

Is the surface layer from hazelnut pollen, which is precipitated by Cuprolinic blue, an effective antigen in hay-fever patients?

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Summary. In electron micrographs it could be shown that hazelnut (*Corylus avellana*) pollen grains are covered on their surface by a diffusible 10 nm thick lamellar layer. On pollen surface as well as in pollen extract this layer could be precipitated and stained by the polycationic dye Cuprolinic blue. By subsequent application of both immunogold labeling with serum from a hay-fever patient allergic to tree pollen grains and histochemical detection with Cuprolinic blue this pollen surface layer proved to be an effective antigen.

Introduction

Hay-fever is a widespread immunodisease of the respiratory tract. It is induced by an individual hypersensitivity of the immunsystem against pollen grains suspended in the air. Diffusion of antigenic substrate from the pollen occurs by contact with the mucous membranes of the respiratory tract (Vithanage et al. 1982; for review see: Knox et al. 1980). This process rapidly induces the symptoms of the so-called hay-fever. Sera of hay-fever patients are therefore characterized by high titers of IgE as well as IgG antibodies in different amounts for one or more pollen grain types.

During electron microscopic preparation procedures the diffusibility of antigens however represents a severe problem in ultra- and immunocytochemical processing of pollen material. The present results show that the detection of pollen antigens in a morphologically distinct structure on the pollen surface could be achieved now with the aid of the colloidal gold technique and by incubation of the specimens with the polycationic dye Cuprolinic blue (Scott 1980; for review see: Roth 1983; De Mey 1983; Romano and Romano 1984).

Materials and methods

Pollen grains (100 mg) from hazelnut (*Corylus avellana*) were suspended in 1 ml phosphate buffered solution (pH 7.2) for 2 min. For immunocytochemical incubation 100 ml serum containing 50 µg protein derived from a tree pollen allergic patient was added to the suspension. This serum was tested before by RIA as well as by ELISA. IgE and IgG antibodies specific for hazelnut pollen

could be detected then. The immunocytochemical incubation of the pollen suspension was continued for 10 min at room temperature. Without intermediate washing a protein-A/gold marker was added to the suspension for another 10 min ((50 µl, containing 12 nm gold particles, prepared according to the method of Frens (1973), enriched by centrifugation, 17 µg protein A per ml, purchased from Sigma (F.R.G.), 5% bovine serum albumin, and 0.025 M HEPES buffer, pH 7.2)). Protein A is known to bind to human IgG but also to a lower extent to IgE.

After short centrifugation (7,000 g) the pellet consists predominantly of pollen grains and the supernatant of diffusible pollen surface material. Both, the pellet and the extract were diluted in 5 ml veronalacetat buffer pH 5.6 containing 0.2 M MgCl₂, 1% Cuprolinic blue (BDH Chemicals, U.K.) and 2.5% glutaraldehyde. After a 30 min incubation in this fixative both samples were washed several times, enriched between each step by centrifugation (7,000 g – 13,000 g), stained en bloc with 0.5% Na₂WO₄, and dehydrated in a graded series of ethanol.

The specimens were embedded in epoxy resin (Spurr 1969). Ultrathin sections, some of them additionally stained with uranyl acetate and lead citrate, were examined in a Philips EM 300 electron microscope equipped with goniometer stage.

The control experiments were performed by the same procedures with serum from a patient not allergic to pollen grains. Corresponding tests by RIA for IgE and by ELISA for IgG did not detect antibodies against hazel pollen.

Results

Electron micrographs of hazelnut pollen grains stained with Cuprolinic blue followed by Na₂WO₄ revealed a 10 nm thick lamellar layer covering the pollen surface (Figs. 1 and 3). Between this layer and the exine of the pollen grain granular material is found which extends into microchannels traversing the exine (Figs. 1–3). The observed staining pattern indicates that the Cuprolinic blue sensitive material of the bacular cavities inside the exine and the granular material outside communicate with one another.

In aqueous solutions as given by the preparation procedures for electron microscopy only a few patches of the lamellar layer remain attached to the pollen surface (Figs. 1, 3, 4, 5). Most of the layer material detaches from the pollen grains and is found after centrifugation in the supernatant as highly folded sheets.

Incubation of both the pollen grains and the diffusible layer material with serum derived from a hay-fever patient

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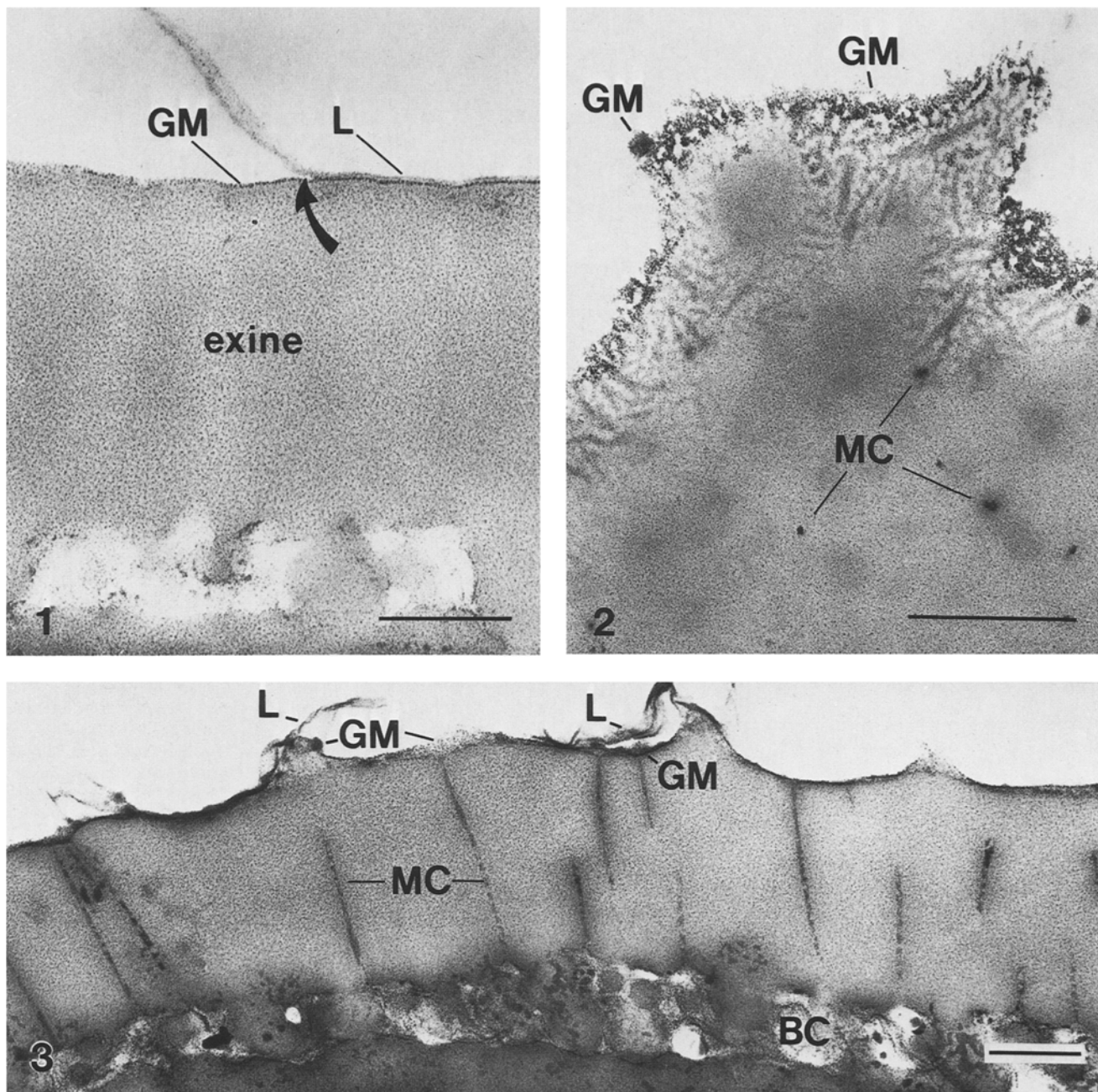


Fig. 1. Detail of the exine of the hazelnut pollen grain covered with a granular material (GM) and with a 10 nm thick lamellar layer (L). Under aqueous conditions this layer detaches from the surface (arrow). Bar: 0.2 μ m

Fig. 2. Tangentially sectioned pollen surface covered with granular material (GM). It remained attached to the exine. The exine is traversed by microchannels (MC) which have been cross-sectioned in this specimen. Bar: 0.5 μ m

Fig. 3. Detail of the pollen grain surface covered with the lamellar layer (L). The bacular cavities (BC) inside the exine communicate with the granular material (GM) outside by microchannels (MC). Bar: 0.2 μ m

reveals a selective binding of antibodies to the surface layer as shown by the protein-A/gold marker (Fig. 4). Whereas the bulk of antigenic material is enriched in the diffusible surface layer only those pollen surface areas still showing the layer are also decorated with the protein-A/gold marker. The granular material underlying the surface layer is completely devoid of label.

For control experiments serum from a non-allergic patient was used. In these experiments the protein-A/gold labeling is negligible indicating that no or only a very few specific antibodies against the pollen surface layer are present in the serum of this patient (Fig. 5). The results show that exclusively in the hay-fever patient the diffusible pollen extract represents an effective antigen.



Fig. 4. Surface from pollen grain incubated with serum from a hay-fever patient and labeled with a protein-A/gold marker. The lamellar layer (*L*) detaching from the pollen surface is selectively stained with colloidal gold particles. This labeling indicates that hay-fever antigens on the pollen surface are exclusively concentrated in this layer. Staining of the ultrathin section with uranyl acetate and lead citrate was omitted. Therefore, this micrograph clearly shows selective staining of the layer (*L*) and of the content of the bacular cavities (*BC*) with Cuprolinic blue. Bar: 0.5 μ m



Fig. 5. Surface of a pollen grain incubated with serum derived from a patient not allergic against hazelnut pollen. No significant immunogold labeling is observed. Additional staining of the ultrathin section with uranyl acetate and lead citrate increased the intensity of the Cuprolinic blue staining (cp. Fig. 4). Bar: 0.5 μ m

Discussion

Immunoglobulins from a hay-fever patient specifically binding to antigenic substrate of pollen grain could be localized now with the aid of immunogold labeling and by the polycationic dye Cuprolinic blue. In hazelnut pollen grains used here this antigenic substrate is a constituent of or is represented even by the lamellar layer which covers the entire pollen surface. Previous investigations have shown that pollen surface material can be preserved for electron microscopic studies by staining with cationic dyes such as cetylpyridinium chloride (Grote et al. 1983) or with phosphotungstic acid (Gaude and Dumas 1984). In our experiments, we instead used Cuprolinic blue, which proved to be superior to other dyes for staining polyanionic components of biological specimens (Scott 1980; Scott et al. 1981; Reale et al. 1983; Völker et al. 1985). The selective staining of the diffusible pollen surface layer with Cuprolinic blue in a critical electrolyte concentration of 0.2 *M* MgCl₂ indicates that the layer is composed of polycarboxylated and/or polysulphated glycoproteins (Scott and Orford 1981; Grote and

Fromme 1984a). An implication of charged sugar residues is also discussed for an IgE-binding glycopeptide of *Parietaria judaica* pollen (Corbi and Carreira 1985). A high density of anionic residues indicates a strong hydrophilic quality of the layer (Howlett et al. 1981). Its diffusibility together with its large surface area must therefore effect in an intensive contact with mucous membranes. Its detachment seems to be mainly promoted by water soaking of the granular material. Not only the application of Cuprolinic blue on plant material but also the immunogold labeling with antibodies not derived from rabbit or other species (Grote and Fromme 1984b) but from an allergic human individual offer an interesting way for investigations on pollen allergens on the ultrastructural level.

Taken together, precipitation and staining with Cuprolinic blue revealed a diffusible structure on hazelnut pollen surface which is obviously very important in binding antibodies in human individuals allergic to tree pollen. The rapid detachment, the high density of antigenic substrate, and the large surface area of this lamellar pollen surface layer may explain why pollen grains by contact with mu-

cous membranes very rapidly induce hay-fever symptoms of the respiratory tract. Further immunolabeling experiments with the aid of the colloidal gold and the Cuprolinic blue technique have to be done now in order to get more information on common features of the site, the structure, the diffusibility, and the immunoglobulin specificity of these pollen antigens.

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