

Brief Report

Immunogold Electron Microscopic Localization of Timothy Grass (*Phleum pratense*) Pollen Major Allergens Phl p I and Phl p V After Anhydrous Fixation in Acrolein Vapor

MONIKA GROTE,¹ CHRISTIANE DOLECEK, RONALD VAN REE,
and RUDOLF VALENTA

Institute of Medical Physics and Biophysics, Münster University, Münster, Germany (MG), Institute of General and Experimental Pathology, University of Vienna, Vienna, Austria (CD,RV), and Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands (RvR).

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We used the vapor phase of acrolein as an anhydrous fixative for timothy grass pollen in an immunogold double-labeling localization study of two different major allergens, Phl p I and Phl p V. More than 48 hr of fixation were needed for the subcellular pollen structures to be satisfactorily stabilized. The immunoreactivity of acrolein-fixed pollen allergens was not destroyed even after prolonged acrolein fixation. By im-

munoblotting, the two allergens differ in their immunological and structural characteristics. Electron microscopic localization traced the allergens at least partially to different subcellular pollen compartments. (*J Histochem Cytochem* 42:427-431, 1994)

KEY WORDS: Timothy grass pollen; Allergens; Phl p I; Phl p V; Immunogold double labeling; Acrolein vapor fixation.

Introduction

Pollen allergens are low molecular weight (~10–30 kD) proteins or glycoproteins which are characterized by extremely high water solubility. Electron microscopic (EM) localization of these allergens in the dehydrated pollen grain requires strictly anhydrous fixation and preparation conditions. Otherwise, in conventional aqueous fixation fluids, rehydration of the dry pollen and diffusion of the allergenic proteins out of the pollen grain take place before the allergens are sufficiently fixed and immobilized for coupling to the antibodies. Therefore, Staff et al. (9) and Singh et al. (8) fixed rye grass pollen in anhydrous glutaraldehyde, whereas Grote solved the problem of immobilizing soluble Bet v I allergen in birch pollen by exposing the pollen grains for several days to *p*-formaldehyde vapor (3).

In the present investigation we wanted to localize by immunoelectron microscopy two allergens of timothy grass pollen. The clinical importance of these allergens is demonstrated by the fact that group I allergens are recognized by 95% and group V allergens by 80% of all patients sensitive to grass pollen allergens (14). This means that a representative localization of the most important grass pollen allergens had to be carried out. In the first instance, the localization of timothy pollen allergens was attempted by one of the hitherto established techniques. However, it proved impossi-

ble to stabilize the ultrastructure in these pollen grains by *p*-formaldehyde vapor fixation, regardless of the duration of exposure. Slightly improved but still unsatisfactory were the results after fixation in anhydrous glutaraldehyde vapor. Encouraged by literature reports on the survival of enzyme activity after fixation in acrolein (e.g., 7), we decided to test this aldehyde fixative, which was first introduced into biological EM by Luft (6) but is rarely used in modern biological EM. In immunoblotting experiments, we characterized the allergenic proteins and the antibodies used: Phl p I, which is homologous to group I allergens of other grass pollen species, and Phl p V, which is homologous to Poa p IX in Kentucky bluegrass and Lol p Ib in rye grass (8).

Materials and Methods

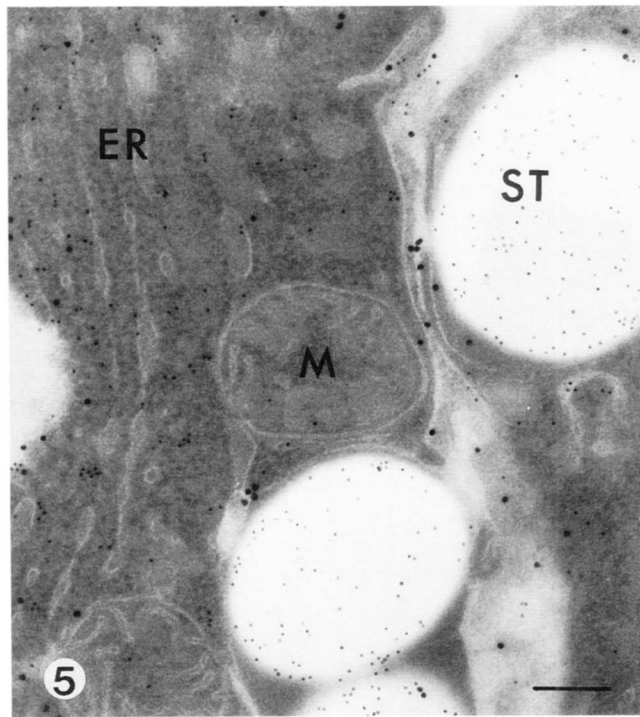
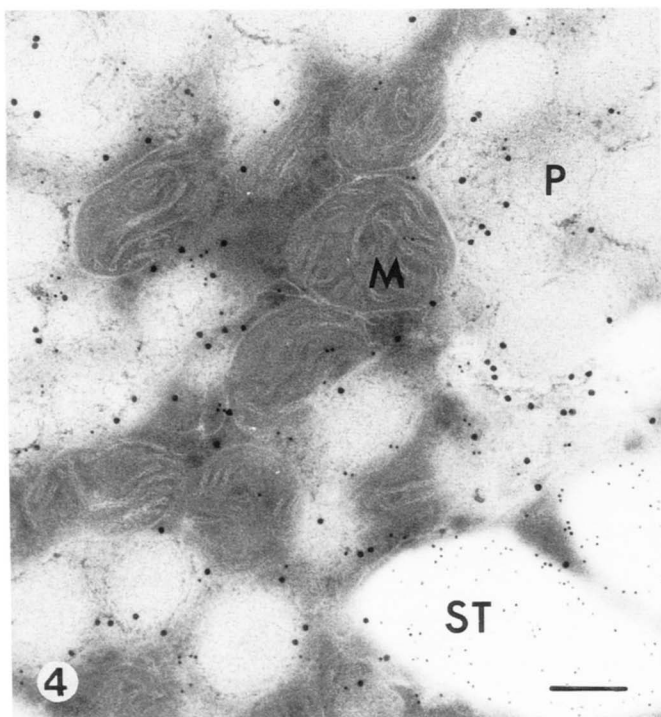
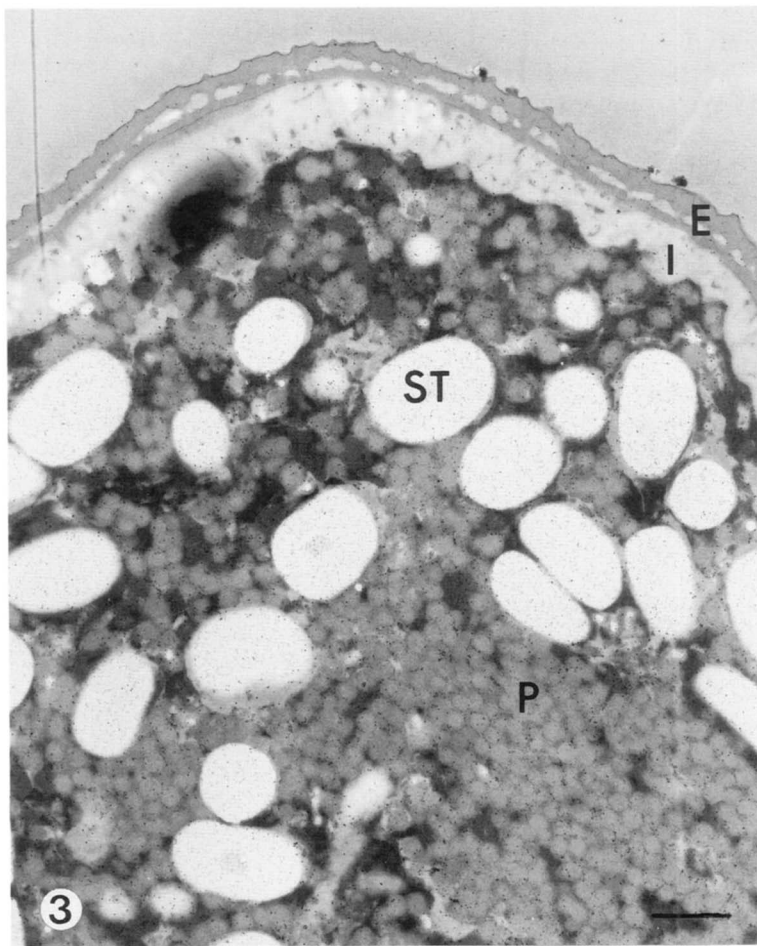
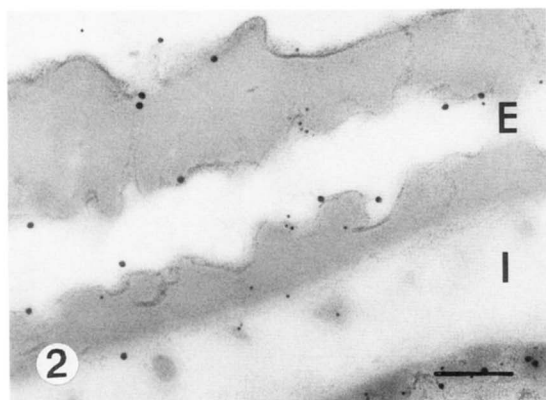
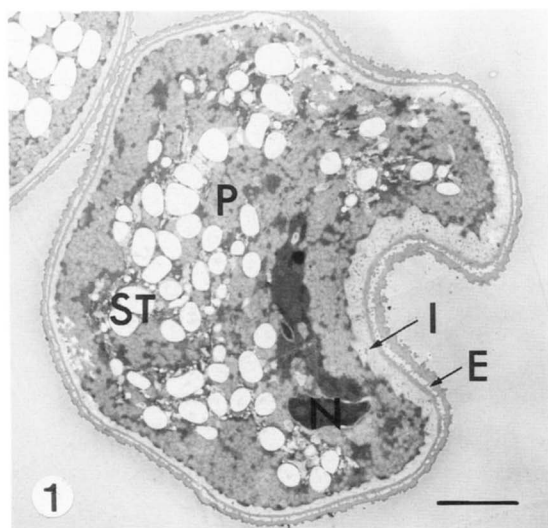
Pollen Material

Pollen of *Phleum pratense* (L) was obtained from Allergon AB (Engelholm, Sweden) and stored in a refrigerator at 8°C until use.

Fixation and Embedding

Pollen grains were dusted onto glass slides to form a "mono-pollen layer" and placed in a desiccator together with a petri dish filled with acrolein liquid (Riedel-de-Haen; Seelze, Germany). The desiccator was tightly sealed and placed in a fume hood at ambient temperature. Pollen samples were taken after 4, 8, 24, 48 hr, and 7 days of fixation. They were dehydrated in dimethoxypropane (DMP) and 100% ethanol for 4 hr each and then

¹ Correspondence to: Dr. Monika Grote, Inst. of Medical Physics and Biophysics, Münster University, Hüfferstr. 68, D-48149 Münster, Germany.



carried through mixtures of ethanol and Lowicryl K4M resin: 2:1 (overnight), 1:1 (8 hr), 1:2 (overnight) at room temperature (RT). They were then transferred into pure Lowicryl resin, where they stayed for 48 hr with two changes of the resin at RT. UV polymerization was carried out at -35°C after the samples had been placed in fresh resin and slowly (over 4 hr) cooled down to -35°C before polymerization started.

Antibodies

Primary Antibodies. For immunoelectron microscopy and immunoblotting, a rabbit antibody raised against Lol p I (10) and a mouse monoclonal antibody (MAb) 4B1 which binds to group V grass pollen allergens (10,11) were used. Immunoblotted timothy grass pollen extracts were also probed with a rabbit anti-recombinant birch profilin antiserum crossreacting with profilin from timothy grass, which was obtained by immunizing rabbits with purified recombinant birch profilin (12) and with an anti-chicken gizzard actin MAb (Chemikon; Temecula, CA). Actin and profilin were included because in previous investigations (13) they were found to be common constituents of pollen extracts. They were used as positive controls to test the validity of the techniques applied. Negative controls were performed with a normal rabbit serum and mouse antibodies without specificity for grass pollen allergens. The antibodies used for EM were purified by protein G affinity chromatography using MAb traps from Pharmacia (Uppsala, Sweden). All antibodies were tested for reactivity with well-characterized recombinant tree and grass pollen allergens (1,12–15).

Secondary Antibodies. Goat anti-mouse IgG and goat anti-rabbit IgG, coupled to colloidal gold particles of different sizes (10 and 20 nm), were obtained from BioCell (Plano; Marburg, Germany).

Immunocytochemistry

Double Labeling for Group I and V allergens. Ultra-thin pollen sections placed on nickel grids were incubated on drops of the following solutions (in a moist chamber, at RT, PBS buffer pH 7.4, TRIS-BSA buffer, pH 8.2): (a) 5% BSA in PBS, 15 min; (b) mouse anti-group V allergen antibody, 1:100 in PBS, 2 hr; (c) PBS, 5 min, Tris-BSA twice for 5 min; (d) goat anti-mouse IgG antibody coupled to 10-nm gold particles, 1:20 in Tris-BSA 1 hr; (e) Tris-BSA 5 min, PBS, twice for 5 min; (f) rabbit anti-group I allergen 1:30 in PBS, 2 hr; (g) PBS, 5 min, Tris-BSA, twice for 5 min; (h) goat anti-rabbit IgG antibodies coupled to 20-nm gold particles 1:100 in Tris-BSA, 1 hr; and (i) Tris-BSA 5 min, distilled water twice for 5 min.

Controls. The following controls were run: (a) replacement of primary antibodies by MAb without specificity for grass pollen allergens and normal rabbit antibodies; (b) double-labeling in reverse order; (c) omission of primary antibodies; and (d) single labeling of each allergen.

Electron Microscopy and Documentation

After immunolabeling, sections were stained in uranyl acetate (6 min) and lead citrate (10 sec). They were analyzed in a Hitachi H 500 transmission EM operated at 75 kV. Photographs were taken with Agfa-Gevaert 23D56 film.

Pollen Protein Extraction, Electrophoresis, and Immunoblotting

Proteins from timothy grass pollen were separated by SDS-PAGE and blotted to nitrocellulose. Strips were incubated (Figure 7) with a mouse anti-chicken gizzard actin antibody (Lane 1), a mouse anti-group V antibody (Lane 2), a rabbit anti-group I antiserum (Lane 3), a rabbit anti-profilin antiserum (Lane 4), a normal rabbit serum (Lane 5), and a mouse MAb without specificity for grass pollen proteins (Lane 6). Lanes 7 and 8 are the buffer controls without addition of primary antibodies (mouse antibodies and rabbit sera, respectively). (For details of the extraction and blotting procedures, see 13.)

Results

Morphology of Timothy Pollen Grains After Acrolein Vapor Fixation

Ultrastructural preservation of the pollen grains was unsatisfactory after 4 and 8 hr of fixation. This was judged from the fact that no section of pollen grain cytoplasm could be obtained. Instead, only the exine was preserved surrounding the empty space where the pollen grain had been. After 24 hr, some pollen grains were sectionable but they revealed a completely unsatisfactory preservation of cell organelles. Most of the pollen grains were sectionable after 48 hr of fixation, but ultrastructural morphology was still poor. After 7 days of acrolein fixation all of the pollen grains were sectionable, with about 75% showing satisfactory preservation of their ultrastructure (Figures 1–6).

At low magnification the dehydrated state of the pollen was clearly recognized from the shrunken appearance of the grains (Figure 1). The pollen grain was surrounded by a two-layered wall, exine and intine (Figures 1–3). Accumulations of amyloplasts (starch grains) and polysaccharide inclusions [P-particles according to Heslop-Harrison (5)] filled most of the interior of the pollen grain. The many electron-opaque, irregularly formed spots between these reserve materials consisted of cytoplasmic substance (Figures 1, 3,

Figures 1–5. Ultra-thin sections of timothy grass pollen grains after fixation in acrolein vapor and double immunogold labeling for Group I allergen (20-nm gold particles) and Group V allergen (10-nm gold particles). E, exine; ER, endoplasmic reticulum; I, intine; M, mitochondrion; P, P-particle (polysaccharide particle); ST, starch.

Figure 1. Cross-section of a whole timothy grass pollen grain at low magnification. Original magnification $\times 2100$. Bar = 5 μm .

Figure 2. Detail of the pollen wall showing exine and intine. Original magnification $\times 36,000$. Bar = 0.2 μm .

Figure 3. Part of the pollen grain showing wall and cytoplasm at medium magnification. Original magnification $\times 10,000$. Bar = 1 μm .

Figure 4. Detail of pollen cytoplasm showing mitochondria, P-particles, and amyloplasts (starch). Original magnification $\times 36,000$. Bar = 0.2 μm .

Figure 5. Detail of pollen cytoplasm showing rough endoplasmic reticulum, mitochondria, and amyloplasts (starch). Original magnification $\times 36,000$. Bar = 0.2 μm .

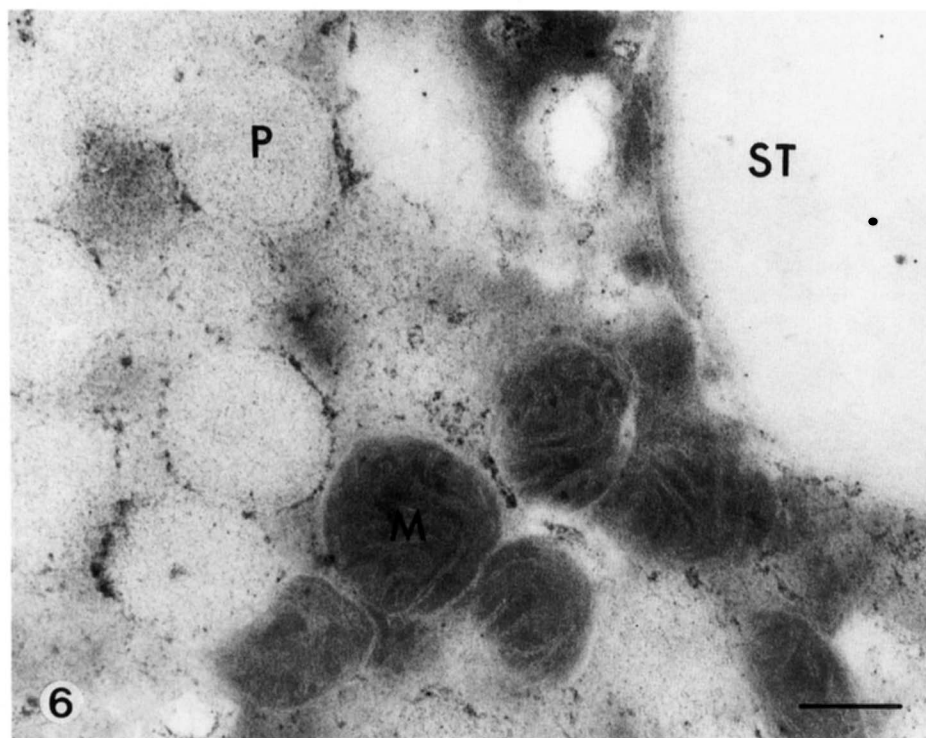


Figure 6. Ultra-thin section of timothy grass pollen grain after fixation in acrolein vapor: control experiment. After replacement of the specific primary antibodies by control antibodies, a low degree of nonspecific staining is observed. M, mitochondrion; P, P-particle; ST, starch. Original magnification $\times 36,000$. Bar = $0.2 \mu\text{m}$.

and 4). Sometimes nuclei of the tricellular pollen grain were visible (Figure 1). At higher magnifications, the good preservation of pollen cytoplasm became evident, with all membranes appearing in negative contrast: large numbers of mitochondria interspersed between the large masses of polysaccharide particles (Figure 4), dictyosomes and, although rather rare, strands of rough endoplasmic reticulum (Figure 5).

Immunocytochemical Labeling

After double immunogold labeling of timothy grass pollen grains fixed for 7 days in acrolein vapor, Group I allergen (large gold particles) was found in the cytoplasmic matrix between the P-particles and cell organelles such as mitochondria. Amyloplasts (starch grains) were not labeled (Figures 3–5). Group I allergen was also found on exine and intine sites (Figure 2).

Group V allergen (small gold particles) was predominantly located in densely labeled amyloplasts (Figures 3–5). It was also detected, together with Group I allergen, in matrix sites between P-particles (Figure 4) and other cell organelles (Figures 4 and 5), and on exine and intine locations (Figure 2).

After replacing the primary anti-Group V MAb by an MAb of irrelevant specificity and the polyclonal rabbit anti-Group I antibody by normal rabbit antibodies (Figure 6), few gold particles were observed in the pollen grains indicating a low degree of nonspecific staining. This was also the case with all other types of control experiments (not shown).

Reactivity of Antibodies with Timothy Grass Pollen Proteins

As shown in Figure 7, a positive reaction was observed in Lane 2 (anti-Group V allergen antibody) and Lane 3 (anti-Group I allergen antibody). The two other pollen proteins, actin and profilin, which were included for comparison (positive control), were also

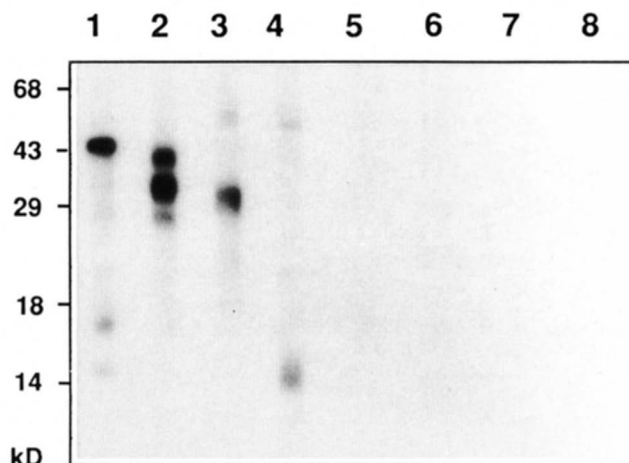


Figure 7. Immunoblot experiment showing the reactivity of antibodies with several timothy grass pollen proteins. See Materials and Methods for explanations.

clearly identified (Lanes 1 and 4). The controls (Lanes 5–8) did not show any reactivity.

Discussion

The use of acrolein as a fixative for morphological purposes in biological EM is recommended only in cases in which glutaraldehyde or *p*-formaldehyde has proven unsatisfactory. Large, dense, or impermeable specimens are reported to be better fixed by acrolein than by glutaraldehyde owing to the faster penetration rate and extreme reactivity of the former (4). However, acrolein, a monoaldehyde, gives less satisfactory morphological preservation than the dialdehyde glutaraldehyde because of a minor degree of cross-linking. This and the unpleasant circumstances of handling acrolein in the laboratory (high volatility, acrid odor, lachrymatory effect, toxicity) have removed this reagent from the list of commonly used aldehyde fixatives in modern labs. The results of the present investigations, however, show that the vapor phase of this fixative can stabilize specimens well and at the same time preserve the antigenicity of proteins for immunocytochemical procedures. The high volatility and reactivity of the fluid, which surely favors this process, have already attracted other investigators in their studies of anhydrous fixation to include acrolein in their considerations. Thus, Frederik et al. (2) tested acrolein vapor in addition to several other inorganic and organic vapor fixatives for preservation of freeze-dried cryosections. It is evident that the findings of our study do not constitute a recommendation for use of acrolein instead of glutaraldehyde or paraformaldehyde in normal immunocytochemical work. However for difficult tissues that do not work with the latter fixatives, the use of acrolein should be considered, not only for morphological preservation but also for preservation of antigenicity.

As shown in the present study, grass pollen allergens seem to belong to a group of proteins whose antigenic conformation can hardly be destroyed by the action of fixatives. This was already observed in the case of allergens from another grass species, *Lolium perenne* (8,9). Some data have been collected in our laboratory concerning possible enzymic or enzyme inhibitory properties of timothy grass pollen allergens (Valenta and co-workers, unpublished observation). Since protein- and carbohydrate-degrading enzymes are comparatively resistant to structural alterations, we speculate that the common biological function may be a characteristic that makes grass pollen allergens comparatively insensitive to harsh fixation conditions. For the subcellular localization of Group I and V allergens in timothy grass pollen, which was first demonstrated in the present study, our results are supported by Staff et al. (8) and Singh et al. (9), who localized homologous allergenic proteins in rye grass pollen in similar pollen compartments.

Acknowledgment

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