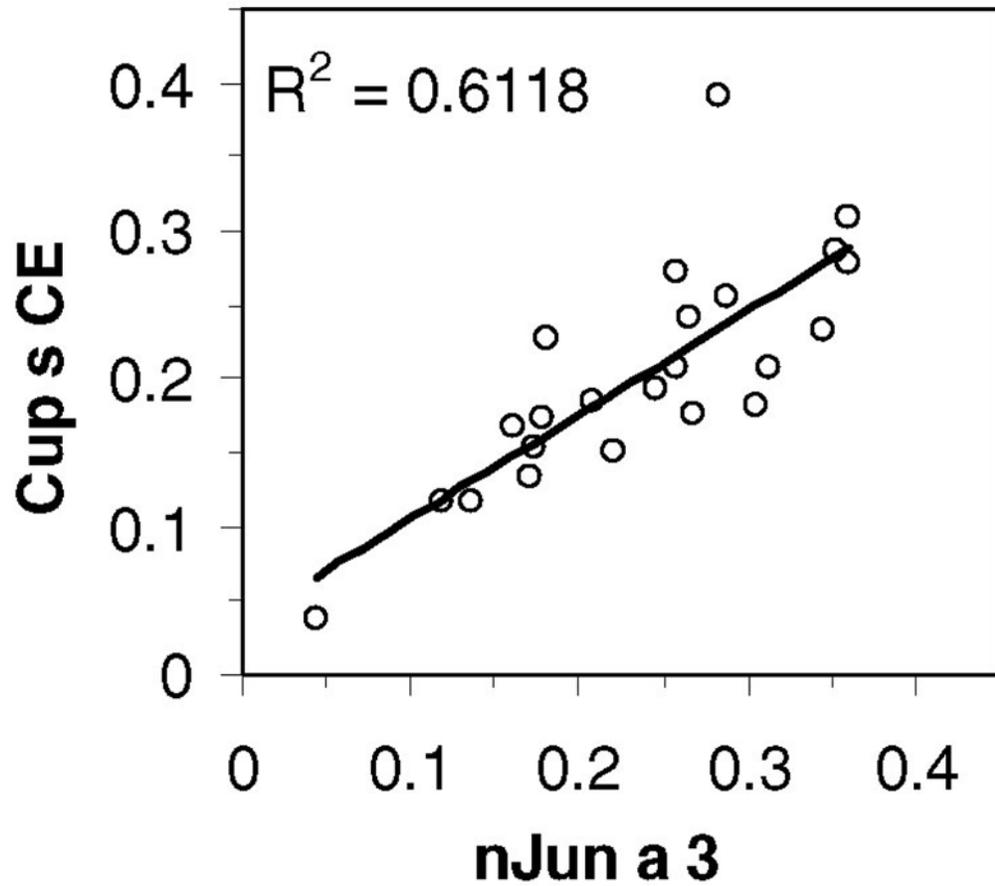


Figure 2. A linear regression analysis of the IgE enzyme-linked immunosorbent assay values against crude extract (CE) of Italian cypress and those against purified native Jun a 3. Each circle represents the values for single Italian cypress-allergic patient.

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Cup s 3.0101          MARVSE LALLLVATSA ISLHMQEAGA -26
Cup s 3.0102          .....L. ....
Cup a 3              *****
Jun a 3              .....F..A..L. ....

Cup s 3.0101 VKFDIKNQCR YTVWAAGLPG G GKRLDQGQT WTVNLAAGTA SARFWGRTGC 50
Cup s 3.0102 .....G .....
Cup a 3 .....K.G .....EF.....
Jun a 3 .....G .....

Cup s 3.0101 TFDWSGTGSC RSGDCGGQLS CTVSGAVPAT LAEYTSQSDQD YYDVSLVDGF 100
Cup s 3.0102 .....K.....
Cup a 3 ...A..K.....
Jun a 3 [..A..K...QT].....

Cup s 3.0101 NIPLAINPTN TKCTAPACKA DINAVCPSEL KVDGGCNSAC NVLQTDQYCC 150
Cup s 3.0102 .....
Cup a 3 .....
Jun a 3 .....AQ.....[.....FK].....

Cup s 3.0101 RNAYVDNCPA TNYSKIFKNQ CPQAYSYAKD DTATFACASG TDYSIVFCP* identity
Cup s 3.0102 ..... 98.9%
Cup a 3 .....N..... 97.0%
Jun a 3 [.....]..... 95.5%
    
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Figure 3. The implied amino acid sequences for group 3 cedar allergens. A TAG stop codon after amino acid 199 is indicated by an asterisk. Sequence identity with Cup s 3.0101 is indicated in the lower right corner. The boxes indicated the IgE epitopes identified by probing proteolytic fragment¹⁸ and overlapping synthetic peptides with the serum samples from patients allergic to mountain cedar. Nucleotide sequences of Cup s 3.0101 (AY353705 and AY353707) and Cup s 3.0201 (AY353706) were submitted to GenBank.

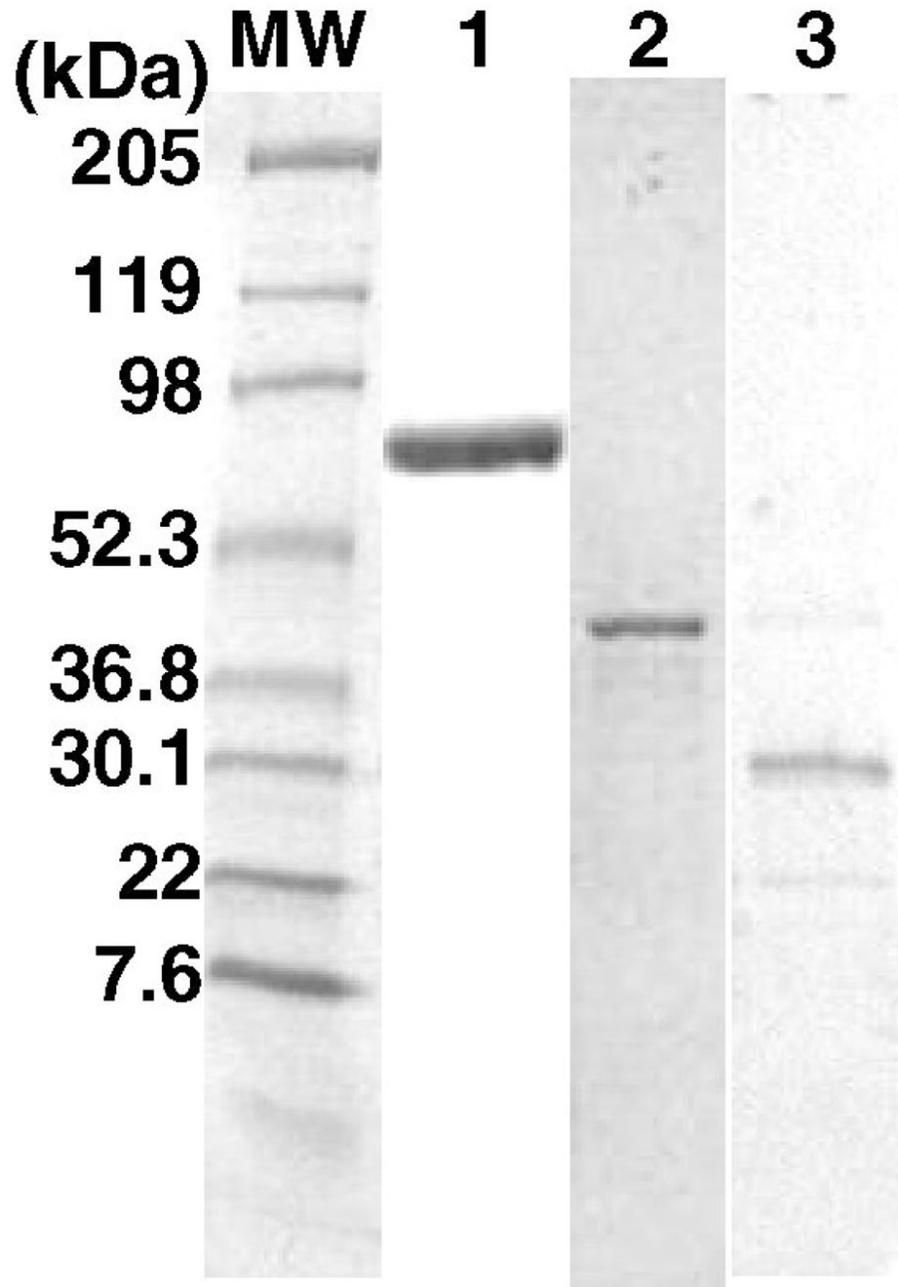


Figure 4. Electrophoresis pattern on 4% to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel for purified maltose-binding protein (MBP) Cup s 3 (lane 1), native Jun a 3 purified by high-performance liquid chromatography (Lane 3), and recombinant MBP (Lane 2). The gel was stained with Coomassie blue stain. Lane MW contains molecular weight markers.

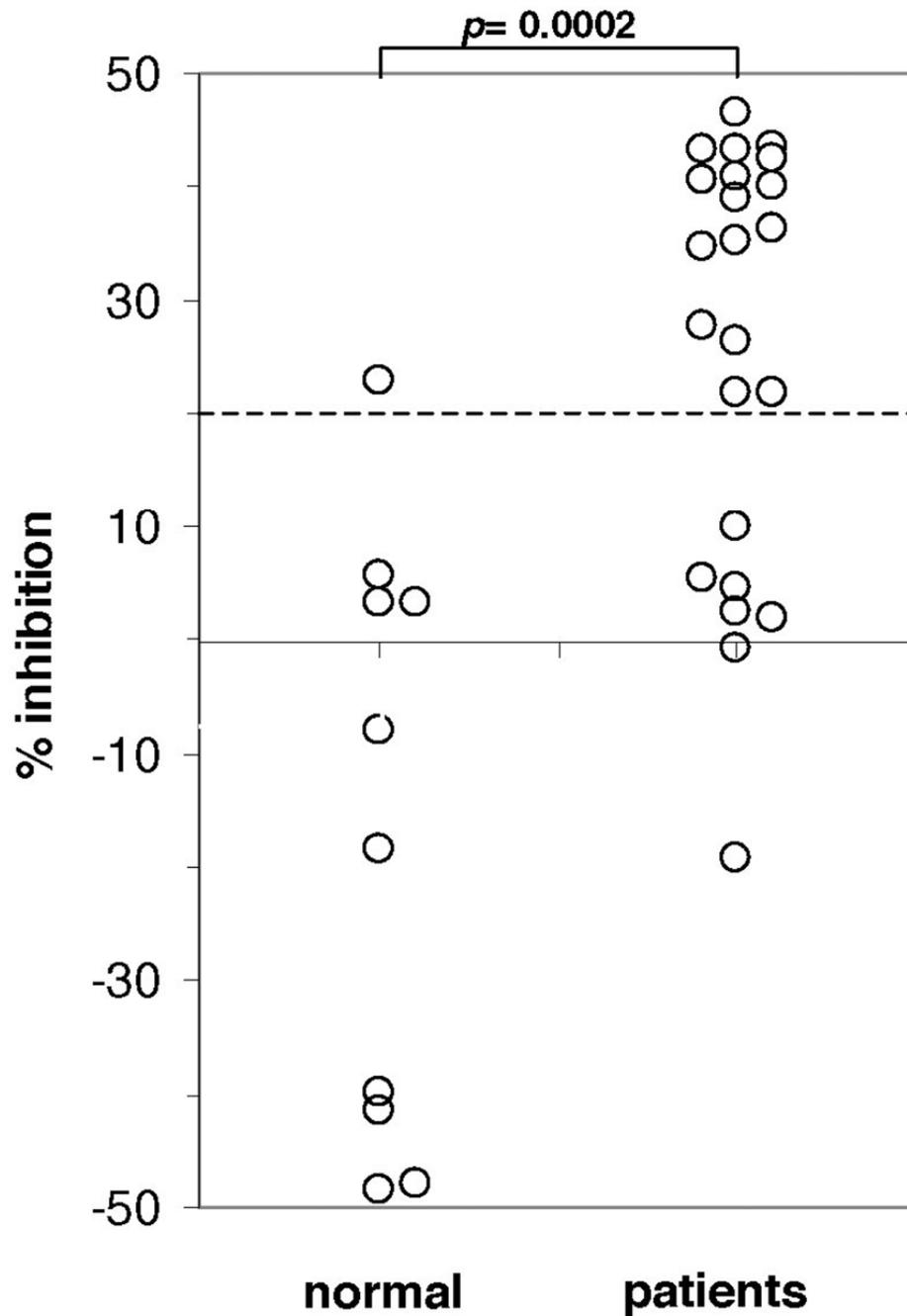


Figure 5.

Maltose-binding protein (MBP) Cup s 3 inhibits IgE binding to crude extract (CE) of Italian cypress pollen. The serum samples from patients and healthy controls were preincubated with 0.5 mg/mL of MBP Cup s 3 or 0.25 mg/mL of MBP before testing in an enzyme-linked immunosorbent assay plate coated with CE. The results are expressed as the percentage of inhibition by MBP Cup s 3 compared with MBP control, as described in the “Methods” section.

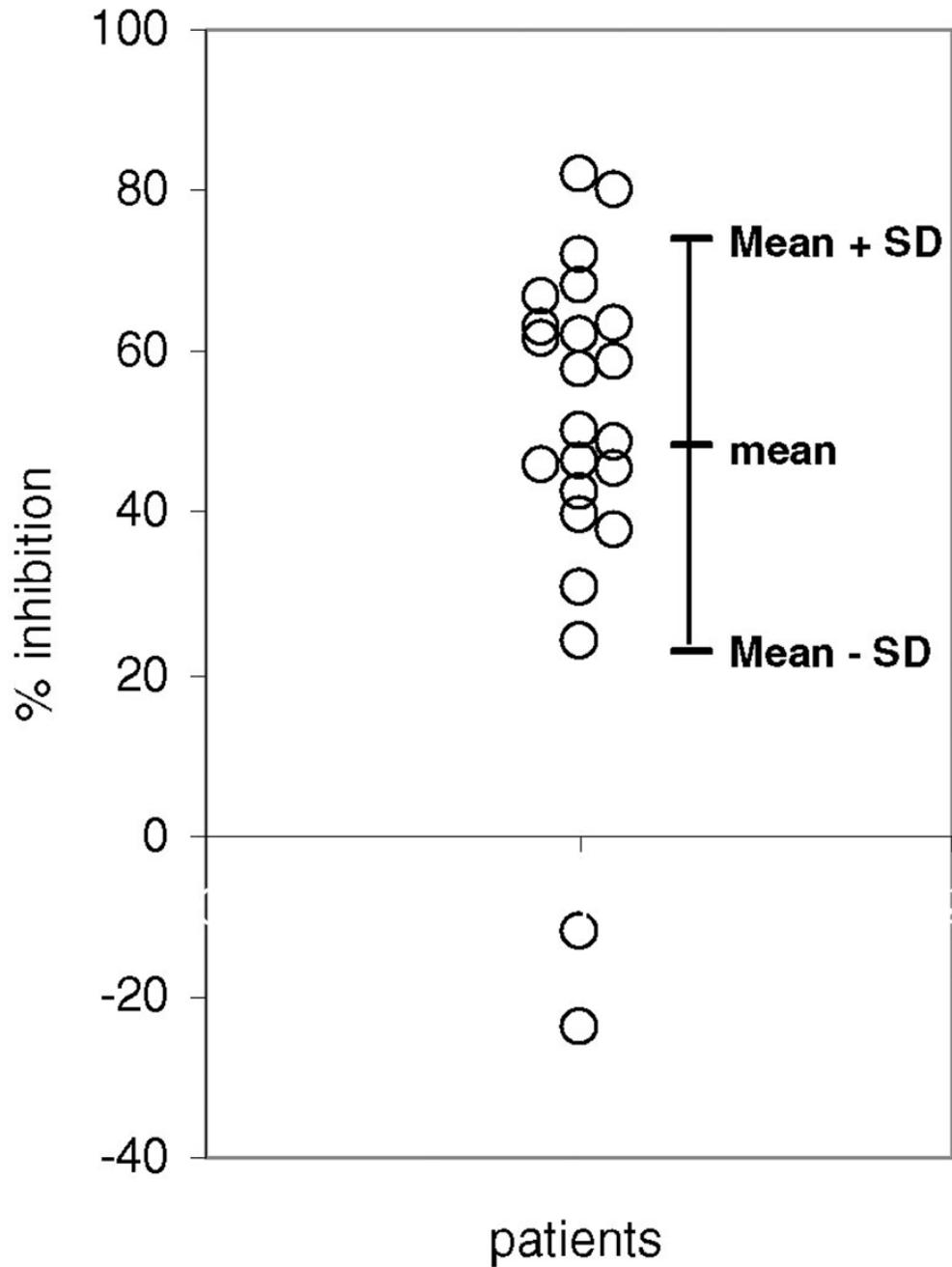


Figure 6.

Inhibition assay of serum IgE to maltose-binding protein (MBP) Cup s 3 by native Jun a 3. The patients' serum samples were preincubated with 0.5 mg/mL of Jun a 3 before testing in an enzyme-linked immunosorbent assay coated with MBP Cup s 3. The results are expressed as the percentage of inhibition by Jun a 3, as described in the "Methods" section.

Table 1

Characteristics of 23 Patients Allergic to Italian Cypress

Patient No./sex/age, y	Clinical history	Skin test result to <i>Cupressus sempervirens</i> , cm*	RAST class to		Other allergies
			<i>Juniperus ashei</i>	<i>C sempervirens</i>	
1/M/22	Asthma	0.5+ 3+	0	3	Grass pollen, <i>Parietaria judaica</i> , <i>Olea europaea</i> pollen, <i>Alternaria alternata</i> , <i>Dermatophagoides pteronyssinus</i> or <i>farinae</i>
2/M/31	AR, conjunctivitis	0.5+ 2+	4	3	Grass pollen, <i>O europaea</i> pollen, <i>Platanus</i> pollen
3/F/37	AR, conjunctivitis	1+ 3+	5	3	<i>O europaea</i> pollen
4/M/30	AR, conjunctivitis, urticaria	1+ 3+	4	3	Household insects
5/M/49	Asthma	1+ 2+	3	2	<i>O europaea</i> pollen, <i>D pteronyssinus</i> or <i>farinae</i>
6/M/21	AR, conjunctivitis	0.5+ 2+	2	0	Grass pollen
7/M/31	AR	1+ 2+	3	2	Grass pollen, <i>D pteronyssinus</i> or <i>farinae</i>
8/F/28	AR, conjunctivitis	1+ 3+	4	3	None
9/F/23	AR, conjunctivitis	1+ 2+	4	3	Grass pollen
10/M/31	AR, conjunctivitis	1+ 3+	4	3	Grass pollen, <i>Alternaria alternata</i>
11/M/34	AR, conjunctivitis	1+ 4+	4	3	Cat, <i>D pteronyssinus</i> or <i>farinae</i>
12/F/47	AR, conjunctivitis, asthma	1+ 4+	4	3	None
13/F/63	Urticaria	1.5+ 4+	3	3	Grass pollen
14/M/40	AR, conjunctivitis, asthma	1.5+ 2+	3	3	Grass pollen, <i>O europaea</i> pollen, <i>Platanus</i> pollen
15/F/41	AR	1.5+ 4+	4	3	Cat, <i>O europaea</i> pollen
16/M/38	AR, conjunctivitis	1+ 3+	3	3	None
17/M/40	AR, conjunctivitis	1.5+ 3+	ND	ND	<i>D pteronyssinus</i> or <i>farinae</i>
18/M/30	Asthma	1+ 3+	ND	2	Grass pollen
19/M/61	AR	0.5+ 2+	ND	1	Grass pollen, <i>O europaea</i> pollen, <i>Platanus</i> pollen
20/F/27	AR	1.5+ 3+	ND	3	<i>O europaea</i> pollen
21/F/35	AR	1.5+ 3+	ND	4	None
22/M/50	Asthma	1+ 2+	ND	2	<i>P judaica</i>
23/F/45	AR, conjunctivitis	1.5+ 3+	ND	2	<i>D pteronyssinus</i> or <i>farinae</i>

Abbreviations: AR, allergic rhinitis; ND, not done; RAST, radioallergosorbent assay.

* Skin test average in centimeters of the wheal and flare.