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Pollen from Different Olive Tree Cultivars Contains Varying Amounts of the Major Allergen Ole e 1

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Key Words
Allergens · Ole e 1 · *Olea europaea* L. · Olive tree · Cultivar · Skin prick test

Abstract
Background: Commercial olive pollen from uncertain cultivar origin is the common material used for clinical and biological studies. We aimed to assess the putative heterogeneity of olive cultivars with regard to the presence of the major pollen allergen Ole e 1 and to determine whether these differences have clinical relevance. ***Methods:*** The Ole e 1 content of several cultivars was determined by immunoblotting and ultrastructural immunocytochemistry and compared to that of a commercially available olive pollen extract designed for diagnosis. Reverse transcription-polymerase chain reaction analysis of Ole e 1 transcripts was also performed. Crude protein extracts were used to carry out skin prick tests (SPTs) on 30 allergic patients in order to evaluate the clinical importance of such differences. ***Results:*** Ole e 1 was present in all cultivars, although significant quantitative differences were detected. Ole e 1 transcripts positively correlated with the amount of the allergen. Significant variations in the average reactivity of allergic patients to SPTs were observed depending on the cultivar considered. ***Conclusions:*** The presence of the Ole e 1 allergen in all the cultivars suggests that this allergen may play an essential biological role. The expression of the allergen is controlled at the transcriptional level. The significant differences in the Ole e 1 content are likely responsible for the different average reactivity exhibited by patients to the cultivars studied, although the role of other allergens cannot be excluded. Our results suggest that the use of the commercial pollen mixtures currently available may lead to mistakes in allergy diagnosis and to limited success in immunotherapy. Therefore, further standardization is strongly recommended.

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Introduction

The olive tree (*Olea europaea* L.) is an important crop in the Mediterranean Basin, where it has been cultivated for several millennia [1]. The extensive culture of this plant has led to the appearance of an extremely wide and varied germplasm. 262 different cultivars have been identified [2] in Spain alone. To date, olive cultivar characterization has been primarily based on agronomic and mor-

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phological traits. However, the use of molecular [polymerase chain reaction (PCR)-based] techniques for this purpose is emerging [3].

On the other hand, olive pollen is one of the main causes of respiratory allergy in Mediterranean countries [4–6]. The major allergen present in this pollen, Ole e 1, is a protein of 20 kD which has been isolated, sequenced, cloned and expressed in *Escherichia coli* [7–9]. It consists of a single 145-amino acid polypeptide chain and contains an N-linked glycosyl moiety. Besides this protein, an increasing number of IgE-binding proteins from olive pollen are being characterized [10–20]. In most of the cases reported in the literature, the working material used corresponds to commercially available mature pollen, composed of an unknown mixture of cultivars and origins. However, the broad spectrum of the olive germplasm suggests that important heterogeneities in both the qualitative and quantitative composition of allergens may be present within the protein extracts corresponding to different cultivars. A pioneer study [21] has shown the existence of significant differences in the allergens present among several olive cultivars after using sera from allergic patients in immunoblot experiments.

The aims of the present study were to analyze the expression of the major olive pollen allergen (Ole e 1) by studying the presence and the localization of Ole e 1 itself and its transcripts in a number of mainly Spanish cultivars of olive of different origins and agronomical characteristics (vigor, drought tolerance, fruit size and oil content), and to determine whether such variations have clinical consequences. In a similar way, a very recent study used skin prick tests (SPTs), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and ELISA inhibition test to confirm the presence of variability in the antigenic and allergenic composition of *O. europaea* pollen extracts in five cultivars of olive tree as well as in Acebuche (wild olive) [22]. The authors also determined Ole e 1 content and total allergenic potency in these olive cultivars.

Materials and Methods

Pollen Samples

O. europaea L. pollen samples were obtained during May and June of 1998 and 1999 from cultivated trees of cultivars for oil production, i.e. Arbequina, Frantoio, Lechín de Granada, Picual and Picudo, and for olive consumption, i.e. Gordal Sevillana, Loaime, Lucio, Manzanilla de Sevilla and Hojiblanca (this last of dual purpose). Pollen samples were collected from numerous branches of at least two trees of each cultivar by shaking flowering shoots inside paper bags. Prior to its storage in liquid nitrogen, the harvested pollen was sieved through a 150-μm mesh in order to eliminate fallen corollas, anthers and other extraneous matter. After light microscopy observation, foreign-species pollen was estimated to be <0.1% and other plant parts <0.5% for all the cultivars used. Most of the cultivars were from different locations in Andalusia (Spain), except Arbequina (Catalonia, Spain) and the Italian Frantoio.

Preparation of Crude Protein Extracts and Ole e 1 Immunoblots

Crude protein extracts were obtained by stirring 1 g of pollen for each cultivar in 10 ml of extraction buffer (0.01 *M* ammonium bicarbonate, pH 8.0, 2 m*M* phenylmethylsulfonyl fluoride) for 8 h at 4°C. After centrifugation (2 × 30 min at 14,000 *g* at 4°C), the supernatants were filtered through a 0.2-μm filter and stored in aliquots at –20°C. Protein concentration in the different samples was measured using the Bio-Rad reagent and bovine serum albumin as standard [23].

Polypeptides (30 μg per lane) and molecular weight (Mw) standards (New England BioLabs Broad Range -Mw_1- and MBI Fermentas -Mw_2-) were separated by SDS-PAGE in 7.5–20% gradient gels [24] in a MiniProtean II system (Bio-Rad). The resulting gels were stained with Coomassie blue or transferred onto BioTrace polyvinylidene difluoride membranes (Pall BioSupport, USA) at 100 V for 1.5 h using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad).

For immunodetection, the membranes were treated with blocking solution (3% w/v bovine serum albumin, 0.5% v/v Tween 20 in Tris-buffered saline) for 2 h and then incubated overnight with a monoclonal antibody to Ole e 1 [25], diluted 1/100 in blocking reagent. A rabbit anti-mouse IgG alkaline phosphatase-conjugate (Promega, USA), diluted 1/2,500 in Tris-buffered saline plus 0.5% v/v Tween 20, was used as the secondary antibody, and the color was developed with the 4-nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate substrate. Densitometric analysis was performed using Scan Pro 5.0 (Sigma, USA) and Quantity One 4.2.0 (Bio-Rad, USA) software.

The same procedure described here was applied to a commercially available extract (Dome Hollister, USA) used for olive pollen allergy diagnosis.

Immunolocalization of Ole e 1 in Mature Pollen from Cultivars with High and Low Ole e 1 Content

Mature pollen samples from Picual (high Ole e 1 content) and Frantoio, Gordal and Arbequina (low Ole e 1 content) cultivars were fixed in a mixture of 4% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde prepared in 0.2 *M* sodium cacodylate buffer, pH 7.2, at 4°C overnight. After washing in the same buffer, the samples were dehydrated in a graded ethanol series, embedded in resin (Unicryl, BioCell International, UK) and polymerized at –20°C for 3 days under UV light. Ultrathin sections (70 nm thick) were cut on an Ultracut microtome (Reichert-Jung, Germany) and mounted on 300-mesh formvar-coated nickel grids.

For immunolocalization, sections were incubated by floating the grids in a blocking solution containing 2% (w/v) bovine serum albumin and 1% (v/v) normal goat serum in PBS for 15 min. This was followed by treatment with an anti-Ole e 1 monoclonal antibody diluted 1/10 in blocking solution for 12 h. After washing in PBS, the grids were incubated with a goat anti-mouse IgG antibody coupled to 20-nm gold particles (BioCell International), diluted 1/50 in PBS, for 2 h. Sections were then washed with PBS and ultrapure water and stained for 30 min with uranyl acetate. Observations were carried out on a Zeiss EM 10C transmission electron microscope operating at

60 kV. Control sections were treated as described above, but omitting the primary antibody.

Reverse Transcription-PCR Analysis of Ole e 1 Transcripts

Total RNA was extracted using an RNeasy Plant Total RNA kit (Qiagen, USA). Samples were ground in a mortar with liquid nitrogen and processed according to the manufacturer's instructions. Both the quality and the concentration of total RNA were assessed by denaturing gel electrophoresis and by measuring the ratio of absorbance at 260/280 nm. The RNA was immediately processed for reverse transcription (RT)-PCR or stored at –80°C.

Reverse transcription of 5 µg of total RNA per sample was carried out using M-MLV reverse transcriptase (Promega, USA) and an oligo dT-adaptor as primer (5'-GACTCGAGTCGACATCGA$(T)_{17}$-3'). PCR was carried out in samples corresponding to 250 ng of the reverse-transcribed RNA (5 µl) by adding 5 µl of 10× PCR buffer, 25 ng of each primer (Ole e 1 and oligo dT-adaptor), dNTPs to a final concentration of 1 m*M* each (including 0.25 m*M* dig-dUTP; Boehringer Mannheim, Germany), 2.5 U of DynaZyme DNA polymerase (Finnzymes Oy, Finland) and water to 50 µl. Samples were denatured for 2 min at 95°C and subjected to PCR amplification for 30 cycles of 94°C for 1 min, 57°C for 2 min and 72°C for 1 min. The oligonucleotide primer was designed based on the reported [8] nucleotide sequence of Ole e 1 (5'-ACCTCCAGTTTCTCAATTTCAC-3'). Reaction products (5 µl) were analyzed by gel electrophoresis and transferred onto Biodyne Plus membranes (Pall BioSupport), and bands were detected using an anti-digoxigenin (Fab) antibody conjugated to alkaline phosphatase, diluted 1/10,000, and disodium 3-(4methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7] decan}-4-yl)phenyl phosphate (CSPD) (both chemicals from Boehringer-Mannheim) as the chemiluminescent substrate according to the manufacturer's instructions. Hyperfilm ECL (Amersham, UK) was used to register the chemiluminescent reaction.

As a reference for controlling the accuracy of equivalent loading of samples, 250 ng of each reverse-transcribed RNA (5 µl) were PCR amplified by adding 5 µl of 10× PCR buffer, 125 ng of each of the following primers designed for amplification of plant ubiquitin (Ubi 1: 5'-ATGCAGAT(C/T)TTTGTGAAGAC-3'; Ubi 2: 5'-ACCACCACG(G/A)AGACGGAG-3'), dNTPs to a final concentration of 1 m*M* each, 2.5 U of DynaZyme DNA polymerase (Finnzymes Oy) and water to 50 µl. Samples were denatured for 2 min at 95°C and subjected to PCR amplification for 10, 20 and 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and 30 s in order to ensure an exponential amplification rate. Reaction products (5 µl) were analyzed by agarose (2%) gel electrophoresis.

Skin Prick Tests

Thirty Spanish patients residing in Eastern Andalucía and allergic to olive pollen on the basis of previous positive skin reactions and clinical symptoms were subjected to SPTs using 10 protein extracts, each one prepared from pollen of one of the well-defined 10 cultivars analyzed. Protein extracts were prepared as described above and diluted in a saline buffer solution containing 0.9% (w/v) sodium chloride and 50% (v/v) glycerol to a final concentration of 10 µg of total protein/ml on the basis of the Bio-Rad protein assay (Bio-Rad, UK). Controls, including a commercial extract from *O. europaea* pollen (Dome Hollister) diluted to a concentration of 30 µg of total protein/ml, a 1 mg/ml histamine solution (positive) and buffered saline (negative), were also performed. Twenty minutes after the inoculation, the wheal surface was quantified using Scan Pro 5.0 (Sigma) software.

Eighteen additional Spanish patients (also from Eastern Andalucía) allergic to olive pollen and currently undergoing immunotherapy were also investigated by SPTs as described above. In these cases, immunotherapy was temporarily suspended several weeks before the tests.

Statistical Analysis

The Shapiro-Wilks test was used to test normality for all variables. In order to determine the existence of significant differences in the average reactivity exhibited by allergic patients (none of them subjected to immunotherapy) depending on the cultivar considered, Friedman ANOVA analysis was performed. Finally, to assess whether these changes were correlated with Ole e 1 levels, the Spearman nonparametric test was used. All statistical analysis was performed using the Statistica 5.1 software (StatSoft, USA).

Results

Protein Analysis in Pollen from Different Cultivars and Commercial Extract

Protein extraction from olive pollen cultivars yielded the following amounts of total protein (mg/ml of extraction buffer): 7.53 (Picual), 5.47 (Loaime), 6.96 (Lucio), 7.66 (Frantoio), 4.87 (Gordal), 6.56 (Manzanilla), 4.83 (Arbequina), 5.38 (Picudo) and 6.62 (Lechín). SDS-PAGE of polypeptides from crude extracts of the different olive cultivars showed numerous bands in all cases after Coomassie staining of gels (fig. 1A). The patterns observed for the major protein species were rather similar for all the cultivars tested, although clear quantitative differences were distinguished, of which the most conspicuous were those in the protein range of 17–20 kD. Proteins within this range were relatively abundant in the extracts corresponding to the cultivars Picual, Loaime, Lucio, Manzanilla de Sevilla, Hojiblanca, Picudo and Lechín de Granada. Densitometric studies indicated that these proteins may represent up to 23% of the total protein (cultivar Lechín de Granada). On the other hand, protein extracts from the cultivars Frantoio, Gordal Sevillana and Arbequina, although also showing these polypeptides, presented a much lower band intensity (the lowest percentage corresponding to the cultivar Arbequina, i.e. 3%). No significant differences in the percentages shown here were observed between the extracts obtained over 2 consecutive years of pollen sampling.

When the commercial pollen extract was assayed by SDS-PAGE, a protein profile similar to the profile corresponding to the individual cultivars was observed, although several bands were absent or poorly resolved. Ole e 1-range proteins represented 25% of the total protein for this extract.

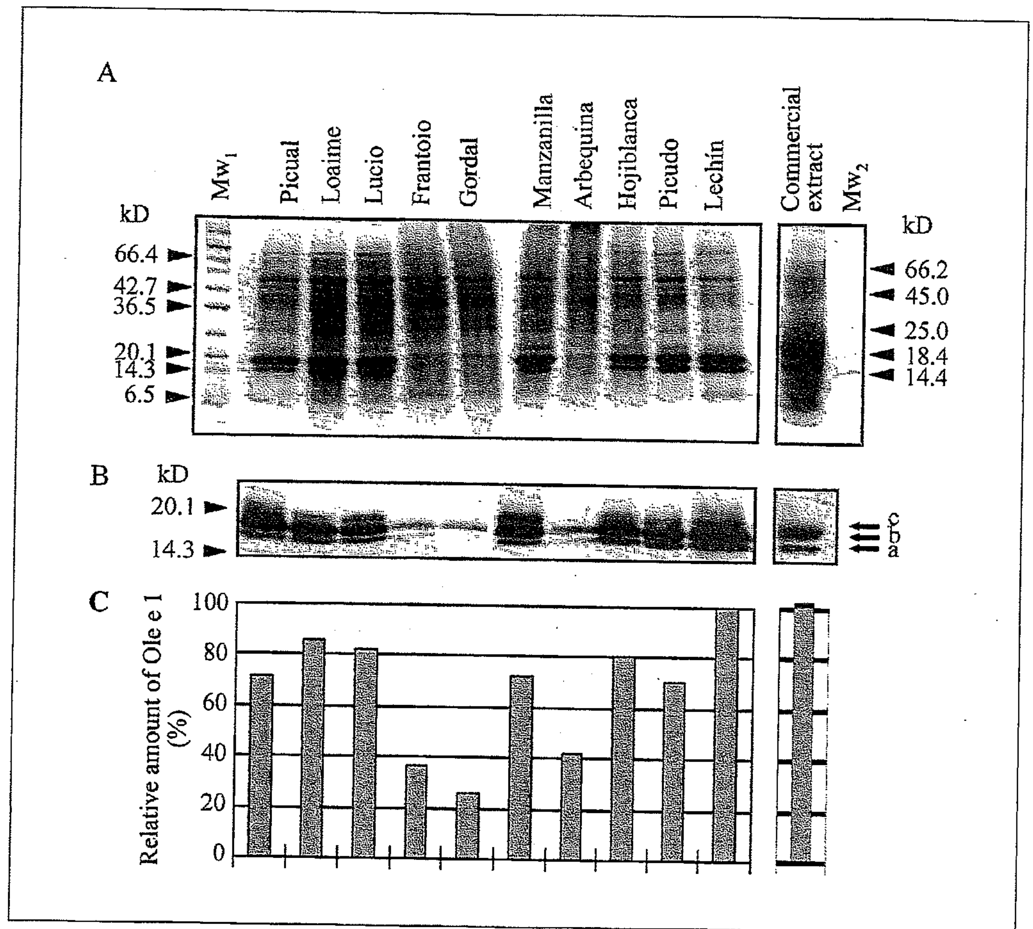

Fig. 1. SDS-PAGE and immunoblot analysis of Ole e 1 protein in crude extracts of pollen corresponding to several cultivars of olive tree and a commercial pollen extract. **A** Coomassie blue staining. Lane 1: Mw standards. Intense bands in the range 18–20 kD were observed, particularly in the cultivars Picual, Loaime, Lucio, Manzanilla de Sevilla, Hojiblanca, Picudo and Lechín de Granada. **B** Immunoblot as above, probed with an anti-Ole e 1 monoclonal antibody. Three reactive bands, corresponding to the nonglycosylated (a) and two glycosylated (b, c) variants of Ole e 1, can be observed in most cultivars. **C** Chart representing relative densitometric data corresponding to the Ole e 1 band from **A**. Data are represented as percentages related to the cultivar Lechín de Granada, which showed the highest optical density (100%).

Reactivity of Protein Extracts to Anti-Ole e 1 Antibody

Figure 1B shows the immunoblot detection of Ole e 1 after using a monoclonal antibody to this protein. Three immunoreactive bands were present in the range of 17–20 kD, corresponding to polypeptides sharing common epitopes and possessing different carbohydrate contents. Although all cultivars and the commercial extract showed reacting bands, their relative intensity varied significantly. The percentages of Ole e 1, in reference to the cultivar with the highest optical density (Lechín de Granada: 100%), are represented in figure 1C. Two main categories were distinguished: the cultivars Picual, Loaime, Lucio, Manzanilla de Sevilla, Hojiblanca, Picudo and Lechín de Granada showed high levels of Ole e 1 allergen (relative percentages of 71.15, 85.31, 81.85, 71.95, 80.17, 70.22 and 100%), whereas the cultivars Frantoio, Gordal Sevillana and Arbequina displayed a reduced level of the protein (relative percentages of 36.95, 26.32 and 41.84%). The commercial extract itself displayed a relative percentage of 101.57% with reference to the cultivar Lechín de Granada.

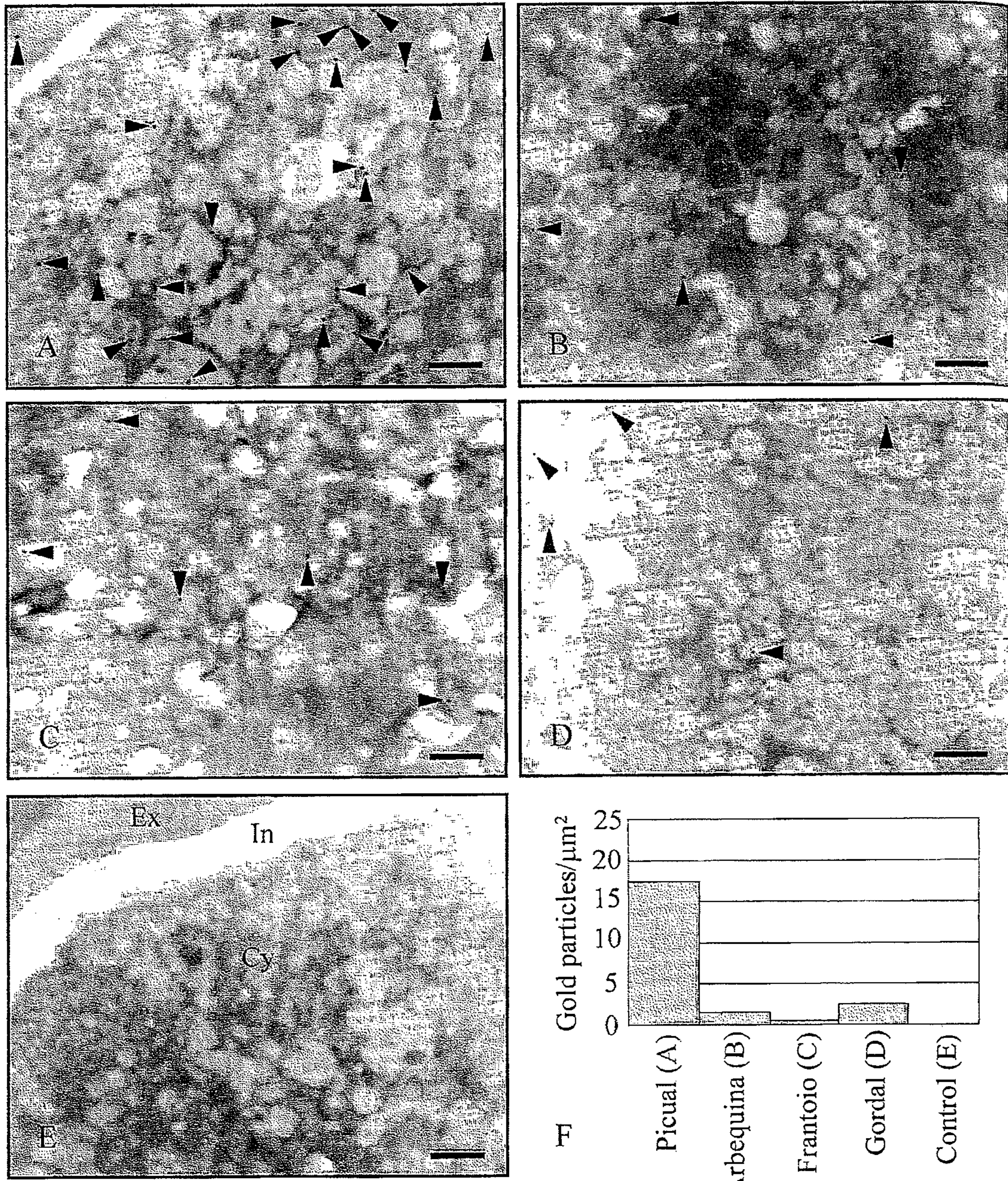

Fig. 2. A–D Transmission electron microscopy immunolocalization of Ole e 1 in mature pollen (arrowheads) from cultivars Picual (**A**), Frantoio (**B**), Gordal (**C**) and Arbequina (**D**), using a monoclonal anti-Ole e 1 antibody. **E** Negative control prepared by omitting the primary antibody (cultivar Picual). Cy = Cytoplasm; Ex = exine; In = intine. Bars represent 0.25 µm. **F** Quantitation of average labeling density (gold particles/µm²).

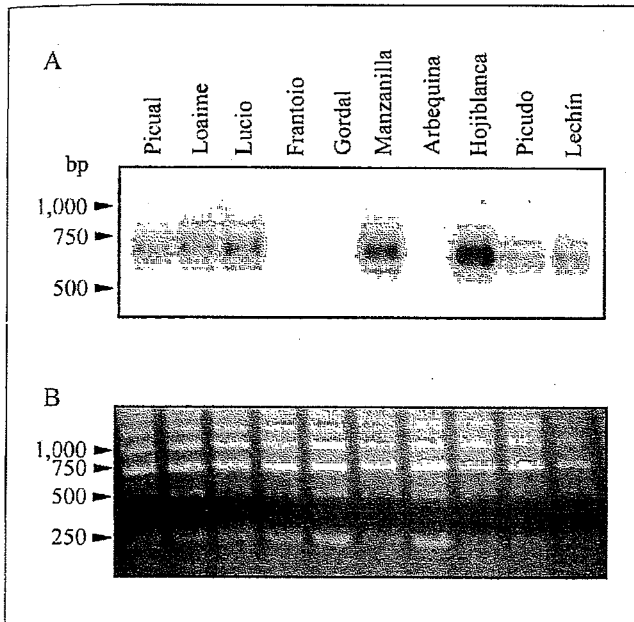

Fig. 3. A RT-PCR analysis of Ole e 1 transcribed in olive pollen from ten cultivars (30 cycles). **B** RT-PCR analysis of ubiquitin monomer to polyubiquitin pentamer transcribed in the same samples as above after 20 cycles of amplification.

Immunodetection of Ole e 1 in Mature Pollen Sections from Low- and High-Content Cultivars

After immunolocalization using an anti-Ole e 1 monoclonal antibody, noticeable gold labeling was detected in the cytoplasm of the vegetative cell in all cultivars analyzed (fig. 2A–D). The vegetative nucleus, organelles such as mitochondria and plastids, the cell wall and the generative cell were devoid of gold particles. Negative controls did not show significant labeling over the background (fig. 2E). After measuring the average gold density resulting from labeling (gold particles/μm^2), significant quantitative differences were observed when cultivars with high (Picual) and low (Arbequina, Frantoio and Gordal) allergen content were compared (fig. 2F).

RT-PCR Analysis of Ole e 1 Transcripts in Olive Cultivars

Levels of Ole e 1 transcripts were analyzed by RT-PCR (fig. 3A) using total RNA extracted from mature pollen of each cultivar. Equal loading of the samples was ensured by the comparable intensity of bands resulting from RT-PCR amplification of ubiquitin monomers to polyubiquitin pentamers in equivalent samples (fig. 3B), as ubiquitin mRNAs display steady-state levels in a number of plant species [26]. A band of 612 bp appeared in those cultivars showing high levels of Ole e 1 allergen. The band was distinguishable in all cultivars when the number of PCR amplification cycles was raised to 40, although undesirable saturation effects were observed for most cultivars (results not shown).

SPTs to Olive Pollen in Allergic Patients

Figure 4 shows a box plot view of the wheal sizes induced by the pollen extracts from ten olive cultivars and the controls in 30 patients allergic to olive pollen. Friedman ANOVA analysis demonstrated the presence of significant differences (ANOVA $\chi^2_9 = 21.81$, $p < 0.009$) in the average reactivity, depending on the cultivar considered (fig. 4). Such variations were significantly correlated with the relative amount of Ole e 1 protein ($r_s = 0.72$, $t_8 = 2.94$, $p = 0.019$), suggesting that the affected skin area depends upon the levels of the allergen Ole e 1 present in the pollen grain.

After detailed study of individual reactions for all patients, we observed some remarkable facts. Figure 5 shows the reactivity of five allergic patients to olive pollen extracts from different cultivars. These patients displayed significantly lower reactivity to the commercial extract than to some extracts in particular (fig. 5A). Moreover, two patients subjected to immunotherapy who did not present any response to the commercial extract showed significant remaining reactivity to some of the cultivars analyzed (fig. 5B).

Discussion

The presence of quantitative intercultivar differences with respect to the olive pollen major allergen Ole e 1, as demonstrated in this paper, suggests the possibility of the pollen from some olive cultivars being more allergenic than others and involves a number of implications for (1) the study of the nature and the biological function of this protein within the pollen grain, (2) the development of clinical diagnostic procedures regarding the determination of patient atopy and immunotherapy and (3) some remarkable environmental implications.

The diverse studies carried out to date in order to characterize Ole e 1 in the pollen of olive and other Oleaceae have shown a high degree of quantitative variability for this allergen within the total protein content of crude extracts [25, 27–30]. These nonhomogeneous results are caused by the use of very different protein extraction procedures and by the use of pollen from commercial sources,

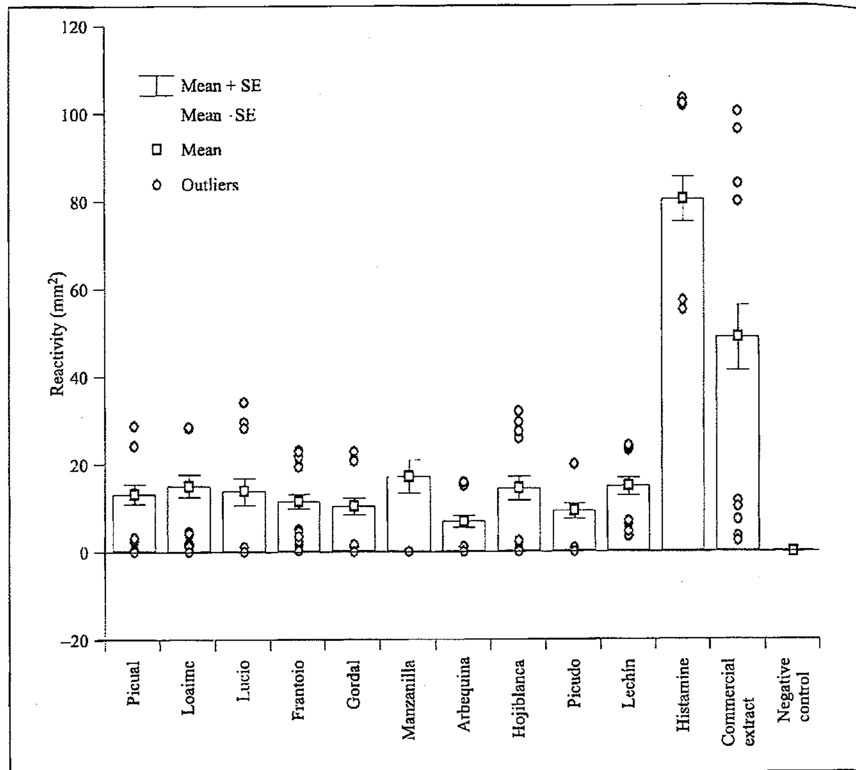

Fig. 4. Box plot view of the wheal areas (in mm^2) induced by extracts prepared from mature pollen of the cultivars analyzed, a commercially available extract, hydrochloric histamine (positive control) and a negative control (buffer) in 30 allergic patients sensitized to *O. europaea* pollen.

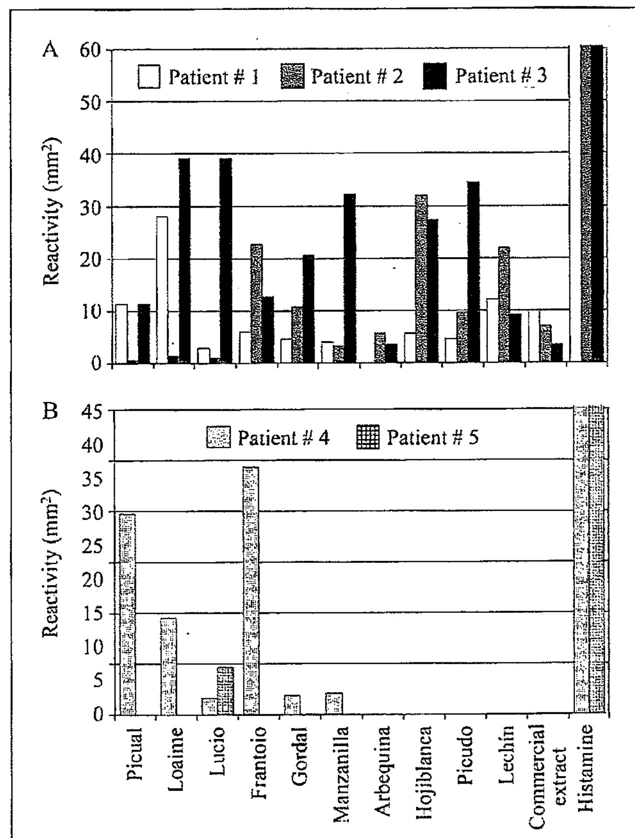

Fig. 5. Individual reactivity of some patients to SPTs. **A** Patients not subjected to vaccination. **B** Patients subjected to prolonged immunotherapy.

corresponding to undetermined cultivars. However, the use of the same protein extraction method and a well-characterized antibody, as shown here, has allowed us to demonstrate the presence of conspicuous differences among the cultivars used. In all the cases reported in this work, and in a pioneer study [21], the protein was present in significant amounts, clearly underlining that Ole e 1 probably plays an essential biological role within the pollen grain.

Although the biological function of Ole e 1 is unknown, some important clues are available. The reported amino acid sequence of the olive pollen major allergen [7] displays substantial identity with the deduced amino acid sequences of the genes *LAT52* from tomato [31] and *Zmc13* from maize [32]. It seems clear for LAT52 that this protein plays an essential role in pollen hydration and/or germination [33], as reduced expression of LAT52 protein induced by antisense repression correlated with abnormal pollen function. Recent evidence obtained by our group suggested that Ole e 1 is involved in germination and/or pollen tube growth rather than in pollen hydration [34]. So far, no detailed studies have been carried out regarding differential pollen hydration in olive cultivars, although pollen from most cultivars seems to perform satisfactorily in commercial orchards. However, more information is clearly needed concerning the role of Ole e 1 in pollen-pistil interaction processes. Striking differences in the allergenic protein content of pollen from *Betula pendula* trees have been described by Hjelmroos et al. [35], depending on the orientation of the branches used to collect such pollen (south-facing branches contain the greatest proportion of allergenic components). Although such differences were not initially detected in olive pollen, a more detailed study discriminating the orientation of the branches, the allergenic content for individual trees of each cultivar and even the correlation between rainfall and allergenic content could be employed to obtain additional data concerning the biological function of allergens and to refine strategies for collecting pollen for allergen extract manufacture.

The ultrastructural localization of Ole e 1 within the pollen grain was similar for the four cultivars used for this purpose, and matched the localization previously reported for this protein [36, 37]. However, the differential labeling densities were in good agreement with the results found after the biochemical analyses. No major ultrastructural differences were found between pollen grains of high and low Ole e 1 content.

Results of RT-PCR analysis of Ole e 1 expression indicate a relatively good correlation between the presence of Ole e 1 mRNAs and the protein itself as detected by the antibody. Slight divergences (i.e. relative intensity of bands in cultivars Hojiblanca, Picudo and Lechín) may be due to differential specificity of the antibody for Ole e 1 in these cultivars, or perhaps to very small differences in loading/setting or time course of the immunoblot and, particularly, the PCR reaction, which could enhance even very slight differences. These results support the presence of a transcriptional mechanism controlling such expression, as suggested during pollen maturation in the cultivar Picual [36, 37].

The presence of quantitative intercultivar differences in the olive pollen major allergen also indicates that qualitative differences are likely to be present among cultivars. Ole e 1 amino acid and nucleotide sequence heterogeneities have already been reported in olive pollen [7, 8]. Preliminary analyses of Ole e 1 DNA sequences obtained by RT-PCR in our laboratory [unpubl. data] have shown that sequence mismatches (some of them also resulting in amino acid changes) are much more frequent between cDNAs from different cultivars than between different cDNAs obtained from the same cultivar. The good correlation observed in this paper between the densitometric data of optical density for 18- to 20-kD polypeptides (mainly Ole e 1 allergen variants) in stained gels and immunoblots suggests that the monoclonal antibody used was probably unable to detect such minor differences in the amino acid sequence among cultivars. However, even small differences in the protein sequence may lead to dramatic changes in the allergenic capacity of this protein.

The present study confirms that patients display different SPT responses when tested with pollen from different cultivar origins. Statistical analysis provides evidence that the variations in the Ole e 1 content in the mature pollen among cultivars are responsible for the different average reactivity exhibited by allergic patients depending on the cultivar considered. However, our study cannot exclude an important role for other allergens other than Ole e 1 in the reactivity of patients. Substantial differences (16–66%) in the percentage of positive skin reactions to olive pollen extracts have also been reported among atopic patients depending on the olive cultivar assayed [38]. In that study, the authors used patient sera, instead of well-defined, specific antibodies to isolated allergens, as was the case for Ole e 1 in the present work. The major advantage of using patient sera is the possibility of evaluating the total allergenic charge of the pollen grain for each variety, although the individual content for each allergen can only be determined using specific antibodies. In future works, we intend to extend the present work to the study

of the allergenic composition of olive cultivars by using antibodies to those allergens showing high prevalence. Preliminary studies carried out by our group using antibodies to Ole e 3 [39] and Ole e 6 [unpubl. results] allergens have shown very minor differences in the expression of both allergens among the pollen of six olive cultivars. However, these studies have yet to be extended to many other cultivars and to most of the well-characterized pollen allergens in order to determine the involvement of so-called secondary allergens in the development of allergy.

The commercial extract used in this work performed better for most patients than the experimental extracts prepared from defined cultivars, as described in figure 4. This can be explained by the higher concentration of total protein present in this extract, its higher relative Ole e 1 content (as reported here) and by its optimization in terms of purity and uniformity.

However, detailed analysis of the response of individual patients, such as those shown in figure 5A and B, emphasizes two important facts: (1) the reactivity of approximately 10% of the patients was, unexpectedly, higher in response to a number of cultivar-defined extracts than to the commercial extract itself, and (2) 10% of the patients subjected to vaccination maintained a persistent reactivity to some of the cultivars analyzed.

Both facts could be explained by the presence of specific epitopes in Ole e I and/or other allergens among the extracts prepared from pollen of specific olive cultivars, which were slightly different (as discussed above) or were even absent from one cultivar to the next, and therefore from the commercial extracts used for diagnosis and vaccines. These differences may or may not be equally recognized by all patients. This suggests that the use of the currently available commercial pollen mixtures may lead to mistakes in allergy diagnosis and also to limited success in immunotherapy at least for a relatively significant proportion of the allergic population. Therefore, this preliminary study illustrates the need to take into account the intraspecific differences in the allergenic content of pollen in order to standardize the extracts used for clinical diagnosis of allergy as well as for the preparation of vaccines. To confirm this point, a larger number of both patients and cultivars should be assayed. Also, a putative linkage between patient origin and the prevalence of allergy to local cultivars deserves closer attention. The use of purified allergens from the main cultivars of the area where the patient lives (and therefore is most likely exposed to), or even recombinant proteins, is strongly recommended.

Finally, our data suggest the possibility of using low-allergenic cultivars as the predominant cultivated variety for new plantations (or at least for ornamental purposes) in densely populated regions, in order to lower the prevalence of sensitization among the population.

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