Purification and characterization of an 18-kd allergen of birch *(Betula verrucosa)* pollen: Identification as a cyclophilin

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Background: Five birch pollen allergens have been identified so far. In a previous study we detected new birch pollen allergens with an isoelectric point in the range 9.0 to 9.3, present only in extracts prepared at controlled basic pH.
Objective: The purpose of the current study was to purify and characterize those allergens.
Methods: The target allergens were purified by ion exchange and hydrophobic interaction chromatography. Analyses were carried out by SDS-PAGE, isoelectric focusing, immunoblotting, and amino acid sequencing. The in vivo reactivity of the allergens was evaluated by skin testing.
Results: An 18-kd protein, which we named Bet v 7, was purified. This 18-kd protein corresponded to 3 bands on isoelectric-focusing immunoblots that probably represent isoforms. On immunoblots up to 20.8% of birch pollen–allergic patients recognized those allergens. The clinical relevance of Bet v 7 was demonstrated by positive immediate-type skin testing on a patient allergic to birch pollen. Sequencing of an internal peptide yielded an amino acid sequence showing high homology with various plant cyclophilins. The rotamase activity of the protein, inhibited by cyclosporin A, further confirmed that Bet v 7 belongs to the group of cyclophilins.
Conclusion: We have purified a novel allergen of birch pollen, Bet v 7, belonging to the cyclophilin family. Because cyclophilins are highly conserved proteins over the phylogeny, we may postulate that Bet v 7 is a member of a new family of panallergens. (J Allergy Clin Immunol 2000;105:286-91.)

Key words: *Allergen, birch pollen, chromatography, cyclophilin, peptidyl-prolyl isomerase.*

Birch pollen is a major cause of allergy in the northern parts of Europe and America and in some areas of Australia, with symptoms occurring in early spring.[1]

Abbreviations used
CsA: Cyclosporin A
IEF: Isoelectric focusing
NFDM: Nonfat dried milk
PB: Phosphate buffer
pI: Isoelectric point
sAAPFn: Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide

Five birch pollen allergens have already been identified so far. The major allergen is Bet v 1, a 17-kd protein consisting of several isoallergens[2] and recognized by IgE antibodies from almost all birch pollen–allergic patients.[3] The Bet v 1 genes show similarity with genes involved in plant defense mechanisms.[4] Proteins that resemble Bet v 1 are found in some fruits and vegetables and are responsible for food-pollen cross-allergies.[5-8] The second well-known allergen is the profilin, Bet v 2,[9] also involved in birch pollen–associated food allergy.[5] Two other allergens, namely, Bet v 3 and Bet v 4, are minor allergens in that they are recognized by a small percentage of sera from birch pollen–allergic patients. Both have been demonstrated to be calcium-binding proteins.[10,11] Recently a 35-kd protein related to the isoflavone reductases has been shown to bind the IgE of birch pollen–allergic subjects.[12] This protein could also be involved in pollen-food cross-allergies.

We have found previously that birch pollen extracts prepared at pH 7.5 or 8.5 under continuous pH control contained 3 unknown allergens with isoelectric points (pI) of 9, 9.1, and 9.3, possibly isoforms of the same protein.[13] They demonstrated reaction with up to 30% of birch pollen–sensitive patient sera. The sequencing of the blotted proteins was unsuccessful, which we attributed to N-terminal blockage.

In this report we describe the purification of this allergen, which we designated as Bet v 7. An internal amino acid sequence analysis showed high homology of this allergen to plant cyclophilins, which was confirmed by rotamase assay and binding of cyclosporin A (CsA). The clinical relevance of Bet v 7 was confirmed by skin testing.

METHODS

Allergen extracts

Betula verrucosa pollen was purchased from Allergon (Engelholm, Sweden). Extraction (1:10 wt/vol) was performed for 3 hours at room temperature in phosphate buffer (PB) 0.01 mol/L, pH 7.5, under continuous pH control and adjustment, as already described.[13]

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The name Bet v 7 has been approved by the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Sub-Committee. The protein sequence data have been submitted to SwissProt (accession number P81531) and PIR (accession number A58996) databases.
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IgE antialbumin antibodies by the huge excess of human albumin in serum. It is equally possible that dog albumin–specific IgE antibodies react primarily with continuous epitopes that are different between dog and human albumin and therefore fail to cross-react with human serum albumin. There is indeed evidence that IgE antibodies of patients allergic to albumin bind to continuous epitopes[35,36] and react preferentially with denatured albumin.[15,37-39]

In conclusion, we believe that the availability of the dog albumin sequence and recombinant dog albumin will contribute to the analysis of interactions of the humoral and cellular immune responses with this allergen. Recombinant dog albumin and genetically engineered hypoallergenic variants thereof may be useful tools for allergy diagnosis and immunotherapy, respectively.

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Extracts were centrifuged (17500*g*, 30 minutes, 4°C) to remove pollen particles and then filtered through a 0.45-μm membrane.

Purification of allergens

The extraction buffer was exchanged for TRIS 0.05 mol/L, pH 8.5. The extract was then incubated with an equal volume of DEAE-Sephacel gel (Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated in TRIS 0.05 mol/L, pH 8.5. The gel was removed by centrifugation (600*g*, 5 minutes) and the supernatant was twice reincubated with fresh DEAE-Sephacel.

The resulting supernatant (fraction A) was dialyzed overnight against 1.4 mol/L ammonium sulfate in 0.1 mol/L PB, pH 7.0, before hydrophobic interaction chromatography in a phenyl Sepharose column (Pharmacia Biotech) equilibrated in 1.4 mol/L ammonium sulfate in 0.1 mol/L PB, pH 7.0. Bound proteins were eluted by application of a gradient of 80% to 0% of 1.4 mol/L ammonium sulfate in the same buffer. Fractions were collected and concentrated by membrane filtration (cutoff value 10 kd), and the buffer was exchanged for PB before the samples were aliquoted and frozen at –70°C until use.

Electrophoresis and immunoblotting

Isoelectric focusing (IEF) was carried out with use of a Multiphor II device for horizontal electrophoresis (Pharmacia Biotech) in 5% thin acrylamide gels containing 7.5% ampholytes with a pH range of 3 to 10 (Isolab, Akron, Ohio) poured on Gelbond PAG film (FMC, Rockland, Me). The running conditions were 1500 V, 25 mA, and 15 W for a total of 1250 Vh. For immunoblotting experiments, proteins were transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, Mass) by press blotting. After they were blocked in 0.2% nonfat dried milk (NFDM) in PBS, membrane strips were incubated overnight at 4°C in patient sera diluted in PBS-NFDM 0.1% (125 μL of serum per strip). Bound IgE was afterward detected by monoclonal mouse antihuman IgE antibodies (CLB, Amsterdam, The Netherlands), followed by peroxidase-conjugated goat antimouse IgG (CLB). Bands were revealed by incubation in the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (KPL, Gaithersburg, Md).

SDS-PAGE was carried out in 13% polyacrylamide gels with 5% polyacrylamide stacking gel with the discontinuous buffer system of Laemmli.[14] Before runs, samples were heated for 5 minutes at 100°C in sample buffer consisting of TRIS 125 mmol/L, pH 6.8, glycerol 17.5%, SDS 4%, β-mercaptoethanol 1%, and bromophenol blue 0.002%. A voltage of 350 V was applied until the bromophenol blue reached the opposite side of the gel.

Microsequencing

Microsequencing was performed by Edman degradation on a pulsed liquid-phase protein sequencer 477/120A (Perkin Elmer, Foster City, Calif). N-terminal amino acid sequencing of the purified protein was unsuccessful, which confirmed the N terminal blockage we had already observed.[13] The protein was therefore digested by the endoproteinase Asp-N for 16 hours at 37°C, and the resulting fragments were separated by reversed-phase HPLC on a C-8 Aquapore RP-300 (1 × 50 mm) column (Perkin Elmer). Similarity searches and alignments of sequences were done by nonredundant searches with use of the Blitz server and the Swissall database.

Skin testing

To ascertain that birch pollen cyclophilin is able to give clinical reactions, skin prick tests with fraction A (extract after DEAE-Sephacel) (80 μg/mL in 50% glycerol) were performed on the forearms of birch pollen–allergic patients selected by clear history and positive skin test to birch pollen until a positive patient was found. Thereafter skin-prick tests with purified Bet v 7 (at 1, 10, and 100 μg/mL in 50% glycerol) were carried out on that patient. A solution of 50% glycerol was used as a negative control.

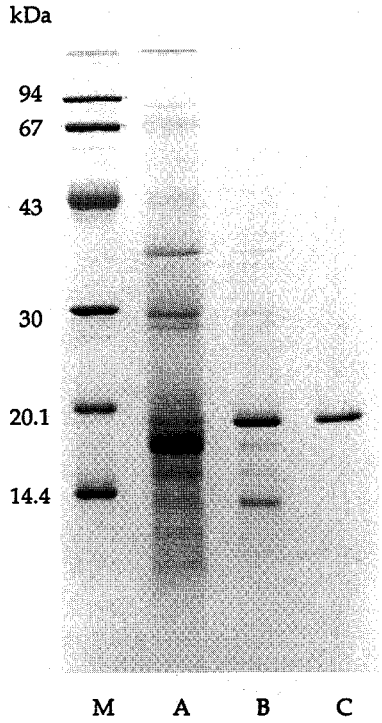

FIG 1. Coomassie blue–stained SDS-PAGE gel showing *A*, total birch pollen extract, *B*, fraction A (see Material and methods), and *C*, pooled fractions 48 to 53 (4 μg of protein) after hydrophobic interaction chromatography. Molecular mass markers shown on *left* are phosphorylase B (94 kd), BSA (67 kd), ovalbumin (43 kd), carbonic anhydrase (30 kd), soybean trypsin inhibitor (20.1 kd), and α-lactalbumin (14.4. kd).

Rotamase assay

Rotamase activity was assayed by a modification of the original method of Fischer et al.[15] In this assay a chromogenic peptide, the succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (sAAPFn, Sigma, St Louis) is cleaved by chymotrypsin, but only when it is in the *trans*-Ala-Pro conformation. More than 80% of the peptide is in this conformation at equilibrium. This fraction will be cleaved almost immediately by chymotrypsin, but the remaining 20% must undergo the relatively slow process of isomerization from *cis* to *trans* before it can be cleaved. Rotamase activity, which facilitates the *cis-trans* isomerization, can then be detected by the acceleration of the rate of peptide cleavage. In our assays, carried out in HEPES buffer 35 mmol/L, pH 8.0, the final concentration of chymotrypsin was 2 μmol/L and that of sAAPFn was 10 μmol/L. Rotamase activity was evaluated for Bet v 7 concentrations of 3 and 30 nmol/L. Absorbance at 390 nm was measured every second in an Ultrospec Plus 4054 spectrophotometer (Pharmacia Biotech). To inhibit rotamase activity, CsA was used at 1 μmol/L.[16] As a negative control for the inhibition of rotamase activity, we used 1 μmol/L FK506, which is known to specifically bind another group of peptidyl-prolyl isomerases, the FK506-binding proteins.[17,18]

RESULTS

Allergen purification

The purification steps are illustrated in Fig 1. Incubation of crude extract with DEAE-Sephacel at pH 8.5

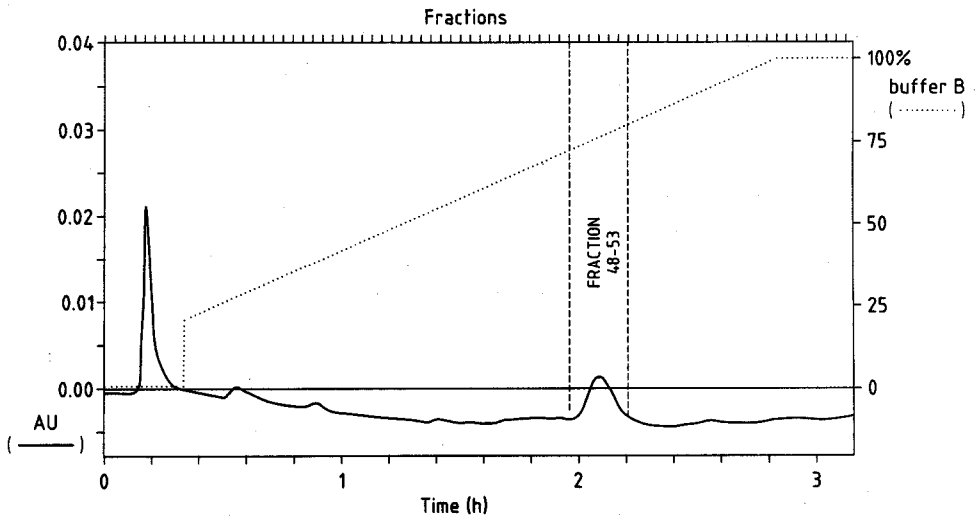

FIG 2. Purification of Bet v 7 by phenyl Sepharose HP chromatography. Fraction A in 1.4 mol/L ammonium sulfate in 0.1 mol/L PB, pH 7.0, was loaded on column and then eluted by 20% to 100% gradient of 0.1 mol/L PB, pH 7.0 (buffer B), at flow rate of 2 mL/min. Absorbance *(AU)* was monitored at 280 nm.

TABLE I. Similarity searches and alignments of sequences* with the sequence DFTAGNGTGGESIYGAK of Bet v 7

Source	Molecular mass (d)	No. of amino acids	Position of corresponding internal sequence	Identity with Bet v 7 sequence (%)	SwissProt accession No.
Foxglove *(Digitalis lanata)*	18055	172	73-89	100	Q96417
French bean *(Phaseolus vulgaris)*	18160	172	73-89	100	Q41119
Rosy periwinkle *(Catharanthus roseus)*	18285	172	73-89	100	Q39613
Tomato *(Lycopersicon esculentum)*	17910	171	73-89	100	P21568
Onion *(Allium cepa)*	16033	150	51-67	100	P34887
Broad bean *(Vicia faba)*	18065	171	73-89	94	O64456
Mouse-ear cress *(Arabidopsis thaliana)*	18492	172	73-89	94	P34790

*All sequences are from plant cyclophilins.

yielded a solution enriched in 18-kd protein (fraction A). The proteins of this fraction were further separated by hydrophobic interaction chromatography, for which a typical elution profile is shown in Fig 2. Screening of the resulting fractions was performed by IEF immunoblotting with sera that were previously shown to recognize protein bands at pI 9.0, 9.1, and 9.3 in an IEF pattern (data not shown). The target basic proteins were found in the fractions 48 to 53. These 48 to 53 fractions gave a single band at 18 kd on SDS-PAGE (Fig 1). A serum reacting with a high number of bands, including the basic ones, was used to probe IEF immunoblot strips of fraction 48 to 53. Only 3 bands at pI 9.0, 9.1, and 9.3 could then be distinguished (Fig 3). This confirmed that several isoforms of Bet v 7 must exist. The protein concentration was determined by absorbance at 280 nm, assuming optical density = 0.6 for 1 mg/mL, which is the absorbance of tomato cyclophilin calculated from its whole sequence.[16] Typically, an amount of 250 μg of purified protein was obtained from 30 g of pollen.

Protein sequencing

Digestion of the homogeneous allergen with the endoproteinase Asp-N yielded several fragments for protein sequence analysis. Two internal sequences could be obtained, DFTAGNGTGGESIYGAK and DXXXXXXXTGPGILSMANAGPGTNG, where *X* designates unreadable amino acids. The results of similarity searches with the former sequence are presented in Table I. Both sequences showed high homology or complete identity with various plant cyclophilins.

Skin testing

After 6 negatively responding patients, 1 patient displayed a clearly positive immediate-type skin test with fraction A and thereafter with the purified birch pollen cyclophilin. Control with 50% glycerol was negative. The sera of the 7 skin-tested patients were used in IEF immunoblots with total birch pollen extract. There was a good correlation between the skin tests and the blots because only the patient who was positive on skin test

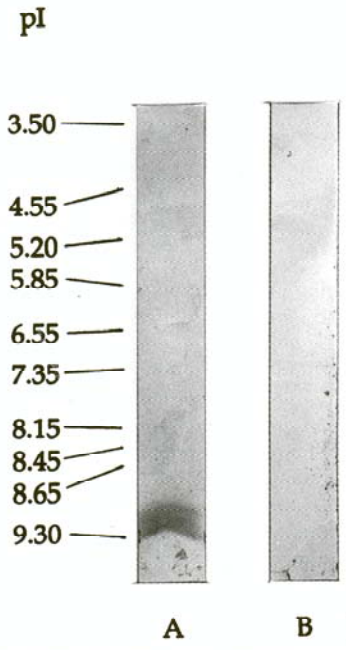

FIG 3. IgE immunoblot from IEF gel of Bet v 7. Pooled fractions 48 to 53 were probed with *A*, serum of patient reacting to high number of birch pollen allergens, including those in the basic range, and *B*, incubation buffer as control. pI markers are indicated on *left*.

for the purified Bet v 7 demonstrated IgE binding to the 3 basic bands on immunoblot (data not shown).

Rotamase activity

Rotamase activity was demonstrated for Bet v 7 in a dose-dependent manner (Fig 4). Furthermore, the 18-kd allergen did bind CsA, which inhibited its rotamase activity, but did not bind FK506 (Fig 4).

IgE prevalence

Screening on IEF immunoblots with 48 sera from birch pollen–allergic patients showed 20.8% of positive reactions (data not shown). Sera were selected from patients with a positive history of birch allergy, associated with either positive skin tests for birch pollen or both positive skin tests and radioallergosorbent tests.

DISCUSSION

An 18-kd protein from birch pollen, which we designated as Bet v 7, has been purified by ion exchange followed by hydrophobic interaction chromatography. This protein corresponded to the 3 bands previously detected on IEF immunoblots of birch pollen extracts prepared at controlled basic pH.[13] Those 3 bands probably represent isoallergens. Screening with 48 sera from birch pollen–allergic subjects demonstrated 20.8% of IgE reactivity to this protein.

Amino acid sequencing of fragments obtained by digestion with endoproteinase Asp-N showed that Bet v 7 has a very high homology (up to 100% identity) with

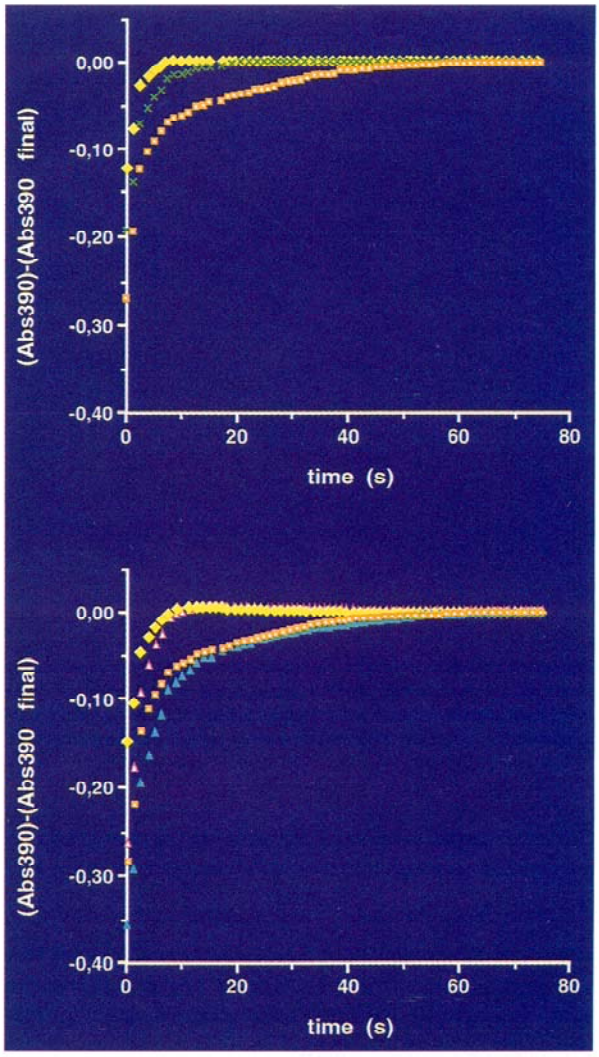

FIG 4. Rotamase assay of purified Bet v 7, involving no Bet v 7 *(orange squares)*, 3 nmol/L Bet v 7 *(green crosses)*, 30 nmol/L Bet v 7 *(yellow diamonds)*, 30 nmol/L Bet v 7 + 1 μmol/L CsA *(blue triangles)*, 30 nmol/L Bet v 7 + 1 μmol/L FK506 *(pink triangles)*. Absorbance at 390 nm reflects cleavage of chromogenic peptide sAAPFn.

cyclophilins of various plants. Plant cyclophilins, accordingly, have a molecular mass of about 18 kd and a basic pI.[19] They are also known to possess a peptidyl-prolyl *cis-trans* isomerase (rotamase) activity and to bind CsA[16,20] like their animal counterparts.[21-23] We have demonstrated in the current study that Bet v 7 possesses rotamase activity, which was inhibited by CsA but not by FK506. This further confirms that Bet v 7 belongs to the cyclophilin family.

Cyclophilins constitute a family of highly conserved proteins and have been found in every organism investigated so far, ranging from bacteria to man. In higher plants they exist in multiple isoforms that are located in different cellular compartments.[24-28] In mouse-ear cress *(A thaliana)*, for example, up to 6 isoforms have been characterized, among them 5 cytoplasmic and 1 chloro-

plastic cyclophilin.[27] Accordingly, in the current study, as in our previous one,[13] we detected 3 potential isoforms of the birch pollen cyclophilin on IEF gels and blots. All 3 bound patient IgE.

The cellular function of cyclophilins remains essentially unknown, although it has been suggested that they can serve as molecular chaperones or folding catalysts[29,30] or be involved in intracellular signaling pathways, reacting with calcineurin, a calcium-binding protein.[31] In plants, their presence has been mentioned in almost all organs studied: roots, leaves, stems, buds, and anthers.[16,27] However, to our knowledge, this is the first report on the presence of cyclophilin in pollen. Because of the poor knowledge of the cellular function of cyclophilins, it is difficult to predict their role in pollen. The synthesis of cyclophilins in maize and bean has been shown to be up-regulated in response to some selective stress conditions, such as heat, exposure to chemicals, and infection by pathogens.[32] Likewise, cold, hormonal, or osmotic stress induced an increasing level of 2 cyclophilins of foxglove.[33] Interestingly, Bet v 1, the major allergen of birch pollen, and homologous proteins are known to be up-regulated by stress.[4,34] This raises the possibility that up-regulation by stress is a common trait of at least some pollen allergens, which would point out the role of pollution in the rising incidence of birch pollen allergy.

Because Bet v 7 had been detected on immunoblots only, it was necessary to investigate its relevance as an allergen in vivo. Indeed, detection with patient serum on immunoblots can be achieved with only one epitope on the antigen, but this would not be sufficient to provoke allergic manifestations, where at least 2 epitopes are needed to cross-link IgE and cause mast cell or basophil degranulation. Therefore we carried out skin tests with purified Bet v 7 on 7 birch pollen–allergic patients. Among them one displayed a clear positive immediate-type response to purified Bet v 7, which indicates that at least two B-cell epitopes are present on this molecule.

Proteins of the cyclophilin family have already been described as allergens in the molds *Psilocybe cubensis*,[35] *Aspergillus fumigatus*,[36,37] and *Malassezia furfur*,[38] where they have been named Pci c 2, Asp f 11, and Mal f 6, respectively. The two internal sequences of Bet v 7 show between 76% and 94% identity with the corresponding sequences of Asp f 11 and Mal f 6 (unpublished data), which is less than the amino-acid identity observed with higher plants (see Table I). Possible cross-reactivity between Bet v 7 and the allergenic mold cyclophilins remains to be studied.

In this report we have described the purification and characterization of Bet v 7, a new birch pollen allergen. It was shown that Bet v 7 is a cyclophilin and that it can induce mast cell degranulation in vivo. Whether it is involved in allergic cross-reactions between pollen species or between food and pollen is currently under investigation. Cyclophilins have already been shown to be involved in immunologic cross-reactions. It was reported that the mold cyclophilins Mal f 6 and Asp f 11 can be cross-reactive.[38] An antiserum raised against cytosolic cyclophilin of mouse-ear cress demonstrated cross-reactivity with a broad range of cyclophilins.[25] Furthermore, human cyclophilin did bind IgE from sera of patients sensitized to *A fumigatus* cyclophilin,[35] which would have implications in patient autoreactivity. Therefore, because of their highly conserved sequences and structures over the whole phylogeny, which is the primary requirement for cross-reactivity through B-cell epitopes, it might be that cyclophilins represent a new family of panallergens, among which Bet v 7 is the first member described in pollen.

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Genetic restrictions in olive pollen allergy

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Background: The major antigen of olive tree pollen, Ole e 1, produces an IgE response restricted by DQ2.
Objective: Our purpose was to further analyze the genetic restrictions associated with IgE and IgG antibodies against Ole e 1 and IgE against the recently described antigen Ole e 3.
Methods: Twenty-two nuclear olive pollen–allergic families (n = 88) were selected. DRB1 and DQB1, TCR-Vα 8.1, the high-affinity receptor of IgE (FcεRI-β) *Rsa* I exon 7 and intron 2 and TNF-β (LTα–*Nco* I) polymorphisms were determined by PCR and analyzed for association with allergic traits by the multiallelic transmission disequilibrium test.
Results: Significant associations were found among HLA-DQB1*0201 (n = 29) and high levels of IgG (*P* = .023) and IgE (*P* = .0136) antibodies to Ole e 1 and with IgE specific to Ole e 3 (*P* = .0368). DRB1*0701 was associated with high levels of total serum IgE (*P* = .04) and IgG against Ole e 1 (*P* = .025). The FcεRI-β *Rsa* I exon 7, allele 1 (n = 39), was associated with high levels of total serum IgE (*P* = .01), IgE antibodies against *Olea europaea* extract (*P* = .004), and specific antibodies to Ole e 1, IgG (*P* = .04), and IgE (*P* = .006). The FcεRI-β *Rsa* I intron 2, allele 2 (n = 33), was associated with IgE antibodies to *O europaea* extract (*P* = .003) and specific antibodies to Ole e 1, IgG (*P* = .025), and IgE (*P* = .05).
Conclusions: We found a new association between IgE antibody response to Ole e 3 and DQB1*0201 and verified the previously reported association between Ole e 1–specific response and DQB1*0201. Also, the association between FcεRI-β and IgE antibodies against Ole e 1 was demonstrated. (J Allergy Clin Immunol 2000;105:292-8.)

Key words: *Olive pollen allergy, Ole e 1, Ole e 3, HLA class II, IgE high-affinity receptor, T-cell receptor and TNF polymorphisms*

Abbreviations used

DARIA:	Double-antibody RIA
dNTP:	Deoxyribonucleoside triphosphate
FcεRI-β:	Chain of the high-affinity IgE receptor
LT:	Linfotoxin
$MgCl_2$:	Magnesium chloride
$(NH_4)_2SO_4$:	Ammonium sulfate

In the Mediterranean area and in California, the olive tree *(Olea europaea)* is widely distributed and its pollen is one of the most important causes of respiratory allergy.[1] The major allergen of this pollen, Ole e 1, is a protein of 18 to 20 kd molecular mass,[2,3] of a heterogeneous nature, that has been cloned and the complete amino acid sequence described.[4,5] Ole e 1 is an acidic protein that exhibits 2 variants of the same 145-residue polypeptide chain, glycosylated (20 kd) and nonglycosylated (18.5 kd). This protein has at least four B-cell epitopes[6] defined by mapping with 6 mAbs and 2 regions around 91-102 and 109-130 amino acids have been defined as immunodominant T-cell epitopes.[7] Finally, the IgE antibody response against this allergen is restricted by DR7-DQ2 antigens[8,9] demonstrated by genetic associations and by restriction of the response of Ole e 1 T-cell line.

Besides Ole e 1, several IgE-binding proteins have been detected in the saline extract of this pollen,[2,10] and some of them have been purified and characterized: Ole e 2[11] is an olive-pollen profilin with a molecular mass of 15 kd and a recognition frequency estimated at 24% of olive-hypersensitive patients; Ole e 3[12] is a protein that consists of a single polypeptide chain of 9.2 kd with a Ca^{++}-binding motif, highly conserved in pollen grains; and Ole e 4 and 5[13] are 2 allergens purified from olive pollen collected in California. Ole e 4 has an apparent molecular weight of 32 kd (without homology with other known proteins) and an IgE binding frequency of 80%. Recently, another group[14] has identified a 36-kd protein with 83% reactivity that could be the same protein as Ole e 4. Ole e 5 has a molecular weight of 16 kd and a high degree of homology with superoxide dismutase of several plants and a recognition frequency of 35%, and finally Ole e 6[15] is a protein with an apparent molecular mass of 10 kd and an acidic isoelectric point of 4.2.

Asthma and allergies are complex diseases involving genetic as well as environmental factors. The immunogenetic mechanisms underlying heightened IgE responsiveness seen in atopic diseases may be divided into 2 types, antigen-specific and nonantigen specific.[16-18]

Strong candidate genes of atopy have been proposed to be located on chromosomes 11q13[19] and 5q31.1[20] where high-affinity IgE Fc receptor β subunit (FcεRI-β)

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