

Monoclonal antibodies against *Olea europaea* major allergen: Allergenic activity of affinity-purified allergen and depleted extract and development of a radioimmunoassay for the quantitation of the allergen

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Several monoclonal antibodies (MAbs) were raised against Olea europaea pollen-extract components. Two of these antibodies, named OL 2 and OL 7, recognize two nonoverlapping, nonrepeating epitopes on the olive-allergen Ole e 1, as demonstrated by different techniques. The allergen was purified in a single step by MAb-based affinity chromatography, and the allergen revealed a band at molecular weight 20 kd as well as a minor band at 18 kd on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The contribution of allergen Ole e 1 to the allergenic activity of O. europaea pollen extracts was determined from the effect of allergen depletion by affinity chromatography on skin reactivity and a histamine-release test. The removal of allergen caused a large reduction in the activity of the preparation in 25 monospecific olive-allergic patients. In agreement, the affinity-purified allergen demonstrated a similar response when it was compared with the whole extract in these assays. The results indicated that Ole e 1 is by far the most important olive-pollen allergen. A two-site solid-phase radioimmunoassay was developed for the quantitation of the allergen Ole e 1 in mass units. The assay was based on the MAbs, OL 2 and OL 7, and had a detection limit in the nanogram range. A good correlation was found between allergenic activity, as determined by RAST inhibition, and allergen content in 18 olive-pollen extracts. This result indicates that the assay can be a good alternative to RAST inhibition for the standardization of O. europaea extracts. (J ALLERGY CLIN IMMUNOL 1992;89:884-94.)

Key words: *Olea europaea*, major allergen, monoclonal antibody, epitopes, allergen depletion, affinity chromatography, radioimmunoassay, standardization, mass units

The identification, purification, and characterization of major allergens is important for clinical reasons and to dissect the immunologic response produced and to study if there is a relationship between allergen molecular structures and allergenicity. In this sense, some previous studies have been performed to identify and characterize the allergenic molecules of *OE* pollen that is an important cause of hay fever (second after

grasses) and asthma in those areas in which olive trees are extensively cultivated (mainly Mediterranean countries).^{1, 2} Thus, Vela et al.³ described major allergens in protein fractions with an MW of 65 kd after Sephadex G-100 gel filtration of *OE*-pollen extracts. Blanca et al.⁴ partially purified an acidic protein of pI 6 consisting of two polypeptide chains of MW 15 and 17 kd, as determined by SDS-PAGE, which appeared to be an important allergen in olive-pollen extracts. Rubio et al.⁵ reported the purification of an olive allergen by HPLC. More recently, Lauzurica et al.⁶ described the purification and partial characterization of two proteins, considered as major olive allergens, which they called *Olea* antigen I and *Olea* antigen II. Antigen I appeared to be composed of two polypeptides of 17 and 19 kd, and antigen II had an MW of 8 kd when these antigens were analyzed by SDS-PAGE. In addition, Villalba et al.⁷ have recently de-

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Abbreviations used

OE:	<i>Olea europaea</i>
pI:	Isoelectric point
Ole e I:	<i>Olea europaea</i> allergen I
MW:	Molecular weight
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
HPLC:	High-performance liquid chromatography
SPT:	Skin prick test
HR:	Histamine release
MAB:	Monoclonal antibody
PBS:	Phosphate-buffered saline
PBS-T:	PBS containing 0.1% Tween 20
BSA:	Bovine serum albumin
PBS-BSA:	PBS containing 1% BSA
OD:	Optical density
PAS:	Periodic acid-Schiff
CIE:	Crossed immunoelectrophoresis
HRU:	Hamlet RAST unit
BU:	Biological unit
IEF:	Isoelectric focusing

terminated the *N*-terminal amino acid sequence of antigen I (*Ole e I*, after the new allergen nomenclature⁸) to which they assigned an MW of 20.5 and 18 kd on SDS-PAGE.

In the previously mentioned studies, the term "major allergen" was used based on serologic investigations, but other studies, such as SPTs and HR, were not performed. In contrast, the contribution of the major allergen to the total allergenic activity of olive pollen is not clear. A good method to analyze this contribution is the study of the effect of the depletion of a major allergen on the biologic activity of the resulting material. This approach has been used with the cat dander and *Dermatophagoides pteronyssinus* allergen systems. In the cat-allergen system, depletion of *Fel d I*, the only major allergen, was reported to induce a significant decrease in skin reactivity.^{9, 10} However, *Der p I* depletion of *D. pteronyssinus* body extracts had no detectable effect on the activity in most patients, and at least 70% of the activity was retained in the depleted extract¹¹ that could be explained by the existence of at least a second major allergen, *Der p II*.

We describe here the obtention of several MABs against OE-extract antigens. Five of these MABs probably reacted with the previously described *Ole e I* allergen, and they defined at least two different epitopes on the molecule. These MABs were used to purify the allergen as well as to prepare allergen-depleted extract to study the clinical relevance of *Ole*

e I. In addition, we have developed a two-site solid-phase RIA based on MABs for the quantitation of the allergen.

MATERIAL AND METHODS

Allergenic extracts

Olive-pollen samples were collected as indicated.¹² Extracts were prepared by extraction for 1 hour at 4° C with 50 mmol/L of NH₄HCO₃, pH 8, at a 10% (wt/vol) ratio. The resulting suspensions were centrifuged at 39,000 g for 20 minutes, filtered through 0.22 µm pore-sized membrane, and stored at -20° C until use. Olive extract, OE III, was prepared in the same way but, after filtration, it was precipitated with 12% trichloroacetic acid, followed by two successive washes with cold acetone. The final precipitate was resuspended in PBS and stored at -20° C. The SDS-PAGE patterns of the starting material and OE III preparation were very similar, and 92% of the allergenic activity was recovered as determined by RAST inhibition. OE III extract was used in mouse immunizations because of its high allergenic potency. Protein content of extracts was determined according to the method of Lowry et al.¹³

Other allergenic extracts were obtained from Alergia e Inmunología Abelló SA, Madrid.

Production of MABs

Three female BALB/c mice were immunized with an intraperitoneal injection of 100 µg of olive extract OE III in Freund's complete adjuvant. Fifteen and sixty days later, mice were boosted with an identical amount of antigen in incomplete adjuvant. Twenty days after the last injection, mouse sera were screened for anti-OE antibodies by ELISA, and 7 days later, the mouse with the highest titer was injected intravenously with 100 µg of OE III in PBS. Three days later, this mouse was killed, and its spleen cells were fused with X63-Ag8.653 myeloma cells, as previously described.¹⁴ Twelve days after the fusion, hybridoma supernatants were screened, and the positive hybrids were cloned and subcloned by limiting dilution.

The isotype of the MABs was determined by immunodiffusion with antimouse subclass antisera (Tago, Inc., Burlingame, Calif.).

For the production of ascites, hybridoma cells (≈2 to 3 × 10⁶ cells per mouse) were injected intraperitoneally into pristane-primed male BALB/c mice, and ascites was collected 7 to 15 days after injection.

Screening assays

Hybridomas were screened for specific antibodies with ability to recognize allergenic molecules in OE extract. Two different screening assays were used. In the first assay, which screened for antibodies recognizing olive antigens (screening I), 96-well plates (high-binding enzyme immunoassay/RIA plate; Costar Corp., Cambridge, Mass.) were coated overnight at 4° C with OE III (50 µl of 10 µg protein per milliliter per well) or *Parietaria judaica* extract (negative control). After saturation with PBS-BSA, the wells were incubated for 1 hour with 50 µl of hybridoma culture su-

pernatant, and after wells were washed four times with 0.1% PBS-T, they were incubated for 1 hour with 50 μ l of biotin-labeled goat antimouse IgG¹⁵ diluted 1:5000 in PBS-BSA. After four more washes, 50 μ l of streptavidin-peroxidase conjugate (Amersham International, Buckinghamshire, England), diluted 1:4000 in PBS-T, was added to each well and incubated for 30 additional minutes. The plates were finally washed with PBS-T and developed with 50 μ l of freshly prepared enzyme substrate (0.03% H₂O₂, and 0.66 mg/ml of o-phenyldiamine [DAKOPATTS, Glostrup, Denmark] in 50 mmol/L of citrate-phosphate buffer, pH 6.0). After 30 minutes, the color reaction was stopped by adding 50 μ l per well of 4 mol/L of H₂SO₄, and the OD of the wells was read at 492 nm.

Positive hybrids in screening I were tested for the capacity to capture protein recognized by specific human IgE in the OE serum pool (screening II).¹⁶ Removable polystyrene wells (Immulon II, Dynatech Laboratories, Alexandria, Va.) were coated with 50 μ l of goat antimouse IgG (10 μ g/ml), and after saturation with PBS-BSA, they were incubated for 1 hour with 50 μ l of hybridoma supernatant. The wells were then washed with PBS-T and incubated with 50 μ l of OE III (50 μ g/ml). Unbound OE-extract proteins were washed with PBS-T, and the wells were incubated overnight with 50 μ l of OE serum pool diluted 1:2 in PBS-BSA. The wells were again washed and then incubated with 40,000 cpm of ¹²⁵I-labeled antihuman IgE (Pharmacia, Uppsala, Sweden) for 3 hours. After a wash, the radioactivity bound to the wells was measured.

In both screening assays, the P3-X63-Ag8 mouse myeloma culture supernatant was used as negative control. This cell line secretes a γ_1 , κ immunoglobulin of unknown specificity, and its secretion level was comparable to that of antilive hybridomas (20 to 40 μ g/ml in a 4-day culture supernatant).

MAB purification and ¹²⁵I-labeling

MAbs OL 2 and OL 7 were purified from ascitic fluid by the caprylic acid method.¹⁷ The purity of the MAb preparation was tested by SDS-PAGE, and protein concentration was determined by OD at 280 nm ($E_{280}^{1\%} = 13.5$ for mouse IgG).

Purified MAB was ¹²⁵I-labeled by the chloramine-T method.¹⁸ Ten micrograms of antibody was labeled with 0.5 mCi of ¹²⁵I Na (Medgenix Diagnostics, Fleurus, Belgium) to a specific activity of about 20 μ Ci/ μ g. Similarly, 20 μ g of OE extract, OE III, was labeled with 0.5 mCi of ¹²⁵I Na with the same method.

SDS-PAGE and immunoprecipitation

SDS-PAGE was done under reducing conditions on a 14% acrylamide gel according to the method described by Laemmli.¹⁹ The Bio-Rad low-range standards (Bio-Rad Laboratories, Richmond, Calif.) were used as MW markers. The electrophoresis was performed in a small vertical slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.), and the protein bands were detected by Coomassie brilliant blue staining. Glycoprotein bands were stained by the PAS technique.²⁰

Immunoprecipitation with antilive MAbs was performed

as follows: Removable polystyrene wells (Immulon II) were coated with 50 μ l of MAbs OL 2, OL 7 or Av1/1 (a MAB specific for *Artemisia vulgaris* allergen I) at 10 μ g/ml, and after saturation with PBS-BSA, the wells were incubated for 1 hour with 5×10^5 cpm of ¹²⁵I-labeled OE III. After three washes with PBS-T and a final wash with water, the radioactivity bound to the solid phase ($\approx 1\%$ of the input) was detached by incubating for 5 minutes at 90° C with 50 μ l of SDS-PAGE sample buffer and then subjected to electrophoresis. Finally, the gels were dried and autoradiographed.

Purification of allergen and preparation of allergen-depleted extract

An immunosorbent column was prepared by coupling MAB OL 2 and OL 7 (30 mg of a 50% saturated ammonium sulfate cut of ascites for each MAB) to 1.5 gm of CNBr-activated Sepharose 4B (Pharmacia). Before use, the column was washed with 3 mol/L of KSCN in PBS and equilibrated with PBS. Then 50 ml (50 μ g allergen per milliliter, as determined by RIA) of OE extract was passed through the column. After a wash with PBS, the bound allergen was eluted with 3 mol/L of KSCN in PBS. Approximately 10 ml fractions were collected, and those fractions containing significant protein content, as determined by OD at 280 nm, were pooled and dialyzed extensively against PBS. The protein content of the preparation was determined according to the method of Lowry et al.¹³ and Waddle's technique,²¹ and the purity of the allergen preparations was assessed by SDS-PAGE and CIE.²² The recovery of allergen was about 30% to 50%, as judged by RIA.

Allergen-depleted OE extract was obtained after passing the extract twice through the MAB immunosorbent column and extensive dialysis against PBS. The allergen content in the final preparation was 0.2 μ g/ml ($<0.5\%$ of the initial allergen concentration) as determined by RIA. SDS-PAGE comparison of whole and allergen-depleted extract revealed an identical pattern except for the presence of the 20 and 18 kd bands.

Allergen RIA

Quantitation of allergen was achieved by a two-site solid-phase RIA as follows: Removable polystyrene wells were coated with 50 μ l of MAB OL 7 at 5 μ g/ml in PBS for 1 hour at 37° C or overnight at 4° C. Wells were incubated for 15 minutes with PBS-BSA to block the remaining adsorptive sites, and then they were incubated for 1 hour at room temperature with 50 μ l of samples diluted in PBS-BSA. After four washes with PBS-T, the wells were incubated for 1 hour at room temperature with ¹²⁵I-labeled OL 2 MAB (100,000 cpm in a final volume of 50 μ l per well). Finally, the wells were again washed, and the radioactivity bound was counted in a gamma counter. Samples were assayed in duplicate at several dilutions. The allergen content, in mass units, was obtained from a control curve with different dilutions (range, 300 to 5 ng/ml) of the affinity-purified allergen. The maximum binding of radioactivity (freshly radiolabeled OL 2) was about 50% of the input, and nonspecific binding with PBS-BSA varied between 200 and 700 cpm.

RAST inhibition

Allergenic activity of *OE*-pollen extracts was determined by RAST inhibition.²³ Paper disks were activated with cyanogen bromide and sensitized with *OE*-pollen extract, as described by Ceska et al.²⁴ A pool of human sera from 30 patients that contained specific IgE to olive-pollen extract was used throughout the experiments. The patients had a positive SPT and clinical history of hypersensitivity to olive pollen. The antihuman IgE MAb used was ¹²⁵I-labeled Hamlet (Alergia e Inmunología Abelló SA). Allergenic activity of *OE* extracts was expressed in BUs per milliliter by comparison with an in-house reference previously calibrated in BUs per milliliter by SPT.²⁵

Specific IgE quantitation

IgE quantitation was performed in human sera from the patients used for skin and HR tests. Informed consent was obtained from all patients. Specific IgE to *OE* extract was determined by the Hamlet method according to the supplier's instructions (Alergia e Inmunología Abelló SA). For the determination of specific IgE to purified olive allergen, a similar procedure was followed, but purified allergen instead of whole extract was coupled to the solid phase. The results were expressed in HRUs per milliliter. A score <0.3 HRU per milliliter was considered negative.

Skin test

Skin tests were performed in 25 patients with clinical history of allergy to *OE* pollen as indicated.²⁶ The whole *OE* extract was tested at three tenfold dilutions containing 45, 4.5 and 0.45 µg of allergen per milliliter, as determined by RIA, and 1.2, 0.12, and 0.012 mg/ml of protein, determined according to the method of Lowry et al.¹³ The allergen-depleted extract was tested at the same total protein concentrations with a remaining allergen content of 0.2, 0.02, and 0.002 µg/ml and the purified allergen at concentrations of 60, 6, and 0.6 µg/ml. The dilution buffer was 0.9% (wt/vol) of NaCl, 0.4% (vol/vol) of phenol, and 50% (vol/vol) of glycerol.

The wheal areas were measured by planimetry, and the mean wheal area of the 25 double determinations was evaluated for each concentration of sample. With the depleted extract, wheal areas were very small, mainly in the most diluted preparations, but they were tentatively included in the calculations. The interpatient wheal size coefficient of variation oscillated between 40% and 70%, and the homogeneity of variances was checked by Bartlett's test. Linear regression was performed with the least-square method, and the model: $\log(\text{area}) = \text{intercept} + \text{slope} \times \log(\text{allergen concentration})$. Regression lines were checked for linearity, and parallelism was checked by analysis of variance. The relative potency of each sample was calculated with the parallel-line assay.²⁷

HR

HR was determined in whole blood with an automated fluorometric method according to the method of Siraganian,²⁸ with the modifications previously described.²⁹ Six fivefold dilutions for each sample, the most concentrated

being equal to the 1:1 dilution used for skin tests, were used. Before allergen challenge, blood samples were challenged with four dilutions of goat antihuman IgE (Tago, Inc.). The total basophil histamine content was measured in all patients by adding perchloric acid to the sample blood at a concentration of 10% (vol/vol). A result >10% release was considered positive.

RESULTS

Analysis and specificity of MAb

From the fusion of spleen cells of a mouse immunized with *OE* III extract and X63-Ag8.653 myeloma cells, we obtained 37 hybrids that were able to secrete significant levels of anti-*OE* antibodies, as determined by screening I. These lines were screened for binding of allergen(s) recognized by human IgE antibodies (screening II). The results of the screening assays for antioleive MAbs selected are presented in Table I. When these MAbs were used in screening I after coating with the allergenic extracts (illustrated in Table III), there was no significant binding of MAbs except for *OE* extract, indicating the specificity of the antibodies obtained. By an immunoprecipitation experiment with cell-culture supernatant of antioleive hybridomas and ¹²⁵I-labeled *OE* III extract, most of the MAbs clearly recognized a protein with an MW of about 20 kd, which corresponded to the MW range described for an important olive allergen.^{6, 7} These MAbs appeared to recognize at least two different epitopes, as determined by a preliminary cross-competition experiment, and two representative hybridomas (OL 2 and OL 7) were selected for additional studies. The SDS-PAGE pattern of the immunoprecipitates obtained with the MAb OL 2 and OL 7 is illustrated in Fig. 1. Both MAbs were able to recognize an *OE* molecule with an MW of about 20 kd.

MAb affinity purification of *Olea* allergen

The allergen defined by MAb OL 2 and OL 7 was purified from *OE* extract by MAb affinity chromatography, as indicated in MATERIAL AND METHODS. The purity of the purified allergen was assessed by CIE and SDS-PAGE. The allergen elicited a single immunoprecipitation line on CIE against an *OE* extract rabbit antiserum (data not presented). On SDS-PAGE (Fig. 2), the material demonstrated a band at MW 20 kd, as well as a minor band with lower MW (18 kd), and the same pattern was obtained on immunoblots with whole *OE* extract probed with MAb OL 2 and OL 7. The fact that the 18 kd is scarcely detected in the immunoprecipitate produced by the MAb (Fig. 1) could be due to a poor ¹²⁵I-labeling of the 18 kd band compared with the 20 kd band in the *OE* III extract. The 20 kd band was stained by the PAS technique that is specific for glycoproteins (Fig.

TABLE I. Screening-assay results for antilive MAbs*

MAb	Screening I		Screening II
	Extract on solid phase (DO ₄₉₂)		
	<i>P. judaica</i>	OE	
			Human serum pool (cpm of ¹²⁵ I-labeled anti-IgE bound)
OL 1	0.06	0.80	3218
OL 2	0.08	1.11	7422
OL 3	0.06	1.10	3428
OL 4	0.07	0.72	1570
OL 5	0.09	0.54	8852
OL 6	0.07	0.69	7250
OL 7	0.07	0.75	6244
OL 8	0.09	1.28	1024
OL 9	0.07	0.69	3602
(-)	0.06	0.07	610

(-), Culture supernatant of mouse myeloma P3-X63Ag8.

*The results of screening assays (see MATERIAL AND METHODS) for selected antilive hybridomas as indicated. All MAbs had a γ_{11}, κ isotype except OL 4 and OL 5, which were γ_{2a}, κ and μ, κ , respectively.

2). In those conditions, the lower MW band was not stained, suggesting that it corresponded to nonglycosylated allergen. The allergen preparation elicited several bands in the pH range from 5 to 7.5 by analytical IEF, indicating heterogeneity of the allergen with respect to pI values. The allergenic activity of the purified allergen was demonstrated by different techniques, such as direct RAST, SPT, and HR, and the results are presented in the next sections.

Clinical relevance of purified allergen

Serologic studies. IgE antibodies to whole OE extract and to purified allergen were measured in sera of a group of 25 monospecific olive-allergic patients. The RAST scores with whole extract and purified allergen (Fig. 3) highly correlated ($r = 0.91$; slope, 0.88). Only two of the patients had a negative RAST to the allergen in spite of having a positive RAST to OE extract. However, in these cases, the RAST score to whole extract was rather low and, in contrast, three patients had a higher RAST score with the purified allergen than with the OE extract.

Skin tests. To study the allergenic activity and clinical relevance of the purified olive allergen, whole OE extract, purified allergen, and allergen-depleted extract, prepared as indicated in MATERIAL AND METHODS at three different concentrations, were assayed by SPTs in 25 monospecific olive-allergic patients. The results obtained with each preparation were compared by parallel-line assay and are indicated in Fig. 4. The purified allergen demonstrated a parallel response and a similar potency estimate (131%) to the whole-extract preparation. All patients tested were sensitive to the purified allergen. In contrast, the allergen-depleted extract was considerably less active

in the skin test than the whole extract (potency estimate, 3%). Ten patients had a wheal area $<10 \text{ mm}^2$ at the highest concentration of depleted extract, and all patients had a much smaller wheal size compared with wheal size of the purified allergen or whole-extract preparations.

HR

The allergen and whole OE extract demonstrated similar results in the HR from whole blood of olive-allergic patients (Fig. 5). The maximum release was similar in both cases and varied between 12% and 54% of total histamine. (A release of 10% is considered positive.) Four patients were nonreleasers for any preparation, including the antihuman IgE antibody. The removal of allergen from OE extract caused a significant change in the HR results. Only four patients were releasers with the depleted extract in the concentration range studied, needing a concentration about 125 times higher than with allergen or whole-extract preparations to produce HR that was always lower.

MAb RIA for allergen quantitation

The obtention of two MAbs with affinity for an important allergen of OE led us to develop an RIA for the quantitation of the allergen. A comparison of different combinations of antilive-allergen MAbs in a two-site solid-phase RIA is presented in Table II. When we used combinations of the same antibody on the solid phase and ^{125}I -labeled, there was no significant binding of radioactivity. However, that was not the case with the heterologous combinations. The results indicated that OL 2 and OL 7 recognized two different nonrepeating epitopes on the olive allergen,

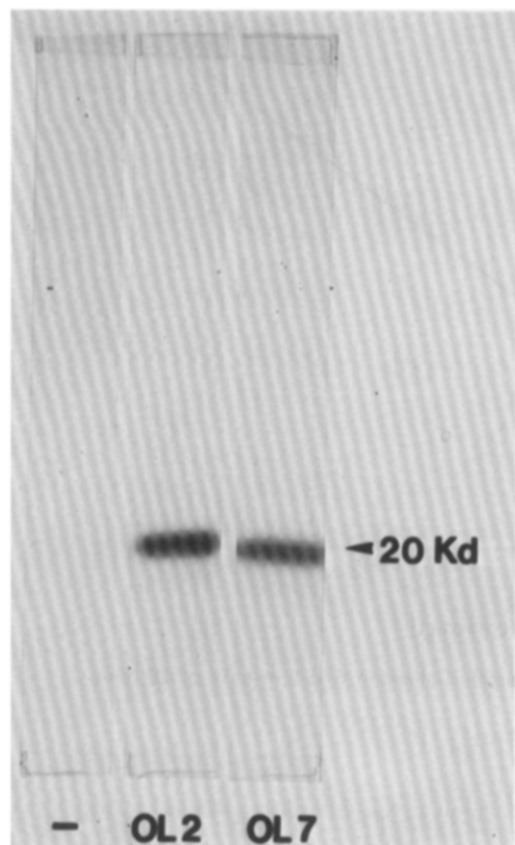


FIG. 1. Autoradiograph of SDS-PAGE analysis of immunoprecipitates obtained with ^{125}I -labeled *OE* III extract and antioleive MAbs OL 2 and OL 7; -, negative control with MAb Av1/1.

and consequently, it was possible to use these antibodies in a two-site RIA to quantitate the allergen. The best results were obtained with OL 7 on the solid phase and ^{125}I -labeled OL 2 as tracer, and that particular combination was used thereafter. The specificity of the assay is presented in Table III. There was no significant binding of ^{125}I -labeled OL 2 to wells incubated with different allergenic extracts, including mite, grasses, weeds, and trees, except in the case of *OE* extract.

The curve obtained with the affinity-purified allergen preparation in the two-site RIA is illustrated in Fig. 6. The assay has a detection limit <5 ng/ml and a practical working range between 5 and 200 ng/ml. The binding curve obtained with an *OE*-pollen extract is also illustrated in Fig. 6. This curve is parallel to the curve of the purified allergen, indicating that the allergen has not been antigenically altered during the purification process and that the assay is measuring the same molecule in both samples. The purified allergen preparation, with a protein content of 260 $\mu\text{g}/\text{ml}$, was used as standard in the two-site RIA for olive-allergen quantitation in mass units.

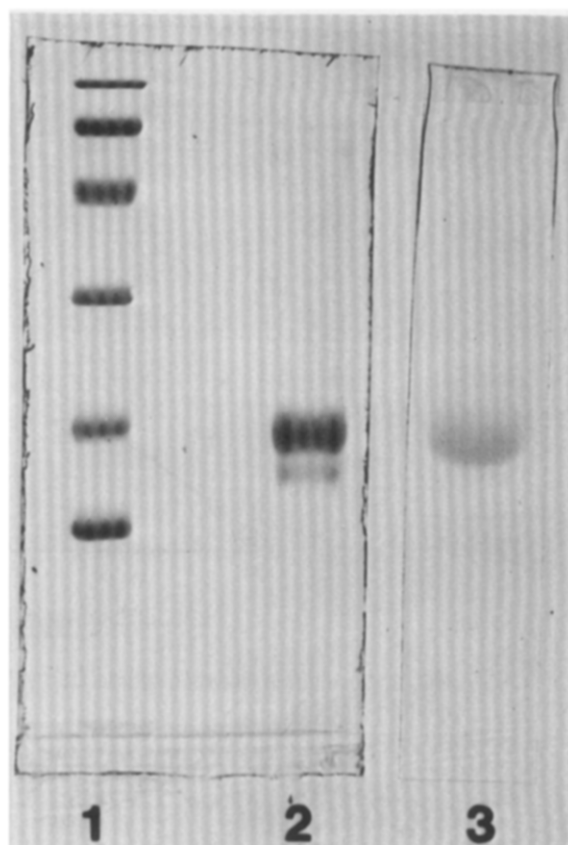


FIG. 2. SDS-PAGE of affinity-purified olive allergen; 1, MW markers: phosphorylase b (97 kd), BSA (66 kd), ovalbumin (43 kd), carbonic anhydrase (31 kd), soybean trypsin inhibitor (21 kd), and lysozyme (14 kd); 2, Coomassie blue staining of purified allergen (20 μg protein applied); 3, PAS staining of allergen (100 μg applied).

TABLE II. Comparison of combinations of antioleive allergen MAbs in two-site RIA

MAb on solid phase*	cpm Bound of ^{125}I -labeled MAb	
	OL 2	OL 7
OL 2	890†	15,450
OL 7	28,900	910†

*Polystyrene wells were coated with 50 μl (5 $\mu\text{g}/\text{ml}$ in PBS) of the MAb indicated. After saturation with PBS-BSA, the wells were incubated for 1 hour with *OE* extract at 20 $\mu\text{g}/\text{ml}$ as source of allergen. After a wash, the wells were incubated for 1 hour with 100,000 cpm of the ^{125}I -labeled MAb indicated, washed again, and the radioactivity bound to the solid phase was determined.

†Nonspecific binding with PBS-BSA was 870 cpm.

The allergen concentration was measured, with the MAb RIA, in 18 *OE* extracts from different pollen batches. The allergen content varied between 2% and 20% of the total protein content, as determined ac-

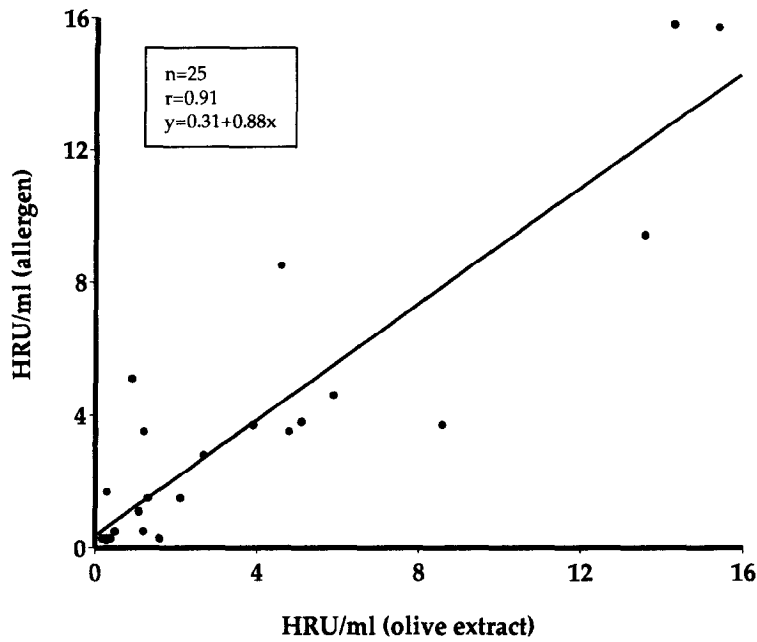


FIG. 3. Correlation between whole *OE* RAST and purified allergen RAST in sera of 25 olive-allergic patients.

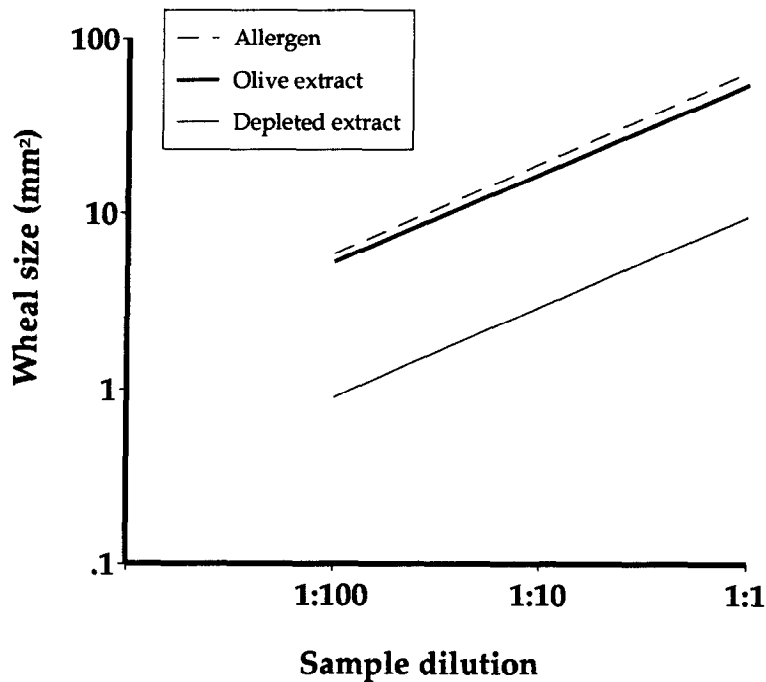


FIG. 4. Skin test activity of whole *OE* extract, purified allergen, and depleted extract in 25 monospecific olive-allergic patients. Regression line obtained with each preparation as indicated in MATERIAL AND METHODS.

cording to the method of Lowry et al.¹³ The total allergenic activity of the olive extracts was also determined by RAST inhibition. That activity was expressed in BUs per milliliter by comparison with an

OE extract in-house reference previously calibrated by SPT (see MATERIAL AND METHODS). The results of the allergen quantitation by MAb RIA and total allergenic activity were compared by linear

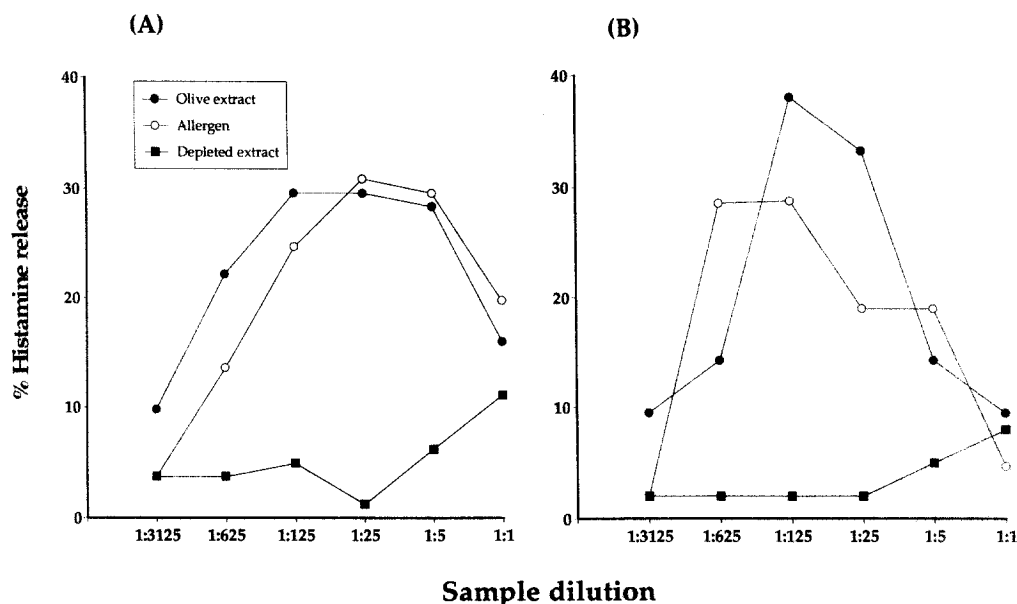


FIG. 5. HR from whole blood of two olive-allergic patients. Dilution 1:1 corresponded to most concentrated preparation used in SPT.

regression analysis (Fig. 7). A good correlation could be observed between both methods ($r = 0.84$; $p = 10^{-5}$) with 1 BU corresponding to approximately 0.6 μg of allergen.

DISCUSSION

In this study, the production of several MAbs specific for olive-pollen molecules is described. Two of these MAbs, OL 2 and OL 7, appear to recognize two different epitopes on the allergen *Ole e* I, as demonstrated by the following experiments: (1) These MAbs were able to recognize, in a specific way, an allergenic structure in OE-pollen extract (Table I). (2) These MAbs immunoprecipitated a protein with MW of about 20 kd (Fig. 1) in the MW range described for *Ole e* I.^{6,7} (3) The material, purified by affinity chromatography, with an immunosorbent column with MAb OL 2 and OL 7, behaved in SDS-PAGE as a double band at about 20 and 18 kd, in agreement with results of Villalba et al.⁷ for the *Ole e* I allergen purified by HPLC. In addition, the 18 kd band was not stained with the PAS technique, suggesting that it could correspond to a nonglycosylated form of the allergen, in agreement with previous studies.⁷ (4) On analytical IEF, the purified allergen preparation elicited several bands in the pH range from 5.0 to 7.5, indicating an heterogeneity of the allergen with respect to pI values that had also been observed by other authors.³⁰ (5) The N-terminal sequence for the first 14 amino acids of the purified allergen (results not pre-

TABLE III. Specificity of the RIA for olive-allergen quantitation

Sample*	cpm Bound of ¹²⁵ I-labeled OL 2
<i>D. farinae</i>	843
<i>Lolium perenne</i>	877
<i>Cynodon dactylon</i>	897
<i>P. judaica</i>	957
<i>Artemisia vulgaris</i>	987
<i>Alnus glutinosa</i>	958
<i>Betula verrucosa</i>	910
OE	25,380

*Polystyrene wells were coated with OL 7 MAb, as indicated in MATERIAL AND METHODS, and they were then incubated with the samples indicated at 1 mg of protein per milliliter, except OE extract at 20 $\mu\text{g}/\text{ml}$. After a wash, the wells were incubated with 100,000 cpm of ¹²⁵I-labeled OL 2. Nonspecific binding with PBS-BSA was 857 cpm.

sented) agrees with the sequence described for *Ole e* I,⁷ except for the absence of heterogeneity in the third position in which saline was found. (6) The RAST scores with whole OE extract and purified allergen were highly correlated (Fig. 3), suggesting that this component is the major allergen of OE pollen, a feature that led to labeling it *Ole e* I.⁷

Barber et al.¹² reported that the allergenic activity of OE-pollen extracts correlated with the presence of a component with MW 55 kd that was composed of protein subunits of 20 kd. This result, in agreement

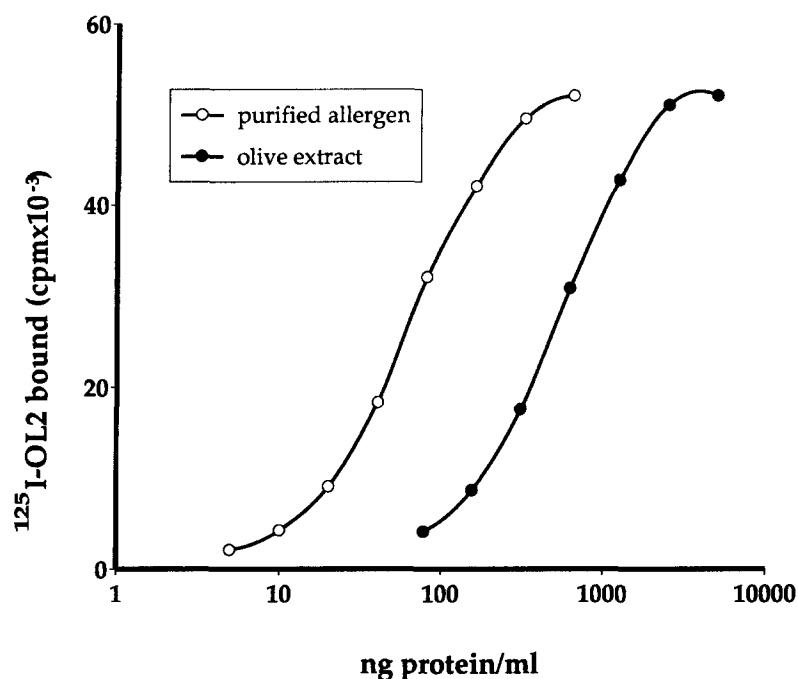


FIG. 6. Dose-response curves in two-site MAb RIA for an *OE* pollen extract and purified olive allergen.

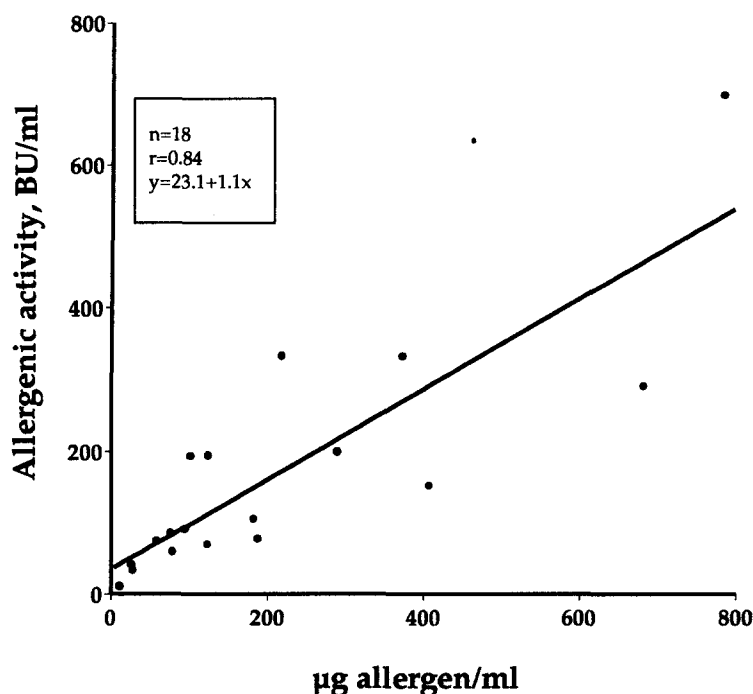


FIG. 7. Correlation between allergenic activity determined by RAST inhibition (BU per milliliter) and allergen content (microgram per milliliter) measured by MAb RIA.

with the works of Vela et al.³ and Wheeler et al.,³⁰ indicates that native *Ole e I* is probably a multimeric structure in which the 20/18 kd subunits are associated in some way.

Anti-*Ole e I* MAbs were used to study the effect of depletion of the allergen by affinity chromatography on the biologic activity of the resulting material. This depletion procedure had been used with the allergens,

Fed d I and *Der p I*,⁹⁻¹¹ and had the advantage over the use of purified allergen of being less aggressive, since during purification of a protein, the different buffers and conditions used throughout the process could affect the biologic activity of the final preparation. The use of affinity chromatography based on MABs appears to be suitable for removal of single components from allergenic extracts. However, depletion with a MAB can be incomplete if there is epitope variation in the population of allergen molecules. In contrast, the use of polyclonal antibodies could lead to the removal of other components from the allergenic extract. In the present study, we have used an immunosorbent column based on two different MABs, recognizing nonoverlapping epitopes, to obtain an *Ole e I*-depleted extract. This situation could be considered intermediate to the use of a single MAB or polyclonal antibodies having potentially the advantages of both approaches. In fact, in our system, the amount of allergen remaining after depletion was <0.5% of the starting amount as determined by the *Ole e I* RIA. In addition, the SDS-PAGE patterns of the whole and allergen-depleted extracts were identical except for the 20 and 18 kd *Ole e I* bands that were not detected in the depleted extract.

Skin test results indicate that *Ole e I* is the most important olive allergen. In fact, all the subjects tested in a group of 25 monospecific olive-allergic patients were sensitive to the affinity-purified allergen, and this preparation demonstrated a similar potency estimate to the whole-extract preparation when it was compared by the parallel-line assay method (Fig. 4). The removal of allergen from *OE* extract caused a large reduction in the biologic activity of the preparation in the same patients, and the potency estimate for the depleted extract is in the order of magnitude of the reduction of allergen content obtained by affinity chromatography. These results fully agree with HR tests and demonstrate that *Ole e I* is the major allergen of *OE* pollen. Other authors^{6, 30} have also found IgE-binding proteins with a different MW from *Ole e I*, and about 10% of olive-allergic patients do not react to *Ole e I*.³⁰ However, in the evaluation of these results, it is necessary to consider the existence of *Ole e I* aggregates and the presence in *OE*-pollen extract of an allergen shared by grasses as González et al.²² have reported. The olive-allergic patients used in the present study were monospecific, but this point is not clear in other studies in the literature. In fact, the allergenic activity of *Ole e I*-depleted extract was higher in patients with positive skin test to both *OE* and grass extracts (Quirce S. Unpublished results).

The existence of two different MABs recognizing two nonoverlapping epitopes on the *Ole e I* molecule was exploited to develop a solid-phase RIA in which

MAB OL 7 was immobilized on the solid phase and MAB OL 2 was used as tracer, the allergen molecule being sandwiched between them. The assay is specific for the *Ole e I* molecule and with a detection limit in the nanogram range. By IEF-immunoblotting³⁰ and reverse-phase HPLC,⁷ (Barber D. Unpublished results) the existence of isoallergenic forms in the *Ole e I* allergen has been pointed out, and it is possible, in principle, that the anti-*Ole e I* MABs were only specific for some isoallergenic forms. However, this finding did not appear to be the case, since the quantitation of allergen by RIA in different reverse-phase HPLC fractions of the allergen elicited similar results to the protein content of the sample (Lombardero M. Unpublished results). Finally, a good correlation was obtained between the content of allergen, as determined by the *Ole e I* RIA, and allergenic activity, determined by RAST inhibition, thus validating the use of the RIA for quality control. This result confirms that *Ole e I* is the most important olive allergen and indicates that such an assay could be very useful for evaluation of the allergenic activity of *OE* extracts, avoiding the use of human serum as well as olive extract-sensitized paper disks that are difficult to standardize. In addition, the developed RIA could be very useful in the standardization in mass units of *OE* preparations intended for clinical use, as well as studies on the stability of the allergen in these preparations. However, we cannot exclude the possibility that, as the allergen denatures, its reactivity with the MAB may decrease at a different rate from reactivity with human IgE, particularly in vivo.

This study illustrates the usefulness of MABs as tools for studying allergens. Thus, the main olive allergen was purified in a single step, and these specific reagents were very effective in preparing allergen-depleted extract. We have also developed a two-site MAB-based RIA for the quantitation of *Ole e I* that, to our knowledge, is the first description of such an assay for a major pollen allergen. Finally, the anti-*Ole e I* MAB available could be helpful in further characterization of the allergen which includes studies of allergenic cross-reactivity in closely related species that can be found in areas in which olive trees are not cultivated, epitope mapping of the allergen, and future cloning work.

We conclude from this study that a single allergen, *Ole e I*, has a very important contribution to the allergenic activity of olive pollen. Consequently, this system could be a good model to study the human immune response to inhalant allergens.

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