Olive Pollen Recombinant Allergens: Value in Diagnosis and Immunotherapy

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Abstract

Olive pollen has a complex allergenic profile, from which more than 10 allergens have been identified and characterized. Some of these belong to well-known protein families and others cannot be included in reported biochemical types. Most of these allergens have been produced by recombinant technology, mainly in *Escherichia coli* or in *Pichia pastoris*, and they are good candidates for diagnostic and therapeutic purposes. Diagnosis and immunotherapy of allergy currently use extracts prepared from homogenates of natural sources, which only allow us to detect sensitivity to the complete source. These extracts can be successfully replaced by mixtures with controlled amounts of specific allergenic proteins obtained by recombinant technology in order to define the sensitization profile of individual patients. Recombinant Ole e 1 can be used as a marker for sensitization to *Oleaceae*. Recombinants Ole e 2 (profilin) and Ole e 3 (polcalcin) can serve as markers of polysensitivity. Finally, recombinant forms of Ole e 6, Ole e 10, and the carboxy-terminal and amino-terminal domains of Ole e 9 would help to detect sensitization to these minority allergens that could be overlooked in the complete olive pollen extract. These recombinant molecules can help provide an accurate diagnosis of sensitivity to individual allergens and, therefore, improve the design of more efficacious allergen-based immunotherapy strategies.

Key words: Olive pollen allergy. Allergen. Recombinant expression. Marker allergen. *Escherichia coli* expression. *Pichia pastoris* expression. Diagnosis. Immunotherapy.

Resumen

El polen de olivo posee un perfil alergénico complejo, a partir del cual se han identificado y caracterizado más de 10 alérgenos. Algunos de ellos pertenecen a familias de proteínas bien conocidas y otros no corresponden a ninguno de los tipos bioquímicos descritos. La mayoría de estos alérgenos han sido producidos mediante tecnología del DNA recombinante, principalmente en *Escherichia coli* o en *Pichia pastoris*, y constituyen buenos candidatos con fines diagnósticos y terapéuticos. La diagnosis e inmunoterapia de la alergia hace uso generalmente de extractos preparados a partir de homogeneizados de las fuentes naturales, los cuales sólo permiten detectar la fuente biológica de sensibilización. Estos extractos pueden ser reemplazados con éxito por mezclas con cantidades controladas de proteínas alergénicas específicas, obtenidas por ingeniería genética, con el fin de definir el perfil de sensibilización de cada paciente. Ole e 1 recombinante puede ser usado como marcador de sensibilización a Oleáceas. Los alérgenos recombinantes Ole e 2 (profilina) y Ole e 3 (polcalcina) pueden servir como marcadores de polisensibilización. Finalmente las formas recombinantes de Ole e 6, Ole e 10 y los dominios carboxilo- y amino-terminal de Ole e 9 podrían servir para detectar la sensibilización a alérgenos minoritarios que podrían pasar desapercibidos en el extracto completo de polen de olivo. Estas moléculas recombinantes pueden proporcionar un diagnóstico preciso de la sensibilización frente a alérgenos individuales y, por tanto, mejorar el diseño de estrategias de inmunoterapia específica más eficaces.

Palabras clave: Alergia a polen de olivo. Alérgeno. Expresión recombinante. Alérgeno marcador. Expresión en *Escherichia Coli*. Expresión en *Pichia pastoris*. Diagnosis. Inmunoterapia.

Olive Pollen Allergens: A Brief Overview

Olive (*Olea europaea*) pollen allergy is one of the most significant pollinoses depending on geographical location. Although the olive tree is grown on all 5 continents, more than 25% of the world's olive tree cultivars are in Spain. After *Gramineae*, olive pollen is the main cause of pollinoses in this country and is becoming the main disease-eliciting pollen in some provinces of Andalusia (Jaén and Córdoba) where pollen counts in the air reach peaks higher than 5000 grains/m^3. Olive pollen has a very complex allergenic profile that can be resolved by electrophoresis in polyacrylamide gels [1,2]. The analysis

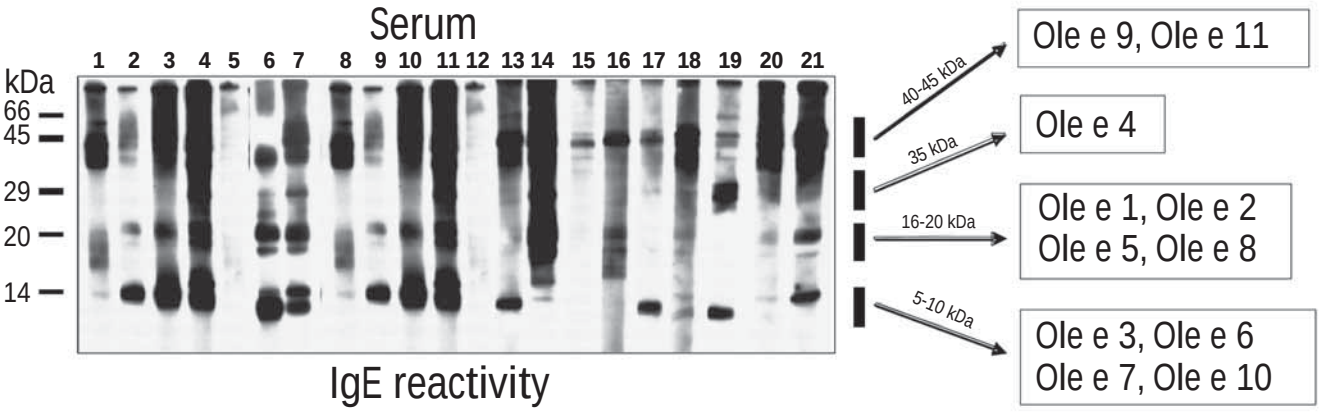

Figure 1. Immunoblotting analysis of individual sera from 21 patients who are allergic to olive pollen. The margin of molecular weights where the olive allergens appear is indicated on the right.

of individual serum samples frequently displays more than 5 concomitant IgE-reactive protein bands [1,2]. To date, 10 allergens have been described from olive tree pollen (Figure 1) and individual frequency of sensitization can vary with the geographical area. Ole e 1 is the most prevalent allergen of olive pollen, often reaching values higher than 70% among olive-sensitive patients [3,4]. It is the single major allergen in regions with low pollen counts, whereas other allergens such as Ole e 6, Ole e 7, and Ole e 9 also affect more than 50% of patients in locations with a high count [5-8].

Ole e 1 constitutes more than 10% of the total protein content of pollen in the most profuse varieties of the *Olea europaea* tree, but it does not exist in fruit, leaf, or stem; therefore, these tissues cannot induce allergy through Ole e 1. It is a single-polypeptide chain glycoprotein of 145 amino acid residues (apparent molecular mass of 20 kDa) with a heterogeneous oligosaccharide at position Asn-111 [9]. In addition, Ole e 1 shows a strong polymorphism in the polypeptide chain that leads to a broad range of pI (from 5.0 to 7.2) [10]. The Ole e 1-like family of proteins comprises allergenic members (Fra e 1, Lig v 1, Syr v 1 from *Oleaceae* species; Pla l 1 from *Plantago lanceolata*; Che a 1 from *Chenopodium album*; Lol p 11 from *Lolium perenne*; and Phl p 11 from *Phleum pratense*), as well as non-allergenic members such as BB18 from *Betula verrucosa*.

Most of the remaining olive pollen allergens belong to families of proteins that have also been reported to be allergenic in different plant sources. Ole e 2 (16 kDa) is a profilin, with a potential role in intracellular signaling and trafficking [11]. Ole e 3 (9.2 kDa and 85 amino acids long) is a 2-EF-hand Ca^{2+}-binding protein whose generic name of polcalcin was proposed because of its specific occurrence in pollen tissue [12,13]. The amino acid sequences of these 2 families of proteins are highly conserved in both taxonomically related and nonrelated species, and this confers strong immunological cross-reactivity to their members (panallergens). Ole e 5 is the superoxide dismutase of olive pollen [14], and Ole e 8 is a Ca^{2+}-binding protein with 4 EF-hand sites and very low prevalence [15]. Ole e 7 belongs to the nonspecific lipid transfer protein family [8], but the data available on its polypeptide sequence suggest a limited similarity with other allergenic lipid transfer proteins from vegetable sources (peach, apple, cherry, apricot, orange, hazelnut) and, therefore, no significant cross-reactivity. Ole e 9 is a 1,3-β-glucanase composed of 2 structurally and immunologically well-defined domains of 334 amino acids (N-terminal domain) and 100 amino acids (C-terminal domain) [7]. The N-terminal domain is involved in IgG and IgE cross-reactivity with other pollens, plant-derived foods, and latex [16], and the C-terminal domain is a glucan-binding module whose sequence is similar to that of Ole e 10 [17,18]. Ole e 10 is a protein that interacts with 1,3-β-glucan and the first reported member of a novel family of β-glucan-binding modules (CBM43) [19,20]. As for Ole e 6, neither biochemical activity nor homology to proteins in databanks has been found for this small allergen (5.5 kDa). It displays a peculiar twice-repeated cysteine motif (CysXXXCysXXXCys) and an interesting 3-dimensional structure that has recently been resolved [5,21]. Finally, some concerns exist about whether Ole e 4 is a genuine allergen since it seems likely to be a proteolytic degradation product of Ole e 9.

Why Should We Use Recombinant Allergens?

One of the main goals in allergy research is to improve tools for diagnosis and specific immunotherapy. Conventional allergen extracts used for in vitro and in vivo diagnosis of allergy are obtained from biological sources and consist of a mixture of allergenic components and high amounts of undesirable products that can interfere with diagnosis. Moreover, they contain variable and unknown concentrations of allergens and can sometimes be damaged by proteolysis or oxidation. By contrast, mixtures of purified allergens can be used within well-defined parameters, as the concentration and integrity—structural and immunological activity—of allergens can be controlled, thus avoiding problems related to the variability of the composition of different batches, low concentrations of many allergenic components, or the prevention of de novo sensitizations [22,23]. Purified allergens can be prepared by isolation from their natural sources or by

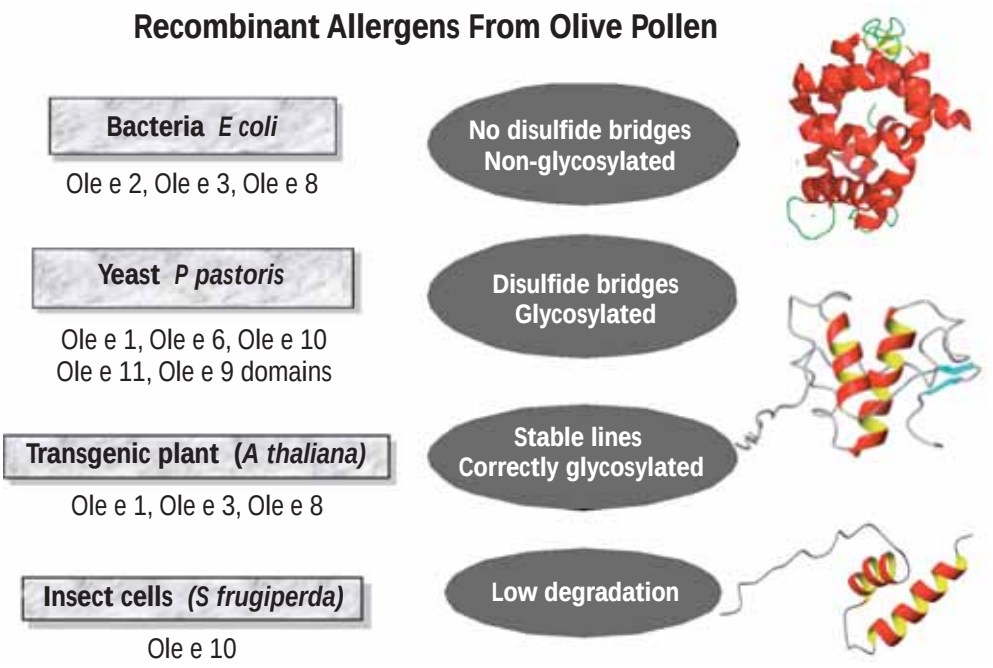

Figure 2. Criteria for selection of hosts for the production of recombinant allergens from olive pollen. Folding of polcalcin, C-terminal domain of Ole e 9 and Ole e 6 are shown.

recombinant production. However, the technical difficulties and low yield in the purification of natural allergens have led us to consider molecular biology methodologies as the best way to obtain unlimited amounts of allergens for diagnosis. In fact, the sensitivity and specificity of molecular and clinical determinations are being improved by replacing natural extracts with recombinant allergens, and these enable the predictive value to be increased. The recently suggested concept of marker recombinant allergens for refining diagnosis may provide complementary and essential information to identify allergy-eliciting proteins and to improve immunotherapy by selection of patients for tailored treatment. Finally, specific cDNA molecules can be changed in order to modify some structural properties of the protein that might increase its stability or reduce its allergenic potential [24-26]. During the last decade, procedures for cloning, sequencing, and recombinant expression have been applied to the study of a large number of allergens, and most of the olive pollen allergens have been obtained using such approaches.

Design and Production of Recombinant Olive Pollen Allergens

Four heterologous systems have been used for the recombinant expression of olive pollen allergens: the bacteria *Escherichia coli*, the methylotrophic yeast *Pichia pastoris*, insect cells of *Spodoptera frugiperda*, and plants of *Arabidopsis thaliana* (Figure 2). The bacteria *E coli* can be successfully used to produce proteins such as Ole e 3 or Ole e 8, which do not need to go through post-translational maturation. However, bacteria do not have the adequate mechanisms for attaching glucans to polypeptide chains, and glycoproteins are expressed as their sugar-free derivatives. Moreover, the reducing environment of *E coli* cytoplasm makes successful arrangement of disulfide bridges difficult. Therefore, allergens such as Ole e 1 (a glycoprotein containing 3 disulfide bonds) and Ole e 6, (a small polypeptide chain containing 3 disulfide bridges) must be produced in eukaryotic cells.

The first attempt to produce rOle e 1 was as a fusion protein with glutathione S-transferase in *E coli*. However, it was unsuccessful [10]. After purification by affinity chromatography and specific digestion with thrombin, free rOle e 1 was obtained as high-molecular-mass aggregates, although a small portion appeared in soluble monomeric form. This poor yield prompted us to express Ole e 1 in *P pastoris*. We transformed *P pastoris* GS115 cells using a pPIC9 expression plasmid, which carried cDNA encoding Ole e 1 in frame with a secretion peptide from *Saccharomyces cerevisiae* [27]. rOle e 1 was efficiently secreted to the extracellular medium as a single overexpressed polypeptide chain. A simple purification procedure yielded the pure and soluble protein. The structural and immunological features of rOle e 1 were equivalent to those of the natural allergen. IgE-binding and IgG-binding capabilities in vitro were found to be identical for natural and recombinant forms of Ole e 1 [27]. P pastoris has also been used to obtain other members of the Ole e 1-like family of allergens such as those from ash, lilac, privet, and chenopod: Fra e 1, Syr v 1, Lig v 1, and Che a 1, respectively [28-30]. These proteins are major allergenic components of the corresponding pollens. Some of these recombinant allergens are used to analyze the 3-dimensional structure of the protein family.

Ole e 3 and Ole e 8 have been expressed in *E coli* [13,15]. These Ca^{2+}-binding proteins do not possess glycan components or disulfide bridges and have been produced as free high-yield polypeptide chains. In both cases, the recombinant products were assessed for their structural and immunological integrity, and their functional ability to bind calcium ions was also demonstrated. Both Ole e 3 and Ole e 8 have also been produced in *Arabidopsis thaliana*, and they retain the biochemical and

immunological integrity of the natural allergens [31]. This plant-based expression system presents production advantages because it possesses cellular post-translational machinery analogous to that of the natural source of olive pollen allergens. It has served as a model for using transgenic plants as an alternative system for producing recombinant allergens. Furthermore, in the future, it would enable plant materials containing recombinant allergens to be included in edible vaccination protocols.

Although the yeast *P pastoris* has been used as a successful expression system for allergens with complex folding and different post-translational modifications, production of Ole e 10, a small protein (10 kDa) with 3 disulfide bridges has been attempted using both yeast [32] and insect cells [33]. Better yields and lower degradation of the recombinant molecule were achieved using Baculovirus/insect cell expression than with *Pichia*, and the allergen was isolated as a stable, soluble, and functional protein.

P pastoris cells have also been used to produce the small Ole e 6 protein [34] and fragments or domains derived from other olive pollen allergens. Despite its short polypeptide chain (50 amino acids long), Ole e 6 has 3 disulfide bonds and the yeast is a suitable host for its production; the recombinant protein was purified at a high yield and properly folded before being used to determine the 3-dimensional structure of the allergen [21]. Furthermore, the C-terminal and N-terminal domains of the 1,3-β-glucanase Ole e 9 have been produced as recombinant proteins in *P pastoris* with a high yield and maintaining their intrinsic properties [16,18].

Finally, genetic engineering has been used to produce hypoallergenic variants of clinically relevant allergens. Hypoallergenic variants of allergens are being studied as therapeutic agents for safer and more effective treatment of allergy, since they exhibit reduced or non–IgE-binding capacity but retain T-cell reactivity [25,27,35]. Three mutants of Ole e 1, whose design was based on the disruption of the immunodominant IgE epitope located at the C-terminal sequence of the molecule, have been produced in *P pastoris*. Their immunologic properties have also been evaluated in vitro and in vivo [36]. Furthermore, using our mouse model of Ole e 1 allergy, we demonstrated that intranasal administration of the best hypoallergenic variant of Ole e 1 was as effective as the whole allergen in preventing the synthesis of IgE antibodies and airway inflammation after subsequent sensitization and respiratory challenge with the allergen [37]. Thus, this mutant could substitute the natural allergen to induce tolerance.

Diagnosis and Immunotherapy With Recombinant Olive Pollen Allergens

Of all the applications of recombinant allergens, their use in diagnosis and immunotherapy is the most interesting for clinical practice. Diagnosis based on individual allergenic components allows specific disease-eliciting allergens to be determined [22]. Taking into account the available recombinant allergens from olive pollen, 3 main goals can be proposed for diagnosis: a) Ole e 1 can be used as a marker for genuine sensitization to *Oleaceae* pollens; b) the panallergens Ole e 2 and Ole e 3 can be used to diagnose polysensitivity; and c) minority allergens such as Ole e 6, Ole e 9, or Ole e 10 serve to identify specific sensitization to these molecules, which are often difficult to detect using currently available allergenic extracts. In addition, it has been suggested that a panel of a few recombinant allergens is sufficient for diagnosis of allergy to olive tree pollen [8].

Ole e 1 Is a Diagnostic Marker for Sensitization to Oleaceae Pollens

It is well known that Ole e 1 belongs to a large family of homologous proteins, which are specifically expressed in pollen tissue [10]. This family comprises both allergenic members such as Ole e 1, Fra e 1, Lig v 1, Syr v 1, Che a 1, Lol p 11, Pla l 1, and Phl p 11, and nonallergenic members such as BB18 from birch. Other members of the family are known through their corresponding nucleotide sequences, and their derived mature proteins have not been isolated or characterized; therefore, their potential allergenicity has not been explored. This is the case of genes from tomato, corn, rice, *Phalaris coerulenscens*, *Sambucus nigra*, or *Arabidopsis thaliana*, which belong to the "Ole e 1-like" family. Therefore, we must ask whether the members of this protein family display immunologic cross-reactivity.

Recently, it has been demonstrated that the epitopes of Ole e 1 are only present in *Oleaceae* pollens [38]. In collaboration with Dr Valenta's group (University of Vienna), we used sera from 2 different European populations–one sensitized to Ole e 1 (patients from Madrid, Spain) and the other nonsensitized to olive pollen (patients from Vienna, Austria)–to perform inhibition assays to bind Ole e 1 to IgE that were preincubated with total extracts from different allergenic pollens. Only pollens from *Oleaceae* were able to inhibit recognition of the protein. Pollens from birch, timothy grass, and mugwort were not able to inhibit the binding of IgE from the sera to Ole e 1. Interestingly, IgE from the sera of patients from Vienna (no contact with olive pollen) recognized Ole e 1. This suggests that the sensitization was induced by a member of the "Ole e 1-like" protein family, probably present in ash pollen. The protein extract prepared from ash pollen completely avoided binding of the IgE antibodies to Ole e 1, which demonstrated the presence of all the Ole e 1 epitopes in ash pollen.

Nevertheless, analysis using the enzyme-linked immunosorbent assay (ELISA) for the recognition of different members of the "Ole e 1-like" family by the IgE from sera of olive-allergic patients demonstrated that allergens from this family belonging to taxonomically nonrelated sources do not share IgE epitopes with Ole e 1. In fact, we obtained very high levels of specific IgE to Ole e 1, whereas the same patients gave negative results against Che a 1, Lol p 11, and Phl p 11. Therefore, the presence of Ole e 1-specific IgE in the sera of sensitized patients does not lead to cross-reactivity with non-*Oleaceae* pollens.

These experimental results may be reasonably explained by comparing the amino acid sequences of the members of this protein family. Figure 3 shows the identity and similarity percentages obtained between the pairs of proteins compared. Identity values greater than 80% (91% to 95% similarity) are obtained for the *Oleaceae* counterparts, whereas the values were lower than 50% (<59% similarity) for the remaining members. Thus, all these analyses support the fact that sensitization to Ole e 1 indicates primary sensitization to *Oleaceae* pollens.

% Identity (amino acid sequence)

% Similarity (amino acid sequence)

S \ I	Ole e 1	Fra e 1	Syr v 1	Lig v 1	Lol p 11	Che a 1	Pla l 1	BB18
Ole e 1	100	87	90	88	27	30	33	29
Fra e 1	93	100	84	91	30	32	34	30
Syr v 1	94	91	100	90	28	31	33	28
Lig v 1	83	95	95	100	28	31	33	29
Lol p 11	44	44	43	43	100	42	25	33
Che a 1	49	48	50	51	54	100	24	44
Pla l 1	49	50	50	52	39	36	100	29
BB18	47	47	46	48	46	59	40	100

Figure 3. Comparison of the amino acid sequences of members of the Ole e 1-like family by pairs. Percentage of identity (I) and similarity (S) is shown.

% Identity (amino acid sequence)

% Similarity (amino acid sequence)

S \ I	Ole e 3	Syr v 3	Bet v 4	Aln g 4	Che a 3	Phl p 7	Cyn d 7
Ole e 3	100	86	77	81	79	63	60
Syr v 3	89	100	72	72	730	65	62
Bet v 4	88	84	100	89	80	62	63
Aln g 4	89	84	96	100	82	63	62
Che a 3	89	86	90	89	100	66	66
Phl p 7	72	76	71	71	74	100	90
Cyn d 7	73	77	71	71	74	92	100

Figure 4. Comparison of the amino acid sequences of members of the polcalcin family by pairs. Percentage of identity (I) and similarity (S) is shown.

Ole e 2 and Ole e 3 as Markers of Polysensitivity

Ole e 2 and Ole e 3 belong to 2 well-known panallergen families named profilins and polcalcins, respectively. They contain a large number of members–profilin from plant and animal tissues, and polcalcins expressed only in pollen from trees, weeds, and grasses. Many members have been reported to be allergenic: profilins from fruits, vegetables, pollens, and latex; polcalcins from *Betulaceae, Oleaceae, Gramineae, Chenopodiaceae,* and *Brassicaceae.* An interesting molecular property of these protein families is the high conservativeness of their amino acid sequences and 3-dimensional structures. More than 70% of sequence identity is obtained when pairs of profilins are compared and more than 60% when pairs of polcalcins are compared (Figure 4). This explains why they are involved in allergenic cross-reactivity processes between plant sources.

We have cloned, isolated, sequenced, and produced recombinant profilins and polcalcins from *Oleaceae* and *Chenopodiaceae* species [13,39]. In 2 earlier experiments, we demonstrated how Ole e 3-specific polyclonal antiserum can recognize protein bands in extracts of pollen from birch, wheat, grasses, lilac, and ash [13], and how these pollen extracts can inhibit the binding of antiserum to Ole e 3. In a more recent study, we obtained similar results by using the profilin from *Chenopodium album* and extracts from a variety of pollen and plant-derived food allergenic sources [40] and suggested recombinant Che a 2, the profilin from chenopod, as a marker for profilin sensitization.

The similarity between profilin amino acid sequences and the similarity between polcalcin amino acid sequences may help to explain a not infrequent result that is obtained in practice: IgE antibodies of sera from patients with a skin prick test that is positive to many pollen sources responds only to a major allergen (Cyn d 1 or Ole e 1) and to profilin or polcalcin when tested against purified allergens. These panallergens are responsible for the positive response of sera to a wide panel of natural extracts in which the corresponding member of the family shares IgE epitopes with the sensitizing molecule. Therefore, these patients were not sensitized by all these pollens but remained sensitive to them. Each profilin and polcalcin, eg, Ole e 2 and Ole e 3, could serve as a marker of polysensitivity.

Detection of Sensitization to Minority Allergens

Most of the olive pollen allergens are contained in the pollen grain at very low levels, as they frequently constitute less than 0.2% of total protein. Ole e 1 is the exception and, although its amount depends on the *Olea europaea* variety, it represents up to 20% of the total protein content. Therefore, when the whole extract is used in ELISA or the coated allergen particle test, IgE reactivity to minority allergens is often not detected. The results obtained in ELISA experiments comparing the response of patients with symptoms of allergy to olive pollen in the pollination season with the response to the whole olive pollen extract and several purified allergens are shown in the table. Whereas the responses to the whole extract and Ole e 1 were very low or negative, the measured levels of specific IgE to Ole e 6 and Ole e 7 were high or very high. In vitro assays with purified natural or recombinant allergens in ELISA, coated allergen particle test, immunoblotting, or microarray technology can detect and assess sensitivity to minority allergens. The production of recombinant allergens provides molecules that are free of allergenic contaminants from the original natural source.

Diagnosis of sensitivity to minority allergens is important when these are the only allergy inducer, as diagnosis of sensitivity to the complete biological source may go unnoticed. In fact, it is necessary in the case of some minority allergens that have been reported to be very prevalent in specific populations with high exposure to pollen or to be associated with some property of the patient's clinical profile. For olive allergic patients, sensitivity to Ole e 7 has been associated with a propensity to adverse reactions [41], and sensitivity to Ole e 10 with severity and persistence of asthma [42]. As Ole e 10 and the C-terminal domain of Ole e 9 are homologous polypeptides, the correlation

Enzyme-linked Immunosorbent Assay Analysis of the IgE Reactivity of the Sera From 7 Patients who Are Allergic to Olive Pollen Against the Whole Pollen Extract and Purified Ole e 1, Ole e 6, and Ole e 7. Values of Optical Density at 492 nm Are Given.

Patient	Pollen extract	Ole e 1	Ole e 6	Ole e 7
1	0.038	0.020	0.350	0.501
2	0.280	0.033	0.891	2.442
3	0.084	0.012	0.512	1.175
4	0.106	0.071	0.565	0.741
5	0.067	0.021	0.402	0.631
6	0.083	0.010	0.501	1.283
7	0.214	0.018	1.124	1.895

between Ole e 10 and asthma symptoms can be extended to sensitivity to the C-terminal domain of Ole e 9. Therefore, the diagnosis of hypersensitivity to these olive allergens may be crucial in clinical practice and can be made using both Ole e 10 and the C-terminal domain of Ole e 9, as they are available by recombinant technology. Therefore, in vitro analysis with these molecules may enable diagnosis to be extended to allergens that are highly prevalent but which have a low concentration in pollen.

Immunotherapy With Recombinant Allergens

Recombinant allergen-based vaccines are free of undesirable components that could induce new IgE reactivities or adverse reactions and can be prepared according to the specific allergen profile of each patient. In addition, hypoallergenic molecules to be used in immunotherapy can be designed to exhibit reduced allergenic activity and the ability to promote synthesis of IgG protective responses. Recombinant wild-type allergens from birch and *Phleum*, as well as genetically modified hypoallergenic derivatives of Bet v 1, have been included in immunotherapy trials and have shown vaccination characteristics and clinical efficiency [23]. Most of the olive pollen allergens are available by recombinant biotechnology and some hypoallergenic derivatives of Ole e 1 have also been produced. Some of these molecules have been proven in a mouse model of allergy with promising results [36,37]. rOle e 1 was included in a prick-test study of reactivity in patients 7 years ago [43]. However, current Spanish legislation does not permit the use of recombinant allergens for in vivo determinations or treatments, although they might be useful for diagnosis and immunotherapy.

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