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Original Article

Immunogold Probes for Light and Electron Microscopic Localization of Ole e I in Several *Oleaceae* Pollens[1]

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We investigated the immunolocalization of the olive major allergen Ole e I and Ole e I-like proteins in pollen from several *Oleaceae* species [olive (*Olea europaea*), ash (*Fraxinus excelsior*), privet (*Ligustrum vulgaris*), lilac (*Syringa vulgare*), and forsythia (*Forsythia suspensa*)]. Crossreactions among different pollens were found in enzyme immunoassays. For immunolocalization with light microscopy we used the silver enhancement technique with three monoclonal antibodies (1D8, 10H1, and 16G2) that recognize three different epitopes of the allergen Ole e I. Our findings show that the silver enhancement technique is very useful when several antibodies are to be used for rapid screening of different materials. MAb 10H1 gave the most precise results and was selected for further immunolocalization studies with transmission electron microscopy. The epitope recognized by this MAb was localized exclusively in the endoplasmic reticulum in olive pollen. In lilac, privet, and ash pollen, most of the reactivity was also seen in the endoplasmic reticulum; however, the 10H1 epitope was not detected in forsythia pollen. (*J Histochem Cytochem 44:151–158, 1996*)

KEY WORDS: Ole e I; Pollen; *Oleaceae*; Immunocytochemistry; Silver enhancement; Immunoelectron microscopy; Endoplasmic reticulum.

Introduction

A number of allergens have been described and isolated from different pollens. The techniques developed by Köhler and Milstein (1975) have made it possible to obtain monoclonal antibodies (MAbs) that recognize many such allergens. MAbs have been used to map epitopes, compare IgE binding determinants, and immunolocalize different proteins (Tovey and Baldo, 1988).

Recently, four groups of MAbs were identified that react with four epitopes of Ole e I, the major allergen of olive pollen (Martín-Orozco et al., 1994). Enzyme-linked immunosorbent assay (ELISA) and immunodetection (IDT) have shown that these MAbs recognize epitopes homologous to Ole e I in proteins from the pollen of other species of the *Oleaceae* family, such as ash (*Fraxinus excelsior*), privet (*Ligustrum vulgaris*), lilac (*Syringa vulgare*), and forsythia (*Forsythia suspensa*) (Martín-Orozco et al., 1994). The crossreactivity among pollens from all species except forsythia is believed to originate from the Ole e I-like proteins described by Obispo et al. (1993) and Batanero et al. (1994).

Because pollen is a major cause of allergic reactions in humans, it is important to know the intracellular localization of its allergens. Earlier studies with light microscopy (LM) and transmission electron microscopy (TEM) have reported the localization of allergens in pollen from ragweed, ryegrass, mugwort, birch, alder, Japanese cedar, and hazel, among other allergenic species (Avjioglu et al., 1994; Grote et al., 1994; Miki-Hirosige et al., 1994; Kos et al., 1993; Vrtala et al., 1993; Singh et al., 1991; Staff et al., 1990).

Classical methods for localizing allergens in pollen with the help of LM are based on immunofluorescence techniques (Takahashi et al., 1989; Vithanage et al., 1982; Howlett et al., 1981; Knox et al., 1970; Belin and Rowley, 1971). Because these techniques can mask the presence of antigens in the pollen cell wall owing to autofluorescence (Knox et al., 1970), we used immunogold-silver enhancement, which has the advantages of indirect immunolabeling originally developed for TEM and makes it possible to visualize gold particles by light microscopy due to the formation of a silver precipitate (Danscher and Norgaard, 1983). In this study we used a polyclonal antibody as well as three MAbs (1D8, 10H1, and 16G2) that recognize three different epitopes of Ole e I. Our results with this rapid, sensitive, and permanent method showed that MAb 10H1

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was the most useful reagent in subsequent immunolocalization studies with TEM. We found that the Ole e I protein in olive pollen, as well as the Ole e I-like proteins in ash, privet, and lilac pollen, were localized with different degrees of intensity in the endoplasmic reticulum.

Materials and Methods

We studied commercially obtained mature pollen grains (Allergon; Välinge, Sweden) from five species of *Oleaceae*: olive (*Olea europaea*), ash (*Fraxinus excelsior*), privet (*Ligustrum vulgaris*), lilac (*Syringa vulgare*), and forsythia (*Forsythia suspensa*).

Polyclonal Antibody. New Zealand rabbits were immunized weekly over 4 weeks using 100 μg of *Olea* crude extract in complete Freund's adjuvant. After this period, hyperimmunization was performed by injecting 50 μg of soluble antigen IV.

Monoclonal Antibodies. The anti-Ole e I MAb 1D8, 10H1, and 16G2 were obtained by the method of Köhler and Milstein (1976), with slight modifications as reported by Martín-Orozco et al. (1994).

Sample Preparation for Light and Transmission Electron Microscopy. Pollen grains were fixed for both LM and TEM in a mixture of 3% (v:v) glutaraldehyde and 4% (w:v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 hr at room temperature (RT). They were then washed in the same buffer, dehydrated in a graded ethanol series, and embedded in Epon (Epon, Epikote 812). Semithin sections for LM or ultrathin sections for TEM were cut on a Reichert Ultracut E microtome.

Immunocytochemical Protocol for Light Microscopy. Semithin sections were incubated with the different MAbs (undiluted) for 1 hr at 37°C in a humid chamber. After several washes in PBS (123 mM sodium chloride, 10 mM potassium phosphate, pH 7.3), the sections were treated with 1:30 goat antimouse IgG or IgM conjugated to 5-nm colloidal gold particles (BioCell; Cardiff, UK) for 45 min at RT. They were then washed in PBS and distilled water, in that order. The silver enhancement technique was used in accordance with the manufacturer's instructions (BioCell): 1 volume of initiator was mixed with 1 volume of enhancer; the mixture was applied and the reaction monitored under LM for 10–15 min. The reaction was stopped by washing the slide in distilled water, after which the sections were air-dried and mounted in DePex. The sections were examined and photographed with a Zeiss Axioplan microscope fitted with an automatic camera. For control, semithin sections of mature pollen grains were processed as described above, except that incubations with MAbs were omitted.

Immunocytochemical Protocol for Transmission Electron Microscopy. Ultrathin sections of pollen from each species were picked up on gold or nickel grids and treated as described above for LM material, except that the primary MAbs were either an anti-Ole e I polyclonal antibody diluted 1:500 in PBS with 1% bovine serum albumin (BSA), or MAb 10H1 diluted 1:20 in PBS. After incubation with the second antibody the sections were contrasted with 5% uranyl acetate for 10 min in the dark and examined with a Zeiss 10C transmission electron microscope. As controls, PBS or an irrelevant MAb (anti-lambda light chain of human immunoglobulin, Lambda-Coulter Clone) were substituted for the primary antibody. The sections were otherwise processed as described above.

Enzyme Immunoassay and Immunodetection. The procedures for enzyme immunoassay (EIA) and immunodetection (IDT) were as described previously (Martín-Orozco et al., 1994).

Results

Table 1 summarizes the results of EIA, IDT (Martinez-Orozco et al., 1994), and immunolocalization with LM. Reactivities of each of the five *Oleaceae* pollens to MAbs 1D8, 16G2, and 10H1 are shown. The epitope recognized by 16G2 was detected in all species, whereas the one recognized by 10H1 was not found in forsythia pollen. The findings with MAb 1D8 varied depending on the technique used. With LM immunolocalization the epitope appeared in all species, with EIA it was recognized only in olive and ash, and with IDT it was not detected in any of the pollens studied here.

Immunolocalization with Light Microscopy

The reactions detected by MAb 1D8, 10H1, and 16G2 were spread throughout the pollen cytoplasm; no precipitate was seen in the cell wall or apertures (Figures 1–7). The intensity of reactivity of

Table 1. *Reactivity of different* Oleaceae *pollens to three anti-Ole e I monoclonal antibodies (1D8, 10H1, and 16G2)*[a]

	MAb 1D8			MAb 10H1			MAb 16G2		
	EIA	IDT	ILM	EIA	IDT	ILM	EIA	IDT	ILM
O	+	−	+	+	+	+	+	+	+
Fr	+	−	+	+	+	+	+	+	+
L	−	−	+	+	+	+	+	+	+
S	−	−	+	+	+	+	+	+	+
Fo	−	−	+	−	−	−	+	+	+

[a] O, olive; Fr, ash; L, privet; S, lilac; Fo, forsythia pollen; EIA, immunoassay; IDT, immunodetection; ILM, immunodetection with light microscopy.

Figure 2. Localization of Ole e I in lilac pollen with MAb 10H1. The cytoplasm was intensely and homogeneously positive, with no clear areas. Some grains were not labeled (star). Original magnification × 400. Bar = 10 μm.

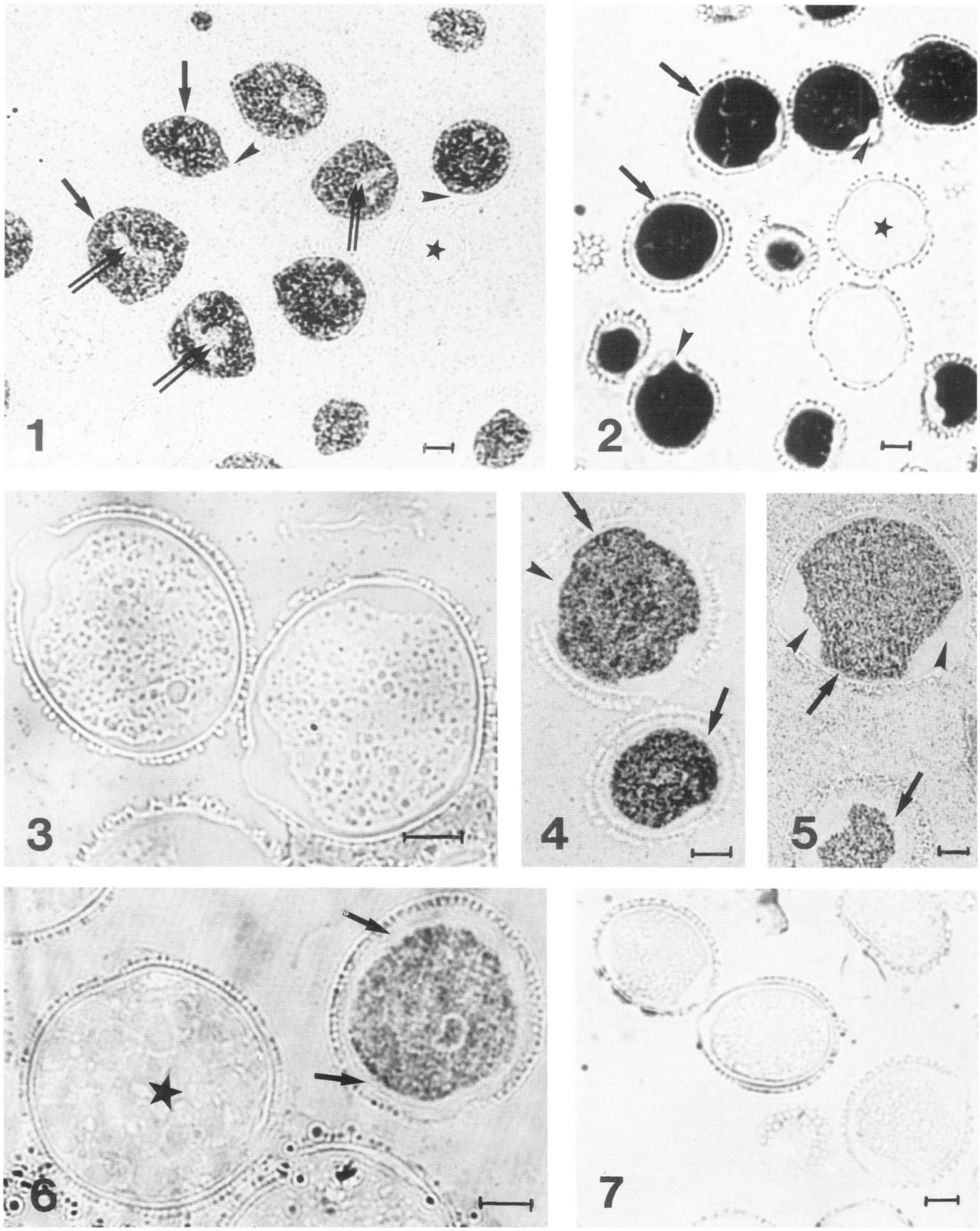
Figure 3. Absence of immunolabeling for Ole e I in forsythia pollen with MAb 10H1. Original magnification × 900. Bar = 10 μm.

Figure 4. Localization of Ole e I in olive pollen with MAb 1D8. The pollen grains were homogeneously labeled with immunoprecipitate. Original magnification × 640. Bar = 10 μm.

Figure 5. Localization of Ole e I in forsythia pollen with MAb 1D8. The immunoprecipitate was seen throughout the pollen grain, but not in the wall. Original magnification × 500. Bar = 10 μm.

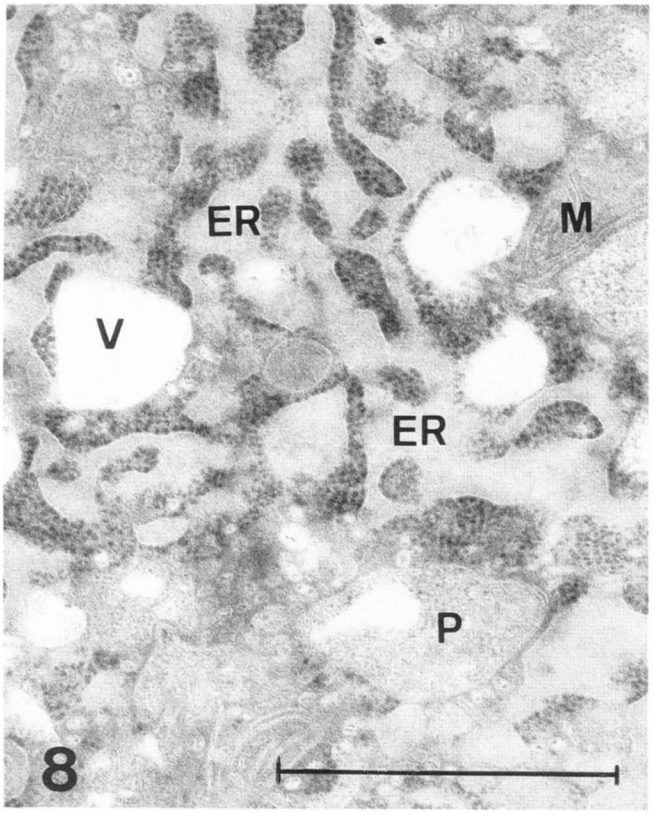
Figure 6. Localization of Ole e I in ash pollen with MAb 16G2. Silver precipitate was seen throughout the pollen grain. Some grains were immunonegative (star). Original magnification × 900. Bar = 10 μm.

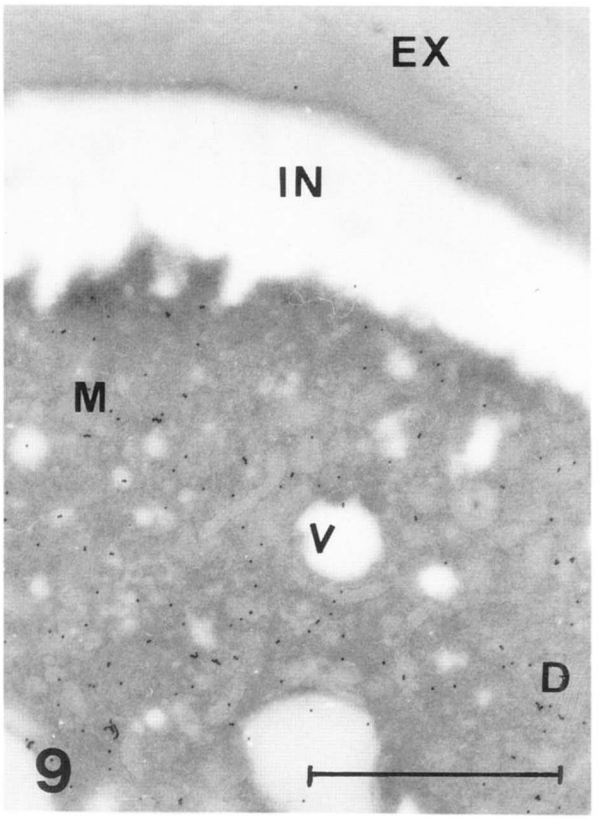
Figure 7. Control olive pollen grains not incubated with any primary antibody did not show silver labeling. Original magnification × 500. Bar = 10 μm.

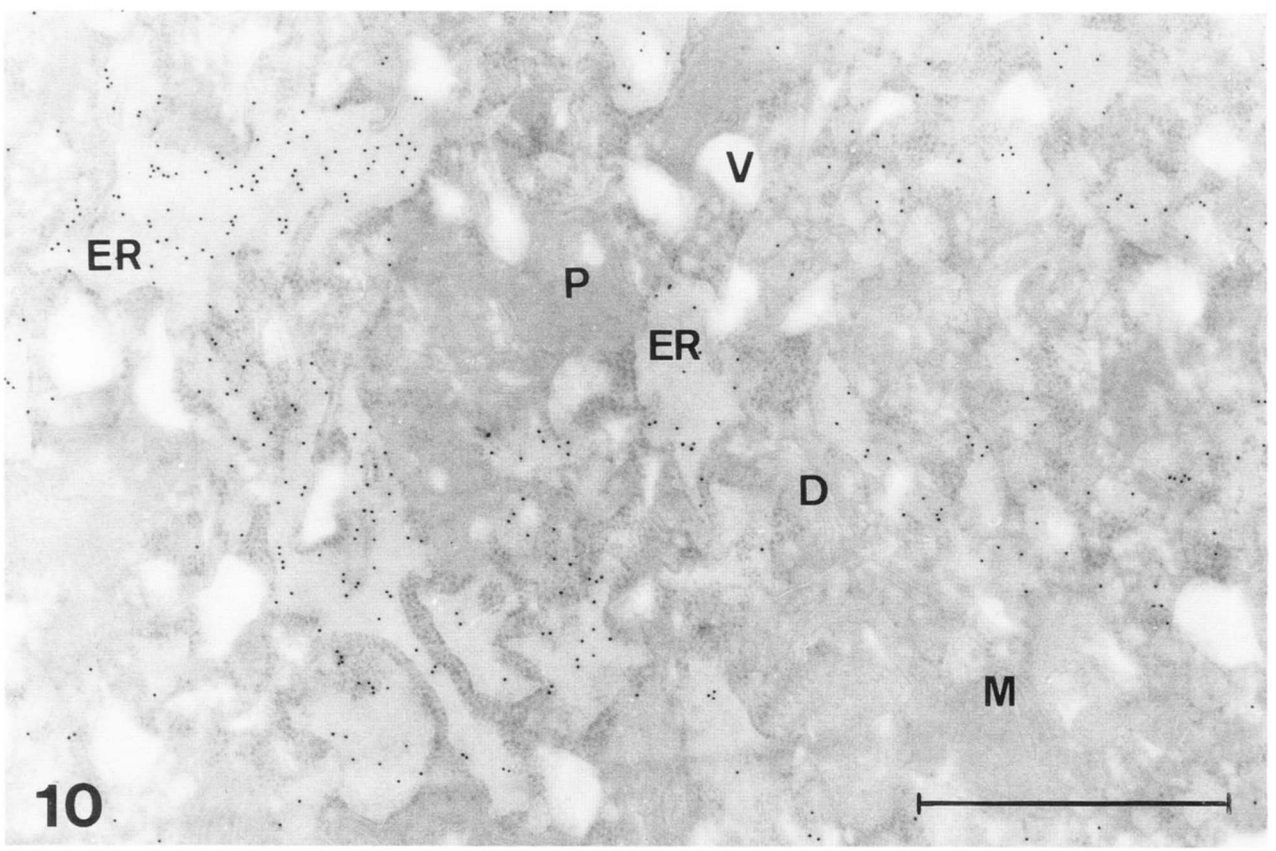


Figures 1–7. Photomicrographs showing immunolocalization of Ole e I in *Oleaceae* pollens. In all species, the pollen wall (arrows) and apertures (arrowheads) were free of silver precipitate.

Figure 1. Localization of Ole e I in olive pollen with MAb 10H1. The silver precipitate was visible throughout most of the cytoplasm in the pollen grain, sparing only the generative cell and the vegetative nucleus (double arrows). Some pollen grains were not labeled (star). Original magnification × 400. Bar = 10 μm.







Figures 8–10. Electron micrographs showing immunolocalization of Ole e I in olive pollen.

Figure 8. Control pollen not incubated with the primary antibody. The cytoplasm was free of precipitate. Cisternae of the endoplasmic reticulum (ER) were dilated and fused. Original magnification × 43,000. Bar = 1 μm.

these antibodies varied from species to species (Figures 1–6). In all pollen except forsythia, the most intense signal after silver enhancement was provided by MAb 10H1. No positivite signal was seen in forsythia with this MAb (Figure 3). Incubation with 1D8 or 16G2 led to positive reactions in all species (Figure 4–6). Although olive pollens were positive with all three antibodies (Figures 1 and 4), it is interesting to note that immunolabeling with 10H1 delimited clear areas in the cytoplasm, where the reaction was absent (Figure 1). In all preparations showing a positive reaction, some apparently normal pollen grains from each species failed to develop the silver precipitate (Figures 1, 2, and 6). When no reaction was visible, longer exposures to the silver enhancement mixture increased the background, but not the reaction product, in these grains. This was also found in control pollen in which incubation with the first antibody was omitted (Figure 7).

Immunolocalization with Transmission Electron Microscopy

In olive pollen, many cisternae of the rough ER were dilated and irregular in outline. Several cisternae were fused, forming complex networks of saccules (Figures 8–10).

Immunolocalization with a rabbit polyclonal antibody against *Olea* pollen allergens in olive pollen showed abundant, scattered labeling throughout the cytoplasm. The cell walls and apertures were not labeled (Figure 9).

After incubation with MAb 10H1, gold particles in olive pollen grains were found only in the ER cisternae (Figure 10). No gold particles were seen in any other organelle or structure. In privet, immunoprecipitation with MAb 10H1 in the ER was similar to that seen in olive pollen, but fewer gold particles were observed (Figure 11). In lilac (Figure 12) and ash (Figure 13), the rough ER vesicles appeared less dilated and were labeled with fewer gold particles than in olive. Control pollen grains incubated without the primary antibody showed no immunolabeling (Figure 8).

Discussion

This study reports the first application of immunogold silver enhancement technique (Danscher and Norgaard, 1983) to localize allergens in pollen by light microscopy. This technique may prove useful in studies that involve several antibodies in different materials. Compared with immunofluorescence, the procedure we used not only obviates the use of cryosections or hydrophilic resins (which can be replaced with Epon when the protein of interest is thermostable) but also avoids the use of fluorochromes. Previous work has shown that autofluorescence of the pollen wall can obscure the localization of antigens present in this structure when immunofluorescence procedures are used (Knox et al., 1970). Another advantage of silver enhancement is that the labeling is permanent. With immunofluorescence, in contrast, antifading chemicals are not entirely able to keep the signal from disappearing. In the present study, we were able to use the same secondary MAbs for both LM and TEM observations. The entire procedure is fast and very sensitive.

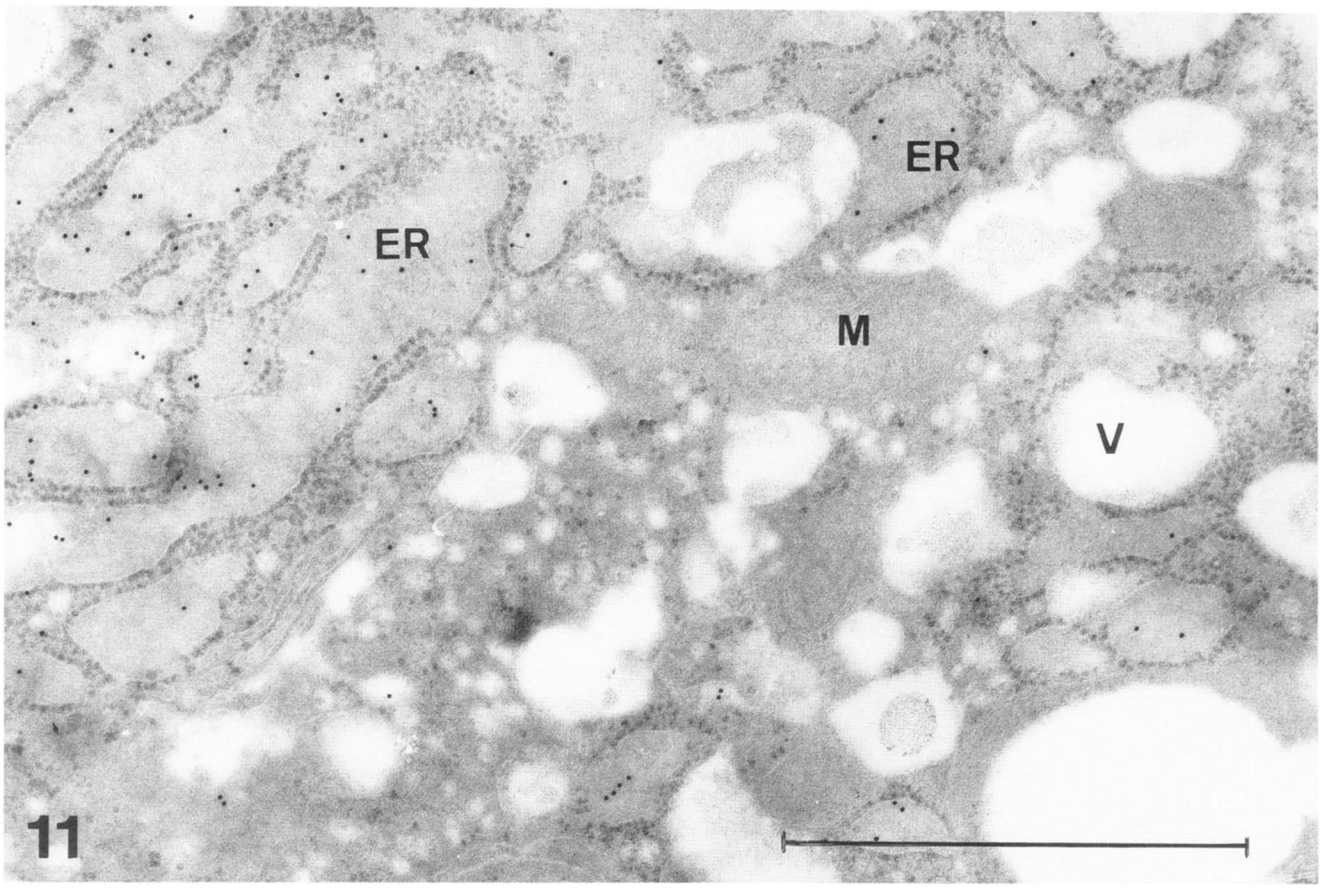
Proteins similar to Ole e I have previously been described in the species of *Oleaceae* we studied, except for forsythia (Batanero et al., 1994; Martín–Orozco et al., 1994; Obispo et al., 1993; Kenerman et al., 1992; Bousquet et al., 1985). The three MAbs used here (1D8, 10H1, and 16G2) recognize different epitopes of Ole e I and also compete to some degree with human IgE. However, only the epitope recognized by 10H1 is considered to be a significant allergenic determinant (Martín–Orozco et al., 1994).

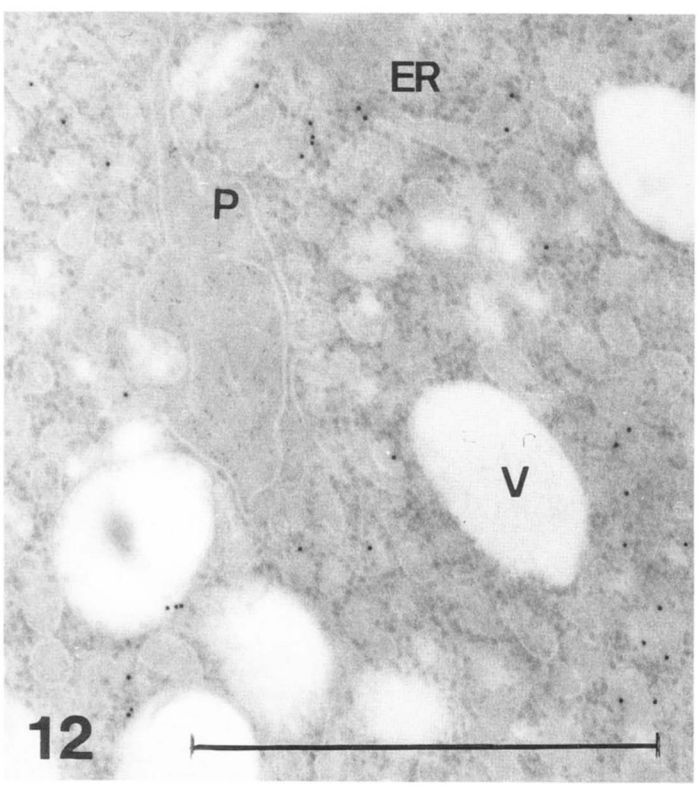
The only MAb that gave variable results depending on the processing technique used was 1D8 (Table 1). The differences between the results with EIA and IDT may have been due to the use of denaturing conditions in the latter procedure, which may have altered the epitopes recognized by 1D8 (Martín–Orozco et al., 1994). The results of immunolocalization with LM, showed that the epitope recognized by 1D8 was present in all species tested here. This finding may reflect the extreme sensitivity of immunolocalization with silver enhancement, a technique able to detect minute amounts of epitope that may be available on the surface of the section. The absence of reactivity with MAb 10H1 in sections of forsythia pollen suggests that proteins bearing this particular epitope are absent in this species, in accordance with EIA and IDT results (Martín–Orozco et al., 1994).

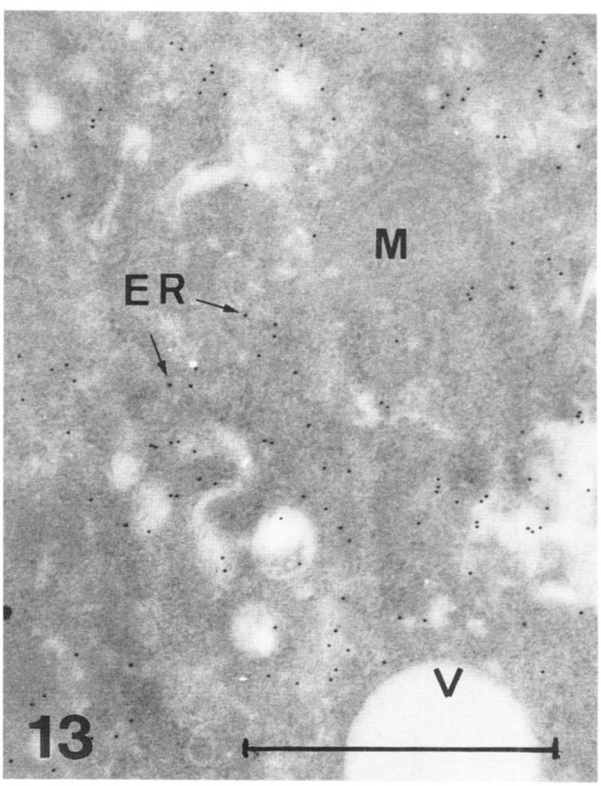
Earlier studies of allergens in pollen from other families of plants have shown these molecules to be low molecular weight, highly water-soluble polypeptides. To obtain a signal strong enough to be detected by immunolocalization, other authors have used anhydrous fixation techniques (Grote, 1991,1992; Grote et al., 1994). Although the Ole e I protein of olive pollen is also a low molecular weight, highly water-soluble molecule (Villalba et al., 1993), we were able to detect this allergen after aqueous fixation followed by incubation with an MAb (Rodríguez–García et al., 1995; Martín–Orozco et al., 1994). This result may reflect the abundance of Ole e I in olive pollen or may mean that it is retained in the ER by specific retention signals (Jackson et al., 1990). We found different epitopes of the allergen in the pollen cytoplasm, but none was detected in the pollen grain wall of any species with the polyclonal antibody or with any of the MAbs used here. The clear areas seen by LM in the cytoplasm after immunolocalization with MAb 10H1 were found only in olive pollen. Because of their size, shape, and location, these regions appear to correspond to the generative cell and vegetative nucleus, an assumption that was confirmed by immunolocalization studies with TEM (Rodríguez–García et al., 1995; Martín–Orozco et al., 1994). This finding may signify that the epi-

Figure 9. Immunolocalization with a rabbit polyclonal antibodiy against *Olea* pollen allergens. Both the intine (IN) and the exine (EX) of the pollen cell wall and vacuoles (V) were free of gold particles, but scattered labeling was evident throughout the rest of the cytoplasm. D, dictyosomes; M, mitochondrion. Original magnification × 32,000. Bar = 1 μm.

Figure 10. Immunolocalization with MAb 10H1. The gold particles were localized specifically in the markedly dilated cisterna of the endoplasmic reticulum (ER). Plastids (P), vacuoles (V), mitochondrion (M), and dictyosomes (D) lacked immunogold labeling. Original magnification × 41,000. Bar = 1 μm.







Figures 11–13. Electron micrographs showing immunolocalization of Ole e I with MAb 10H1 in different species of *Oleaceae*.

Figure 11. In privet, as in olive, the Ole e I epitope was also localized in dilated, fused cisternae of endoplasmic reticulum (ER), although fewer particles were visible. Mitochondrion (M) and vacuoles (V) were practically free of immunolabeling. Original magnification × 60,000. Bar = 1 μm.

tope recognized by MAb 10H1 is present only in proteins of the olive pollen cytoplasm. In lilac, privet, and ash this MAb may also have detected other determinants in proteins present not only in the cytoplasm but also in the vegetative nucleus and generative cell. The MAbs 1D8 and 16G2 also recognized proteins in these areas in all species tested here (Figures 4–6). This nuclear labeling, likewise observed in rye grass pollen (Staff et al., 1990) and birch pollen (Grote 1991), may reflect the nonspecific attraction of the gold probe to nuclear material (Staff et al., 1990). Alternatively, nuclear labeling may be considered evidence of free diffusion of the small allergen molecule into the nucleus via the nuclear pores, as suggested by Grote (1991). However, the diffusion hypothesis cannot be supported by our results. After treatment with the same antibody and under the same experimental conditions for all species studied here, we found no label in the nuclei of any olive pollen grains.

We encountered nonreactive pollen grains in the samples of all five species (Figures 1, 2, and 6), a finding that may reflect the loss of viability in some grains during storage. In commercially obtained olive pollen, we confirmed the immunolocalization of Ole e I in the ER reported for fresh pollen collected from trees (Rodríguez–García et al., 1995). In privet, ash, and lilac, Ole e I-like proteins are also concentrated in the ER, and are sometimes found in close association with the ER membranes. These results suggest that the ER is the site of storage or synthesis (or both) of these proteins.

The fact that immunolocalization in ER was more evident in olive pollen than in the other species of *Oleaceae* suggests that the epitope of the Ole e I molecule recognized by MAb 10H1 was more accessible in olive pollen than were the Ole e I-like proteins detected in other species. Ole e I-like proteins may be less abundant or more widely dispersed in the pollen grain than Ole e I itself.

In summary, MAb 10H1 was found to give the best results with our immunolocalization procedure for the detection of Ole e I, the major allergen of olive pollen. The epitope detected by this antibody was localized predominantly in the ER of olive; weaker reactivities were found in pollen from lilac, privet, and ash, and no reactivity was detected in forsythia pollen.

Acknowledgments

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Figure 12. In lilac, small, rounded vesicles of endoplasmic reticulum (ER) showed moderate immunolabeling. P, plastid; V, vacuole. Original magnification × 60,000. Bar = 1 μm.

Figure 13. In ash, small vesicles of the endoplasmic reticulum (ER) showed slight immunolabeling. V, vacuole; M, mitochondrion. Original magnification × 40,000. Bar = 1 μm.

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