

Immunological Activity of Recombinant Ole e 1 in Patients with *Olea europaea* Pollinosis

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Key Words

Pollinosis · *Olea europaea* · Recombinant allergens · Diagnosis

Abstract

Background: Recombinant allergens have potential advantages over conventional allergenic extracts. However, these recombinant allergens should be evaluated for their antigenic activity and compared with their natural counterparts before being used for clinical purposes.

Methods: We studied 33 patients with seasonal rhinitis and/or bronchial asthma and a positive skin prick test to *Olea europaea* pollen extract, 10 atopic patients with no history of pollinosis and a negative skin prick test to *O. europaea* extract and 10 healthy controls. Skin prick tests and determination by ELISA of specific IgE to natural Ole e 1 (nOle e 1) and recombinant Ole e 1 (rOle e 1) expressed in *Pichia pastoris* were performed in all patients and controls. Inhibition assays were performed between nOle e 1 and rOle e 1 by ELISA. **Results:** All patients with *O. europaea* pollinosis had positive skin test responses to both commercial *O. europaea* extract and nOle e 1 allergen, and all reacted to rOle e 1 on the

skin prick test. The nonatopic and atopic control subjects with negative olive pollen skin test results did not react to rOle e 1 on the skin prick test, even at the highest concentrations, confirming the specificity of this test. We found a weak correlation between the wheal surface area produced by the prick test with nOle e 1 and the wheal surface area produced by rOle e 1 at 10 µg/ml ($r = 0.42$, $p < 0.05$). Comparison of specific IgE against both nOle e 1 and rOle e 1 in the patients did not reveal any significant difference. There was a strong correlation between the amount of specific IgE against nOle e 1 and rOle e 1 ($r = 0.99$, $p < 0.01$). The two proteins displayed the same extent of binding inhibition to IgE antibodies in ELISA inhibition experiments. **Conclusions:** These results confirm the immunological activity of rOle e 1 expressed in *P. pastoris* and indicate that Ole e 1 is one of the major allergens in *O. europaea* pollinosis as evaluated by skin prick test and serological methods. The correlation between rOle e 1 and nOle e 1 in skin test results and serologic data indicates the potential of recombinant allergens for clinical applications and diagnosis of *O. europaea* pollen allergy.

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Introduction

Current standard diagnostic methods in allergy, including the skin prick test and quantification of specific IgE antibodies in human sera, utilize crude pollen extracts which contain a complex mixture of allergenic and nonallergenic proteins. Recombinant DNA technology offers the possibility of producing well-defined allergens in large quantities for use as diagnostic and therapeutic agents [1]. These recombinant allergens produced in high-level expression systems should be evaluated for their antigenic activity and compared with their natural counterparts before being used for clinical purposes.

Olive tree (*Olea europaea*) pollen is an important cause of allergy in countries on the Mediterranean coast during pollination season [2]. Ole e 1 is one of the major allergens of *O. europaea* extract, with an IgE-binding frequency near to 80% among patients who are sensitive to olive pollen. Ole e 1 is a well-characterized protein that is present in the olive pollen in two main forms, glycosylated and nonglycosylated, with apparent molecular masses of 20.0 and 18.5 kD, respectively [3]. We established its amino acid sequence: a single polymorphic polypeptide chain of 145 amino acid residues with a calculated molecular mass of 16.3 kD [3, 4]. It has been found that Ole e 1 contributes significantly to the total allergenicity of olive pollen extract, and its concentration is closely related to the allergenic reactivity of the whole pollen extract [5]. The expression of Ole e 1 in *Escherichia coli* as a fusion protein rendered a low yield of soluble and functional allergen [4]. However, this protein has recently been expressed in the yeast *Pichia pastoris* [6], a heterologous expression system that had been shown to be very efficient for the production of well-folded cys-containing proteins [7]. At present, the recombinant Ole e 1 (rOle e 1) expressed in *P. pastoris* displays a conformational structure, as well as IgG- and IgE-binding capabilities in vitro identical to those of the natural protein isolated from the pollen [6]. However, the induction of skin reactivity with rOle e 1 in patients with olive pollinosis has not yet been analyzed. The aim of this study was to evaluate the immunological activity of rOle e 1 obtained in *P. pastoris* in a population of patients suffering *O. europaea* pollinosis, using the skin prick test as well as an allergen-specific ELISA and ELISA inhibition assays.

Patients and Methods

Patients

This study included 33 consecutive patients who fulfilled the following criteria: (1) seasonal rhinitis and/or bronchial asthma from late April to June; (2) a positive skin prick test response to *O. europaea* extract (Abelló Lab Madrid, Spain); (3) positive skin prick test and specific IgE antibodies to natural Ole e 1 (nOle e 1), and (4) no previous *O. europaea* immunotherapy. Ten healthy subjects and 10 atopic patients (4 monosensitized to mites and 6 to animal dander) were also included as a control group. Ethical approval for skin testing with recombinant allergens in human subjects was obtained from the Ethical and Research Committee of our hospital. A full explanation of the procedure was given to all subjects, and written consent was obtained.

Purification of nOle e 1 from Olive Pollen Extract

Olive tree pollen was obtained from Allergon AB, Sweden. The pollen (5% w/v) was extracted with 50 mM ammonium bicarbonate, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride. Isolation of nOle e 1 was carried out according to the method of Villalba et al. [3]. Protein concentration was determined by amino acid quantification of hydrolyzed samples with 5.7 N HCl at 105°C for 24 h. Hydrolyzed samples were analyzed on a Beckman system 6300 amino acid analyzer.

Production and Purification of rOle e 1

Ole e 1-encoding complementary DNA (cDNA) was expressed in *P. pastoris* using the pPIC9 vector, as previously described [6]. Briefly, a cDNA encoding Ole e 1, fused to a *Saccharomyces cerevisiae* alpha-mating factor prepropeptide, was inserted into the yeast genome using the pPIC9 vector, under control of the *AOX1* promoter. After induction with methanol, the protein secreted to the extracellular medium was purified by ion-exchange and size-exclusion chromatographies. After purification, protein concentration was determined as described above.

Skin Testing

Skin prick tests with nOle e 1 (60 µg/ml) and rOle e 1 were performed in duplicate by puncture with a Morrow-Brown needle (Bayer SA Lab, Madrid, Spain) on the volar side of the forearm. Histamine phosphate (10 mg/ml) was used as positive control and physiologic saline solution was used as negative control. The test sites were placed 3 cm apart to avoid false-positive results. From an initial solution of rOle e 1 of 1 mg/ml, four serial 10-fold dilutions (from 100 µg/ml to 0.1 µg/ml) of this allergen were made in sterile physiologic saline solution. The reactions were recorded after 15 min by measuring the maximal longitudinal (D) and transversal (d) diameters of the wheal reaction. The surface area of the wheal was calculated according to the following formula: $[(D+d)/2]^2$ [8]. Patients were regarded as being sensitized to specific olive pollen allergen if the wheal had a surface area of at least 9 mm².

Detection of Specific IgE Antibodies to Ole e 1

IgE-binding measurements of allergic sera were performed by the titration of solid phase-fixed purified allergens. ELISA plates were coated overnight at 4°C with 0.1 ml of antigen (1 µg/ml) in PBS, pH 7.2, in each well. Wells were washed with PBS and 0.5% (v/v) Tween 20, then saturated with PBS containing 3% (w/v) nonfat milk and 0.1% (v/v) Tween 20. Plates were incubated with the human sera

diluted 1:10 followed by 100 µl of mouse anti-human IgE diluted 1:5,000 in blocking solution. Afterwards, plates were incubated with horseradish peroxidase-labeled goat anti-mouse IgG (1:5,000 dilution). Finally, the peroxidase reaction was developed using fresh enzyme substrate (0.03% (v/v) H₂O₂ and 0.63 mg/ml of *o*-phenylenediamine in 0.1 mM sodium citrate, pH 5.0) and the optical density was measured at 492 nm.

Inhibition Assays

ELISA inhibition assays were performed as previously described [9]. After being coated with 100 µl of antigen (1 µg/ml), the plates were incubated with the sera (diluted 1:10), previously incubated with different concentrations of nOle e 1 or rOle e 1 (0.01–1,000 ng) as inhibitors; this was followed by incubation with mouse anti-human IgE and horseradish peroxidase-labeled goat anti-mouse IgG.

Data Analysis

Statistical analysis was performed with the two-tailed Mann-Whitney U test. Differences associated with probability values of $p < 0.05$ were considered significant. The correlation between the wheal surfaces of nOle e 1 and rOle e 1 was analyzed by the Spearman rank correlation test, and the correlation between the amount of specific IgE against nOle e 1 and rOle e 1 was determined with Pearson's linear regression.

The diagnostic value of the prick test with rOle e 1 was evaluated in terms of sensitivity and specificity. The sensitivity of the diagnostic test was defined as the proportion of truly diseased persons in the population correctly identified by the prick test. The specificity was defined as the proportion of nondiseased persons correctly identified as negative by the test.

Table 1. Values of specific IgE antibodies against nOle e 1 and rOle e 1 compared with skin prick test results in patients with *O. europaea* pollinosis

Patient	rOle e 1 prick test (mean wheal surface area in mm ²)				ELISA, OD units	
	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	nOle e 1	rOle e 1
1			90	211	1.35	1.17
2			64	82	2.5	2.5
3			77	157	1.91	1.77
4	17	10	77	241	0.39	0.34
5			69	222	1.27	1.25
6			77	156	2.5	2.5
7	15	15	57	196	1.02	0.72
8			46	272	0.10	0.12
9	9	28	36	134	0.76	0.66
10			52	81	0.49	0.45
11			64	170	0.36	0.31
12			36	155	1.23	1.22
13			69	197	0.18	0.16
14			109	100	0.23	0.25
15			100	649	0.05	0.04
16		18	24	197	2.07	1.97
17		12	106	207	0.36	0.29
18		14	23	204	0.58	0.46
19			61	164	0.17	0.14
20			158	385	0.73	0.60
21			50	90	0.44	0.38
22			53	104	0.23	0.20
23			52	105	2.43	2.18
24			39	165	0.29	0.24
25			61	122	0.53	0.46
26			138	253	0.16	0.15
27			58	127	0.28	0.25
28			129	676	0.26	0.22
29			68	212	0.11	0.09
30			45	121	0.14	0.14
31			77	68	0.64	0.56
32			49	116	1.73	1.54
33			42	336	2.5	2.5

OD = Optical density.

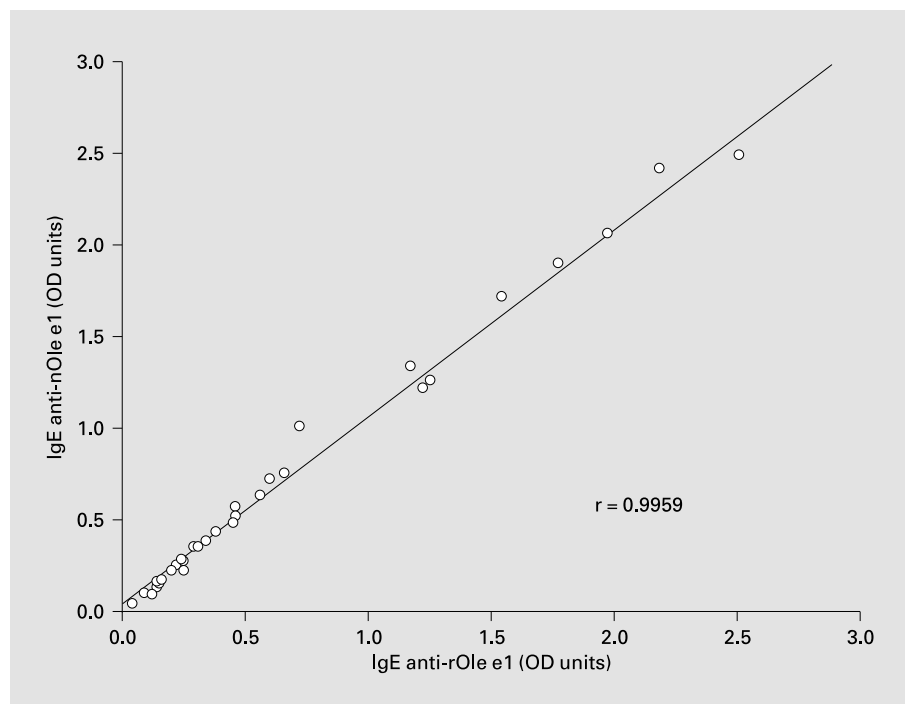


Fig. 1. Correlation between the amount of specific IgE antibodies against nOle e 1 and rOle e 1 measured by ELISA. OD = Optical density.

Results

Skin Test with nOle e 1 and rOle e 1

The 33 patients with either seasonal rhinitis or bronchial asthma included 18 women and 15 men ranging in age from 11 to 34 years (mean age 21.2 years). All patients had positive skin test responses to both commercial *O. europaea* extract and nOle e 1. As shown in table 1, all patients reacted to rOle e 1 at different dilutions. Among them, 3 patients (9%) reacted at 0.1 µg/ml, 3 patients (9%) reacted at 1 µg/ml and the remaining 27 patients (82%) showed positive responses at both 10 and 100 µg/ml of rOle e 1. Thus, the accuracy of establishing Ole e 1 sensitization was 100% when rOle e 1 was used. Moreover, there was no positive reaction to rOle e 1, even at the highest concentrations, in 20 control subjects with negative olive pollen skin test results, confirming the specificity of this test. For the patients who reacted to rOle e 1 at 10 µg/ml, we found a weak correlation between the wheal surface area produced by the prick test with nOle e 1 and the wheal surface area produced by recombinant allergen ($r = 0.42$, $p < 0.05$).

Additionally, we also calculated the quantity of rOle e 1 that produces a wheal surface area of the same size as the median wheal surface area produced by histamine (10 mg/ml), according to Nordic guidelines of allergen

standardization [10, 11]. The geometric mean of the wheal sizes at corresponding dilutions of rOle e 1 was calculated. Linear regression was performed with the least-square method and the following model: $\log(\text{area}) = 0.805 + 0.727 \times \log(\text{allergen concentration})$. The concentration of rOle e 1 that induced a wheal surface area of the same size as the median wheal surface area produced by histamine (10 mg/ml) was 31.6 µg/ml.

Allergen-Specific ELISA

Using an allergen-specific ELISA, we analyzed the binding of IgE to nOle e 1 and to the rOle e 1 used in skin testing (table 1). The presence of specific IgE against nOle e 1 could be detected in all *O. europaea*-allergic patients who responded on the skin prick test to natural allergen. Comparison of specific IgE against both nOle e 1 and rOle e 1 in the patients did not reveal any significant difference ($p > 0.05$, Mann-Whitney U test). With most of the sera, the optical density obtained for the binding of the IgE antibodies to rOle e 1 was slightly lower than that obtained for the binding to nOle e 1. However, as shown in figure 1, there was a strong correlation between the amount of specific IgE against nOle e 1 and rOle e 1 ($r = 0.99$, $p < 0.001$).

IgE Binding Inhibition Assays

IgE binding to rOle e 1 and nOle e 1 was quantitatively evaluated in ELISA inhibition experiments, by using the sera from two patients whose specific IgE antibodies had been determined. The assays were performed by coating the wells of the plates alternatively with each of the allergens (fig. 2). The two proteins, nOle e 1 and rOle e 1, displayed the same extent of binding inhibition to IgE antibodies, indicating that they share allergenic determinants and so are equivalent at an immunological level.

Discussion

Experimental data suggest that recombinant allergens, which are produced by appropriate strategies and systems of recombinant cDNA methodology [6, 12–14], can be used for the diagnosis of pollen, mite, mold and hymenoptera-induced allergies [1, 15]. However, a few allergens have been validated by skin testing in defined allergic populations, as for example rBet v 1 and rBet v 2 [16], rDer p 2 [17], rApi m 1 [18] and *Aspergillus fumigatus* allergens, rAsp f 1 [8] and rAsp f 3 [19]. As for other allergens, the success of recombinant production of Ole e 1 is highly dependent on the system used. The very low level of soluble and well-folded recombinant olive allergen obtained by using *E. coli* cells compelled us to achieve its expression in the yeast *P. pastoris* [6]. In this work, we have demonstrated that the recombinant form of Ole e 1 expressed at high levels in this yeast possesses an immunological activity in skin prick tests comparable to its natural counterpart, without any adverse effect in the control or patient population studied. The patient group in this study was selected on the basis of a sensitization to nOle e 1. Our results demonstrate that the sensitivity in establishing the diagnosis of olive pollinosis in patients with a positive response to nOle e 1 is 100% when this rOle e 1 is used. In the control patients with negative olive pollen results, there was no positive reaction to rOle e 1, confirming the 100% specificity mentioned above.

Skin testing has been proposed as the basic technique for the unitage of relative extract potency for many years. In Europe, we use the Nordic standardization system which is based on an evaluation of the wheal induced by a prick test [10, 11]. Thus, we calculated the concentration of rOle e 1 (31.6 µg/ml) that produces a wheal of the same size as the median wheal produced by histamine (10 mg/ml). Evaluation of the immunological activity of other recombinant olive tree pollen allergens is also now in progress.

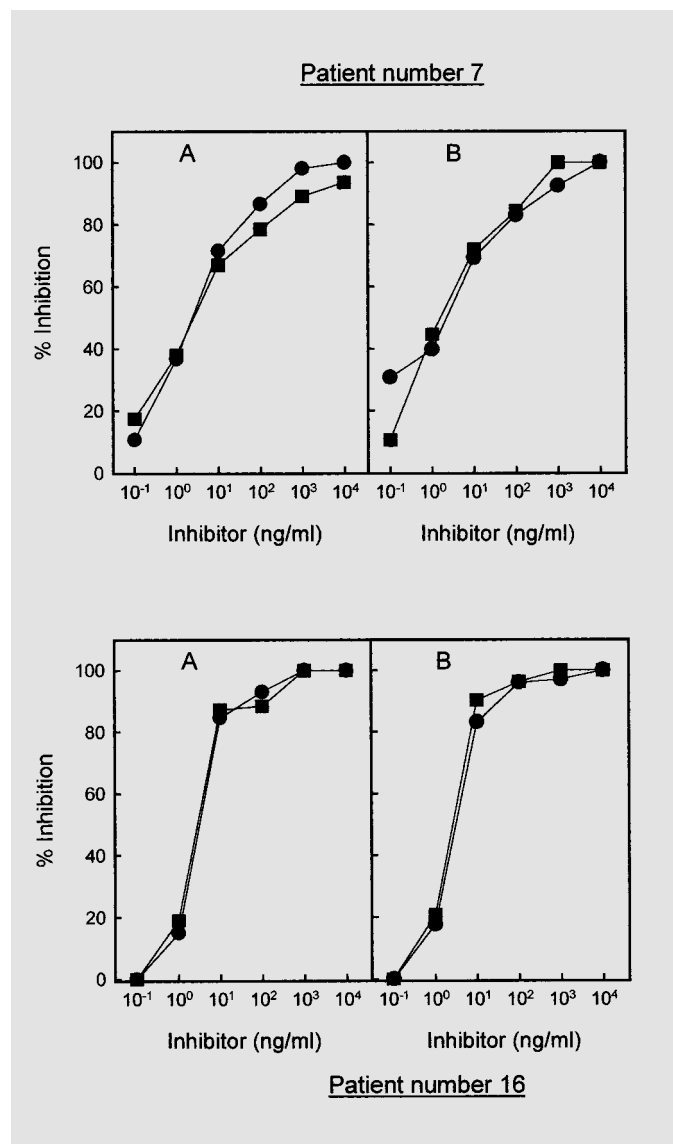


Fig. 2. ELISA inhibition assays. The binding of IgE from sera of allergic patients to nOle e 1-coated (A) and rOle e 1-coated (B) wells was assayed. nOle e 1 (●) and rOle e 1 (■) were used as inhibitors at the concentrations indicated.

The equivalence of nOle e 1 and its recombinant form obtained in yeast was also demonstrated by quantifying the specific IgE values which were present in the sera from the olive-allergic patients as well as by inhibition assays between these two forms of the allergen. The latter analyses showed the inhibition capacity possessed by each form against the other, indicating that they can saturate the IgE epitopes of each other. Small discrepancies were observed between the values obtained for the IgE responses against

nOle e 1 and rOle e 1 in ELISA. The optical density values of the positive IgE reactivity to rOle e 1 of most of the sera (26/33) were between 85 and 100% of those obtained for allergen isolated from the pollen. A minor proportion of these sera (3/33) did not reach 80% of the IgE response with rOle e 1. This could be due to the absence of the glycan epitope contained in nOle e 1. In fact, nOle e 1 is a single polypeptide chain which contains a N-linked carbohydrate moiety with allergenic activity [20, 21]. In the mid-nineties, this allergen was cloned and expressed in *E. coli* cells. The production of Ole e 1 in this bacterial system resulted in a nonglycosylated protein [4, 20, 21]. Therefore, the absence of the adequate glycan moiety in this bacterially expressed rOle e 1 decreased its binding capability to the IgE from the sera of patients which recognize the glycan epitope. Later, we used a new method for producing a soluble and proteolytically stable form of rOle e 1 by means of heterologous expression in the methylophilic yeast *P. pastoris* [6]. rOle e 1 expressed in this eukaryotic system appeared to be properly folded, as it displays spectroscopic properties (circular dichroism and fluorescence), as well as IgE- and IgG-binding capacities, which were indistinguishable from those of the natural protein [6]. *P. pastoris* is able to glycosylate proteins after gene translation, but it lacks the enzymes that incorporate type-complex glycans with the typical features of plant glycoproteins [22, 23]. Therefore, although rOle e 1 expressed in *P. pastoris* does contain a glycan component, it does not have the structure of the allergenic glycan present in the natural form of the allergen [6]. This could explain the small discrepancy between the specific IgE values of the sera from olive-allergic patients against nOle e 1 and

rOle e 1. Another possibility to be considered is the high polymorphism of nOle e 1 [4], in contrast with the single molecular specie produced as rOle e 1. As some heterogeneous positions of an epitope can be involved in antibody recognition [24], the IgE reactivity can be directed only against specific isoforms of the allergen. Thus, the absence of some amino acid residues in the expressed isoforms could be detected by the IgE antibodies, giving a decreased reactivity as observed.

In conclusion, this study has proven the diagnostic efficiency of rOle e 1 used in skin testing and ELISA for assessing olive tree pollen allergy. We think that the accuracy of tests with recombinant allergens will significantly improve the diagnosis and treatment of olive pollinosis. It is possible that certain *O. europaea* allergens could be responsible for some defined clinical syndromes (as for example bronchial asthma), and the use of single major and/or minor recombinant *O. europaea* allergens will possibly allow the establishment of an individual IgE reactivity profile for patients [1, 15], and thus, we will be able to design specific immunotherapy for each particular patient in the near future.

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