Allergens, IgE, mediators, inflammatory mechanisms

Molecular characterization of a cross-reactive *Juniperus oxycedrus* pollen allergen, Jun o 2: A novel calcium-binding allergen

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Background: Species belonging to the Cupressaceae family are a relevant source of allergens that are present in a wide number of countries.
Objective: We sought to identify, purify, and characterize recombinant allergens from *Juniperus oxycedrus*, a species belonging to the Cupressaceae family.
Methods: Double-stranded cDNA was synthesized from mRNA and cloned into the lambda-ZAP expression vector. IgE screening of the library was performed with a pool of sera from subjects allergic to Cupressaceae. A recombinant 6×His-tagged *Juniperus oxycedrus* allergen, Jun o 2, was expressed in *Escherichia coli* and purified by Ni^{2+} affinity chromatography. It was studied further by immunoblotting inhibition with pollen extracts from other Cupressaceae, Oleaceae, Urticaceae, and Graminaceae. The role of protein-bound calcium on the allergen's IgE-binding capacity was tested in a plaque assay in the presence or absence of EGTA.
Results: A cDNA coding for a newly identified *Juniperus oxycedrus* pollen allergen, rJun o 2, was isolated. The deduced amino acid sequence contained four typical Ca^{2+} binding sites and showed a significant sequence similarity to calmodulins. Depletion of Ca^{2+} in the plaque assay led to a loss of IgE-binding capacity of rJun o 2. Immunoblotting inhibition revealed that *J. oxycedrus*, *J. ashei*, *Cupressus arizonica*, *C. sempervirens*, *Parietaria judaica*, *Olea europaea*, and *Lolium perenne* pollen extracts were able to inhibit IgE binding to blotted rJun o 2 at different concentrations.
Conclusion: rJun o 2 contains IgE-binding epitopes shared by taxonomically unrelated species, and therefore it can be regarded as a new panallergen. These findings could contribute to an explanation for the phenomenon of multiple positive test results in polysensitized patients and the potential symptom-eliciting role of allergenic sources previously not encountered. (J Allergy Clin Immunol 1998;101:772-7.)

Key words: *Cupressaceae, Juniperus oxycedrus*, cDNA cloning, IgE, calcium-binding protein, cross-reactivity

Abbreviations used

CaE:	*Cupressus arizonica* pollen extract
CsE:	*Cupressus sempervirens* pollen extract
EGTA:	Ethylenebis (oxyethylenenitrilo)-tetraacetic acid
JaE:	*Juniperus ashei* pollen extract
JoE:	*Juniperus oxycedrus* pollen extract
LpE:	*Lolium perenne* pollen extract
OeE:	*Olea europaea* pollen extract
PjE:	*Parietaria judaica* pollen extract
SDS-PAGE:	Sodium dodecylsulfate–polyacrylamide gel electrophoresis
UTR:	Untranslated region

Allergenic extracts isolated from natural sources are complex mixtures of proteins and other molecules. Their composition differs greatly in allergenic, as well as nonallergenic, material. Basic allergy research is often hindered by the low amount of allergenic material available. Major problems are also encountered in the diagnosis of some pollinosis, which is related to the poor quality of extracts available for in vitro and in vivo testing.[1] To overcome these limitations, interest has been focused in recent years on recombinant DNA technology as an effective alternative.[2] mRNA isolated from different allergenic sources is commonly used to generate cDNA libraries, and several recombinant allergens have been produced.[2]

Pollen from the Cupressaceae family is an important cause of worldwide winter respiratory allergies.[1] Cypress pollinosis is gaining more attention because of the increasing number of species described as sensitizers belonging to the Cupressaceae family or to closely related cross-reactive families[3-7] and because of the widespread use of these plants for anthropic purposes.[8] Characterization of allergenic pollen extracts from *Juniperus* spp., which belong to the Cupressaceae family, has been reported for *Juniperus ashei*.[9] *Juniperus oxycedrus*, a Mediterranean species, has been described as a symptom elicitor in patients allergic to cypress.[10] In this article we describe the cloning, expression, and sequencing of cDNA coding for a newly identified *J. oxycedrus* pollen allergen, designated rJun o 2, which displays sequence similarities with a family of Ca^{2+}-binding

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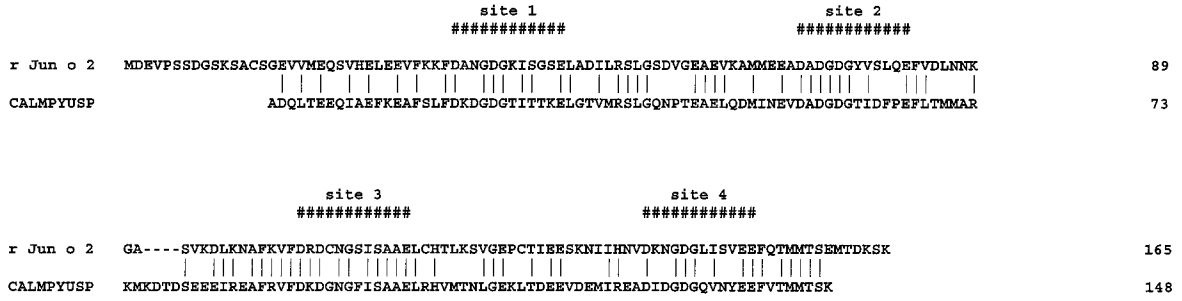

FIG. 1. Results of comparative analysis between rJun o 2 and CALMPYUSP, calmodulin from sea squirt (EMBL accession no. P11121). Residues forming calcium-binding motifs are indicated with *# . Bars* represent gaps that have been introduced just to align similar portions (calcium-binding sites) and to ensure maximum homology. Identical and similar residues between two sequences are indicated with *vertical bar.*

proteins and bearing epitopes shared by pollens from unrelated families.

METHODS

Pollen

Pollen from *J. oxycedrus* was collected in November from plants growing along the coastal area of the Circeo National Park in central Italy. Source plants were identified by means of botanical criteria.[11] Pollen contamination was excluded by controlled collecting conditions and by microscopic analysis (purity >99%). Pollen was dried and stored at −80° C until use.

Human sera

Human sera were obtained from patients sensitive to Cupressaceae, as determined by skin prick test results and specific IgE in vitro detection. These patients were screened on the basis of previously reported criteria.[1] Sera from three of these subjects, chosen on the basis of their high specific IgE levels, were pooled and used for cDNA library screening. A panel of 41 sera was tested for recognition of blotted rJun o 2. Individuals had not received specific immunotherapy, and their informed consent to participate in the study was obtained. Serum samples to be used as controls were collected from nonatopic volunteers.

Pollen extracts

J. oxycedrus and *J. ashei* pollen extracts (JoE and JaE) were prepared as previously described.[9, 12] *Cupressus arizonica* and *C. sempervirens* pollen extracts (CaE and CsE) were prepared according to Di Felice et al.[13] *Parietaria judaica* and *Olea europaea* pollen extracts (PjE and OeE) were prepared as previously described.[14, 15] *Lolium perenne* pollen extract (LpE) was purchased from Greer Laboratories (Lenoir, N.C.).

Total and poly(A)+ mRNA preparation

RNA was isolated by using the guanidinium thiocyanate method of Chomczynski and Sacchi.[16] Poly(A)+ mRNA was enriched by affinity chromatography with oligo-dT-cellulose (Sigma, St. Louis, Mo.).[17]

cDNA library immunologic screening and cDNA sequencing

Double-stranded cDNA was synthesized from mRNA and cloned into EcoRI and XhoI sites of the lambda-ZAP expression vector, according to the Stratagene cDNA cloning kit (Stratagene, La Jolla, Calif.). The primary phage stock contained 5×10^5 independent clones. Sixty thousand plaques were immunologically screened by using the pool of sera previously described. One positive clone, hereafter referred to as OK1, was identified by peroxidase-labeled anti-human IgE (KPL, Gaithersburg, Md.) and studied further. The nucleotide sequence of the cDNA insert was determined on both strands by the dideoxy-chain termination method with Sequenase (USB, Cleveland, Ohio). Northern blotting was performed on total RNA from pollen of *J. oxycedrus* according to the method of Sambrook et al.[17] RNA was transferred onto nylon membrane, and filters were hybridized with a ^{32}P-labeled OK1 insert as a probe. The probe was radiolabeled by the random primer method.* Sequence analysis was performed with the Genetics Computer Group package (version 9.0) (Genetics Computer Group Inc., Madison, Wis.).[18] The FASTA[19] and BLAST[20] programs were used to search the EMBL (Release 48.0) and GenBank (Release 97.0) nucleotide databases and the PIR (Release 50.0) and SwissProt (Release 34.0) protein databases for the sequence similarity to the OK1 nucleotide and amino acid sequences, respectively. The alignment between proteins was performed by using the Clustal IV program. PROSITE[21] database was searched to identify motifs in the OK1 amino acid sequence.

IgE-binding assay and Ca^{2+} influence on IgE binding

Twenty thousand plaques of the positive clone OK1 were seeded according to the manufacturer's instructions (Stratagene). Nitrocellulose filters were cut into sections and incubated with the most reactive patient serum either in the presence or in the absence of 10 mmol/L ethylenebis (oxyethylenenitrilo)-tetraacetic acid (EGTA) (pH 7.5),[22] and bound IgE antibodies were detected with ^{125}I-labeled anti-human IgE (Bio-Allergy, Rome, Italy).

Cloning of cDNA insert into pQE vector and purification of the recombinant allergen

The cDNA insert from the lambda-ZAP clone was cut after in vivo excision (with suitable restriction endonucleases) and subcloned into pQE 31 expression vector containing the sequence encoding the 6×His affinity tag (QIAGEN GmbH, Hilden, Germany). The recombinant clone was expressed in *E. coli* M15 strain. The recombinant allergen was purified by

*The nucleotide and deduced amino acid sequences of the OK1 cDNA have been submitted to the GenBank database with the accession number AF031471.

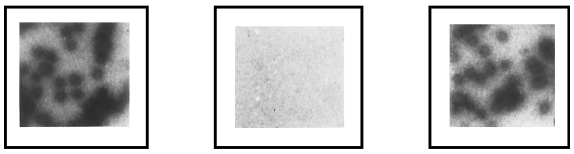

FIG. 2. Effect of protein-bound Ca^{2+} on IgE-binding capacity of rJun o 2. Plaque-lift nitrocellulose sectors containing rJun o 2 were incubated with serum IgE from individual allergic to Cupressaceae. Filters were incubated either with serum only **(A)** or with serum in presence of EGTA **(B)**. After development of both filters with ^{125}I-anti-human IgE, filter B was probed with newly added human serum in absence of EGTA and further developed with ^{125}I-anti-human IgE **(C)**.

affinity chromatography to a Ni^{2+}-charged resin according to the manufacturer's instructions (QIAGEN). The concentration of the recombinant allergen was determined according to the method of Bradford.[23]

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE and immunoblotting were carried out as previously described[13, 24] in 15% polyacrylamide gels. The blotted nitrocellulose strips were incubated overnight with individual human sera or with pooled sera. IgE-allergen complexes were detected by ^{125}I-labeled anti-human IgE (Bio-Allergy).

IgE-immunoblotting inhibition

Inhibition was carried out as previously described.[24] Inhibition of specific IgE binding on recombinant blotted allergen was performed by incubation of the human serum used in the plaque assay diluted 1:10 with a final concentration of 50, 25, 5, 1, and 0.25 μg/ml protein of JoE, JaE, CaE, CsE, PjE, OeE, and LpE. Bound serum IgE were detected as described above.

Plaque hybridization with ^{32}P-labeled insert of clone OK1

To investigate the 5'-untranslated region (UTR) of the gene, the cDNA library (10^6 plaques of the amplified library) was screened by plaque hybridization with ^{32}P-labeled insert of clone OK1.

RESULTS

Molecular cloning of rJun o 2 and sequence analysis

The poly(A)+ mRNA, which was isolated from *J. oxycedrus* pollen, was used as a template to synthesize the cDNA used for preparing the cDNA library in the expression-vector lambda-ZAP. One positive clone, designated as OK1, was found by screening about 6×10^4 plaques. The purified clone gave no signal when tested with nonallergic human sera. Clone OK1, encoding for this allergen molecule, was named rJun o 2 in accordance with International Union of Immunological Societies nomenclature.*[25]

Sequence analysis revealed a 1002 bp cDNA clone with an open reading frame of 495 bp. The deduced amino acid sequence corresponds to a protein of 165 amino acids, with an estimated molecular weight of 18 kd (Fig. 1). The rJun o 2 sequence also contains 195 bp 3'-UTR with a canonical poly(A)+ tail at the end. Screening of the library with a ^{32}P-labeled insert of clone rJun o 2 allowed the identification of the longest 5'-UTR (309 bp). A Northern-blot analysis was performed on total RNA from *J. oxycedrus* pollen by hybridization with a ^{32}P-labeled DNA fragment from the rJun o 2 insert, and a single 1.0-kilobase RNA species was detected (data not shown).

The predicted rJun o 2 protein sequence contains four typical Ca^{2+}-binding motifs,[21, 26] and screening of protein and nucleic acid databases by FASTA[19] and BLAST[20] programs revealed a significant similarity between rJun o 2 and calmodulins, with the percentage of similarity ranging from 55.4% to 52.9% (sea squirt and yeast calmodulin, respectively).

Calmodulin is a highly conserved protein in eukaryotes, with 98% similarity between vertebrates and plants.[27] The alignment between rJun o 2 and sea squirt calmodulin shows that the two proteins have similar length, contain the same number of calcium-binding sites, and that the similarity is not limited to the common Ca^{2+} binding sites, but it is extended in other portions of the two sequences (Fig. 1). Because the similarity between rJun o 2 and the calmodulins (52.9% to 55.4%) is lower than the similarity within the calmodulin family (90%), we hypothesize that rJun o 2 is a calmodulin-related protein rather than a calmodulin itself.

Several other allergens have been demonstrated to be calcium-binding proteins.[22, 28-32] A comparative analysis did not reveal significant similarity between rJun o 2 and these proteins outside the common calcium-binding motifs.

Role of Ca^{2+} in IgE binding to rJun o 2

Several authors reported that the binding of specific IgE against some calcium-binding recombinant allergens require Ca^{2+}.[22, 29] We therefore tested whether the IgE binding in plaque-lifts assay could be affected by the depletion Ca^{2+} by using EGTA. The depletion of Ca^{2+} led to a loss of the IgE-binding capacity, which could be restored by subsequent incubation with newly added serum (Fig. 2).

Immunologic characterization of rJun o 2

The cDNA clone OK1 was subcloned into the pQE 31 expression vector. The recombinant product was constituted by the allergen rJun o 2 plus 25 amino acid residues peculiar to the expression vector (including a six consecutive histidine residues tag) at the NH_2 terminus. Affinity-purified recombinant allergen showed an apparent molecular mass of 29 kd in SDS-PAGE (Fig. 3, *A*, *lane 4*). This molecular size is larger than the calculated molecular mass of 18 kd for rJun o 2 because of the presence of the 6×His tag that influences the migration of protein in SDS-PAGE. Immunoblotting analysis, per-

*The allergen reported has been submitted to the World Health organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee for approval of a new name.

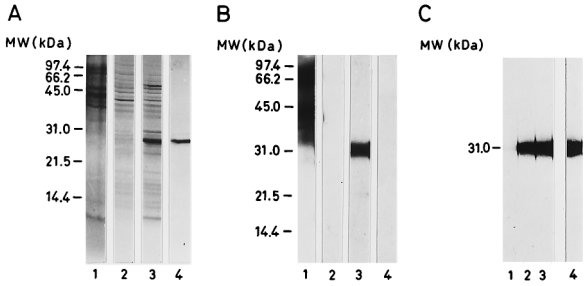

FIG. 3. A, SDS-PAGE analysis of JoE *(lane 1),* noninduced cell lysate *(lane 2),* isopropyl β-thiogalactoside–induced cell lysate *(lane 3),* and affinity-purified rJun o 2 *(lane 4).* **B,** Immunoblotting analysis of transferred JoE *(lane 1),* crude noninduced cell lysate *(lane 2),* affinity-purified rJun o 2 *(lane 3)* developed with pooled human sera from subjects allergic to Cupressaceae, and immunoblotting analysis of transferred affinity-purified rJun o 2 developed with human serum from a nonallergic individual *(lane 4).* **C,** Immunoblotting inhibition pattern of rJun o 2 after incubation of human serum from one subject allergic to Cupressaceae with 25 *(lane 1),* 5 *(lane 2),* and 1 *(lane 3)* μg protein/ml of JoE. *Lane 4,* No inhibitor added. *MW,* Molecular weight.

formed with a pool of allergic sera, showed that specific IgE bind affinity-purified rJun o 2 (Fig. 3, *B*, *lane 3*), whereas the purified molecule did not react with a nonallergic human serum (Fig. 3, *B*, *lane 4*). Specific IgE did not recognize an irrelevant affinity-purified recombinant protein expressed in the same vector used for rJun o 2 (data not shown). IgE binding to affinity-purified rJun o 2 was analyzed by immunoblotting assays with human sera obtained from 41 subjects allergic to Cupressaceae, and six of the test samples displayed IgE reactivity.

When whole JoE was blotted, IgE binding for a component with a molecular weight corresponding to the calculated molecular weight of rJun o 2 could not be observed (Fig. 3, *B*, *lane 1*). However, when the binding of Jun o 2–specific human IgE to the blotted rJun o 2 was inhibited by JoE, a total inhibition was obtained with 25 μg protein/ml of whole extract (Fig. 3, *C*, *lane 1*), thus indicating the presence of rJun o 2 epitopes in the extract.

Cross-reactivity between rJun o 2 and pollen extracts from other Cupressaceae or unrelated families

To demonstrate whether the natural counterpart of rJun o 2 was present in other Cupressaceae and in species from unrelated families, blotting inhibition experiments were carried out (Fig. 4). Pollen extracts from Cupressaceae or from unrelated families were all able to inhibit, to a different extent, the IgE binding to blotted rJun o 2. When JaE was employed as an inhibitor, a complete binding inhibition was achieved by 25 μg protein/ml of whole extract (Fig. 4, *A*, *lane 1*), whereas pollen extracts from *Cupressus* spp. were not able to provide complete inhibition (Fig. 4, *A*, *lanes 4* and *9*), even when used at the highest concentration of 50 μg protein/ml. Among extracts from the unrelated families,

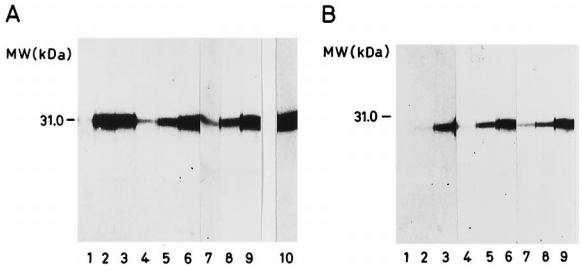

FIG. 4. Immunoblotting inhibition pattern of IgE binding to rJun o 2 inhibited with pollen extracts from Cupressaceae **(A)** or from taxonomically unrelated families **(B).** Amounts of inhibitors are 25, 5, and 1 μg protein/ml of JaE (**A**, *lanes 1* to *3*); 50, 25, and 5 μg protein/ml of CaE (**A**, *lanes 4* to *6*) and CsE (**A**, *lanes 7* to *9*); 5, 1, and 0.25 μg protein/ml of PjE (**B**, *lanes 1* to *3*); 50, 25, and 5 μg protein/ml of OeE (**B**, *lanes 4* to *6*); and 50, 25, and 5 μg protein/ml of LpE (**B,** *lanes 7* to *9*). *Lane 10* **(A),** No inhibitor added.

PjE was able to reach a complete inhibition with only 5 μg of proteins (Fig. 4, *B*, *lane 1*), whereas different levels of inhibition were obtained with OeE (Fig. 4, *B*, *lane 4*) and LpE (Fig. 4, *B*, *lane 7*).

DISCUSSION

In this study the cloning and expression of a *J. oxycedrus* pollen allergen, rJun o 2, a species belonging to the Cupressaceae family and characteristic of the Mediterranean area, is reported. The rJun o 2 cDNA encoded a protein of a calculated molecular weight of 18 kd, with four calcium-binding sites and with a significant sequence homology with calmodulins.

Cupressaceae allergy is a worldwide pollinosis caused by different species. Cross-reactivities within the Cupressaceae family have been described,[1, 24] and recent data indicate that JoE might play a very peculiar role in this context in relation to its early-fall pollinating period and because of its cross-reactivity with other Cupressaceae.[10] Data on cross-reactivity between extracts prepared from pollen of Cupressaceae and closely related families (Taxodiaceae[33] and Podocarpaceae[34]) or from taxonomically distinct pollens have also been reported.[35] The presence of cross-reactive epitopes in rJun o 2 and in pollen extracts from *J. oxycedrus*, as well as from species taxonomically related and unrelated to *J. oxycedrus*, demonstrated by immunoblotting inhibition experiments supports these findings. Although all the pollens tested were able to inhibit the IgE binding to rJun o 2, CaE, CsE, and LpE were less potent inhibitors than the other pollens because they were not able to provide a total inhibition at the highest concentration tested.

However, IgE-binding components corresponding to the molecular weight of rJun o 2 were not detectable when whole JoE was blotted. These findings can be explained assuming that the molecular weight of the native molecule in the extract is different because of either glycosylation[36] or aggregation occurrence.

Sugar moieties present on glycoprotein allergens have been reported to contribute to cross-reactivity between allergenic molecules from related, as well as unrelated,

species.[37, 38] However, some cross-reactions have been explained on the basis of the presence of common molecules (panallergens) that are shared between various extracts. Profilin is an important and well-defined panallergen involved in cross-reactions among pollens, vegetables, and fruits.[39] In addition to profilin, other proteins contained in pollen from different species that have conserved sequences could be responsible for allergic sensitization in subjects not previously exposed to a given biologic source. In this context, calcium-binding proteins could play an important role because of the presence of calcium-binding sites, which are highly conserved.

A similar situation for rJun o 2 could be suggested by the results of inhibition experiments in which binding of IgE to Jun o 2 was inhibited by taxonomically unrelated pollen extracts. The classification of Jun o 2 itself as a panallergen will be further investigated by assaying a wider number of species from different sources in the inhibition test and by enlarging the number of allergic subjects.

The relevance of Ca^{2+} in the IgE recognition of Jun o 2 could be explained by two hypotheses: the Ca^{2+} binding sites may be identified themselves as the IgE epitopes, or on the other hand, the presence of bound Ca^{2+} may affect the three-dimensional structure of the molecule and thereby its ability to bind IgE. The actual role of Ca^{2+}-binding sites should be investigated in our system by testing peptides containing only the Ca^{2+}-binding domain in the inhibition of the IgE binding to the whole molecule.

The diagnostic features of the patients with positive reactions to rJun o 2 clearly support the clinical relevance of rJun o 2. All of them had positive reactions to more than 10 different pollen species belonging to unrelated families from either Gymnospermae and Angiospermae, whereas subjects monosensitized to Cupressaceae were never able to recognize blotted rJun o 2. Although some of the tested pollen species are of poor or no aerobiologic interest for positive subjects on the basis of airborne pollen sampling (data not shown), the related extracts used in skin prick testing were able to elicit a positive reaction. Thus the use of rJun o 2 will be useful to explain the phenomenon of multiple positive test results in polysensitized patients and the potential symptom-eliciting role of allergenic sources previously not encountered.

Because Ca^{2+}-binding proteins working as calcium flux regulators are ubiquitous in eukaryotic cells, Jun o 2–homologous molecules could be identified in higher organisms, as previously demonstrated for other recombinant molecules.[40, 41]

Ca^{2+}-binding proteins from different pollens have been reported recently as targets for IgE antibodies (e.g., *Betula verrucosa*,[22] *Cynodon dactylon*,[29] *Brassica* spp.,[30] and *O. europaea*[28]).

This finding could be related to the important role played by Ca^{2+} metabolism in pollen germination and pollen-tube growth.[42] Molecules such as Jun o 2 could then be involved in cellular processes that affect the reproductive pollen function.

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