

Immunoelectron-microscopic localization of diffusible Birch-pollen antigens in ultrathin sections using the protein-A/gold technique

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Summary. Pollen grains from *Betula pendula* were fixed in a mixture of p-formaldehyde and cetylpyridinium chloride (CPC) for the precipitation of soluble pollen glycoproteins. After dehydration and embedding at low temperatures in the water-soluble resin, Lowicryl K4M, ultrathin sections of the pollen grains were incubated using specific antibodies against birch-pollen extract and protein-A/gold complexes. Antigen activity was found in the CPC-precipitated surface material and within the exine (bacular cavities) and the cytoplasm (except for starch grains and lipidic droplets). There was no labelling within the intine. The region of the germinal aperture also showed a very low degree of antigen activity. The control sections were almost completely free of background staining.

Introduction

Among scientists engaged in the study of pollen biology, histochemistry and allergy, it is well known that, in many pollen species including the highly allergenic ones, soluble proteins and glycoproteins rapidly diffuse from the pollen grains when they are brought into contact with a moist medium. Thus, in (immuno)cytochemical investigations using aqueous fixation fluids, there is a danger of losing the material which is needed for the reactions with cytochemical markers or specific antibodies.

This problem has long been recognized, and therefore most investigators have used anhydrous fixation conditions, usually cryotechniques, i.e. freeze sectioning (Knox and Heslop-Harrison 1970), freeze drying (Vithanage et al. 1980, 1982; Howlett et al. 1981) or fixation in absolute methanol (Vithanage et al. 1980). However, it should be borne in mind, that during the incubation necessary for cytochemical or immunocytochemical reactions, even samples prepared by totally anhydrous methods are brought into contact with aqueous fluids. In this context, the labelling of thick, plastic-embedded sections which, in turn, are re-embedded and cut into ultrathin sections for electron microscopy (Vithanage et al. 1980, 1982; Howlett et al. 1981) is certainly a step into the right direction; however, this method has the disadvantage of only labelling the cut surfaces of the samples, but not the deeper-lying, intra- or extracellular parts of the specimens.

Because of these difficulties, Grote et al. (1983) have returned to the method of aqueous fixation of pollen grains, but with the addition of precipitating agents. They have shown that, when the quaternary ammonium compound, cetylpyridinium chloride (CPC) is added to aqueous aldehyde fixation medium, it can prevent highly soluble proteins or glycoproteins from diffusing out of the pollen grains and thereby becoming inaccessible for cytochemical analysis and localization (Grote et al. 1983).

In view of the significant morphological alterations of the glycoproteins which could be expected from a precipitation using a cationic compound such as CPC (Reale et al. 1983), it seemed rather doubtful that sufficient cytochemical binding sites or the antigenicity of pollen proteins for immunocytochemical labelling would be retained. In an attempt to clarify this question, the cytochemical procedures of periodic-acid/thiocarbohydrazide/silver-protein (PA-TCH-SP) staining were combined with CPC-aldehyde fixation. It has been demonstrated that there are carbohydrates with vicinal glycol groups within the precipitated, CPC-reactive material coating pollen grains (Grote and Fromme 1984a). Using lectin labelling, individual sugars, such as glucose and mannose (Grote and Fromme 1984b) or *N*-acetylglucosamine (M. Grote and H.G. Fromme, unpublished results), have been identified in the precipitated material.

The present study deals with an attempt to elucidate whether the antigenicity of pollen samples is influenced by the precipitation method. In order to expose all of the intra- and extracellular compartments of the pollen grains to the labelling agent, we chose a post-embedding technique which involved the labelling of ultrathin sections with commercially available antibodies against birch-pollen extract.

Materials and methods

Fixation and embedding. Mature pollen was collected from the flowering catkins of *Betula pendula* trees in spring 1984 and stored until use in the freezing compartment of a household freezer at 250 K.

Frozen samples of the pollen grains were placed in 0.071 M cacodylate-buffered, freshly prepared p-formaldehyde, pH 7.4, containing 0.5% CPC (Serva, Heidelberg, FRG). The pollen grains were fixed overnight at 277 K and then washed for 1 h in cacodylate buffer. For dehydration and embedding in Lowicryl K4M (Carlemalm et al. 1982), we used a cooling chamber of our own design which operates in the temperature range of between 273 and 223 K. In short, the dehydration and embedding of the pollen

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grains were performed as follows: 30% ethanol for 60 min at 273 K; 50%, 70%, 96%, and $2 \times 100\%$ ethanol for 60 min at 253 K. There were three intermediate ethanol/Lowicryl mixtures (2:1, 1:1, 1:2). In each mixture, the samples were kept at 253 K for 6 h. There were three successive stages of degassed pure resin, in each of which the pollen grains were kept at 253 K for 24 h. Polymerization was achieved by ultraviolet irradiation (2×15 W; distance 25 cm; maximum of radiation at 360 nm wavelength) at 253 K for 48 h. During the impregnation and subsequent polymerization procedures, the polymerization-inhibiting oxygen was replaced by flooding the cooling chamber with nitrogen. The polymerized blocks were kept dry in a desiccator until sectioning.

The controls for CPC were carried out by fixing pollen in buffered p-formaldehyde as already described, but without the addition of CPC. Ultrathin sections of both types of specimen were cut on a Reichert-Om-U3 ultramicrotome and placed on pioloform-coated nickel grids.

Immunocytochemical reagents. Antibodies raised in rabbits against the pollen extract from *Betula verrucosa* were purchased from the Allergologisk Laboratorium (Copenhagen, Denmark). Normal rabbit IgG was obtained from Dynatech (Denkendorf, FRG). Protein-A from *Staphylococcus aureus* was supplied by Sigma (Munich, FRG). The protein-A/gold complex was prepared as described by Roth (1982), using trisodium citrate as the reducing reagent of the chloroauric acid. The mean diameter of the gold granules was about 15 nm.

Immunocytochemical labelling. The labelling was carried out according to Roth (1982), but with some modification of the blocking reaction; bovine serum albumin (BSA) was used instead of ovalbumin which was found to be less effective in blocking non-specific binding. The BSA concentration was raised to 5%, and the blocking time was extended to 10 min. The nickel grids with the sections face downwards were successively floated on drops of the solutions described below. All incubations were done at room temperature. The washing steps were performed by immersion and spraying with a plastic bottle.

The blocking reaction was performed using 5% BSA in PBS, pH 7.4, for 10 min. The antibody reaction was performed in a moist chamber for 2 h; the antibodies were diluted (1:30) in PBS, pH 7.4. The sections were then washed three times (5 min each) in PBS, pH 7.4. The protein-A/gold reaction was performed in a moist chamber for 1 h. The sections were again washed three times (10 min each) in PBS, pH 7.4, and once (5 min) in distilled water. Finally, the sections were stained with uranyl acetate for 3 min.

Electron microscopy. The sections were viewed and photographed with a Hitachi-H-500 transmission electron microscope operated at 75 kV using Agfa-Gevaert-23D56 films.

Controls. The following controls were performed: a) substitution of the specific antibodies by normal rabbit IgG, and b) incubation with the protein-A/gold complex alone.

Results

Reaction with cetylpyridinium chloride

After fixation in p-formaldehyde/CPC, the surface of the birch-pollen grains was covered with a distinct layer of amorphous material of medium electron density (Fig. 1a, b). In controls which had been fixed in p-formaldehyde alone, the surface of the grains was free of such deposits (Fig. 2a, b, showing the region of a germinal aperture).

Immunocytochemical labelling

After the labelling of ultrathin sections of birch-pollen grains with anti-birch-pollen-extract antibodies, gold markers were found in the CPC-reactive surface material covering the exine and within the exine itself. Here, we found a distinct accumulation of gold granules within or near the bacular cavities, whereas the remainder of the exine was only weakly labelled. There were no gold markers within the intine (Fig. 1a). Within the region of the germinal aperture (Fig. 2a), only few gold granules were found; these were mainly located in the inner part of the voluminous thickening of the intine. In the cytoplasm, a large number of gold granules was visible, but there was no labelling of most of the starch grains and lipidic droplets (Fig. 3a). The corresponding control experiments (Figs. 1b, 2b, 3b), in which incubation was carried out with normal rabbit IgG, showed a very low degree of background labelling. In the samples incubated with the protein-A/gold complex alone (micrographs not shown), even fewer gold granules were observed.

Discussion

The results of the present study clearly show that, even after the precipitation of glycoproteins with CPC, sufficient antigenic binding sites are available for the antigen/antibody reaction. In the case of allergenic pollen, this means that the highly soluble antigenic and allergenic proteins are retained, and a good ultrastructural preservation of the whole grain is achieved due to the use of aldehyde fixation. Furthermore this type of fixation could be combined with further cytochemical analysis of the localization of allergenic and antigenic compounds. Therefore, this kind of preparation must be considered to be a viable alternative to cryo-methods which often result in the poor preservation of cellular details. Moreover, during immunolabelling, even cryo-fixed and/or freeze-dried specimens are necessarily brought into contact with aqueous incubation or washing media for shorter or longer periods of time; this always means

Fig. 1a, b. Ultrathin sections of birch-pollen grains after embedding at low temperatures in Lowicryl K4M. Detail from the pollen wall after fixation with p-formaldehyde/CPC. The surface of the pollen grain is covered with electron-dense, precipitated material (pm). **a** After incubation with specific antibodies, most gold granules are associated with the CPC-precipitated material (pm) covering the exine (e), the bacular cavities (bc) within the exine and the cytoplasm (cy). There is no antibody binding in the intine (i). $\times 55,000$. **b** After incubation with normal rabbit IgG, almost no gold granules are visible on the CPC-precipitated material (pm), the exine (e) with bacular cavities (bc), the intine (i) or the cytoplasmic structures (cy). $\times 48,000$

Fig. 2a, b. Region of the germinal aperture after fixation with p-formaldehyde without the addition of CPC. There is no material precipitated on the surface of the pollen wall. **a** After incubation with specific antibodies, the exine (e) and cytoplasmic structures (cy) are heavily labelled. The outer layer of the intine (oi) shows no antibody activity, whereas the inner intine thickening (ii) exhibits a low degree of specific labelling. $\times 15,000$. **b** After incubation with normal rabbit IgG, the exine (e) and intine [outer layer (oi) and inner thickening (ii)] together with the cytoplasm (cy) show negligible background staining. $\times 15,000$

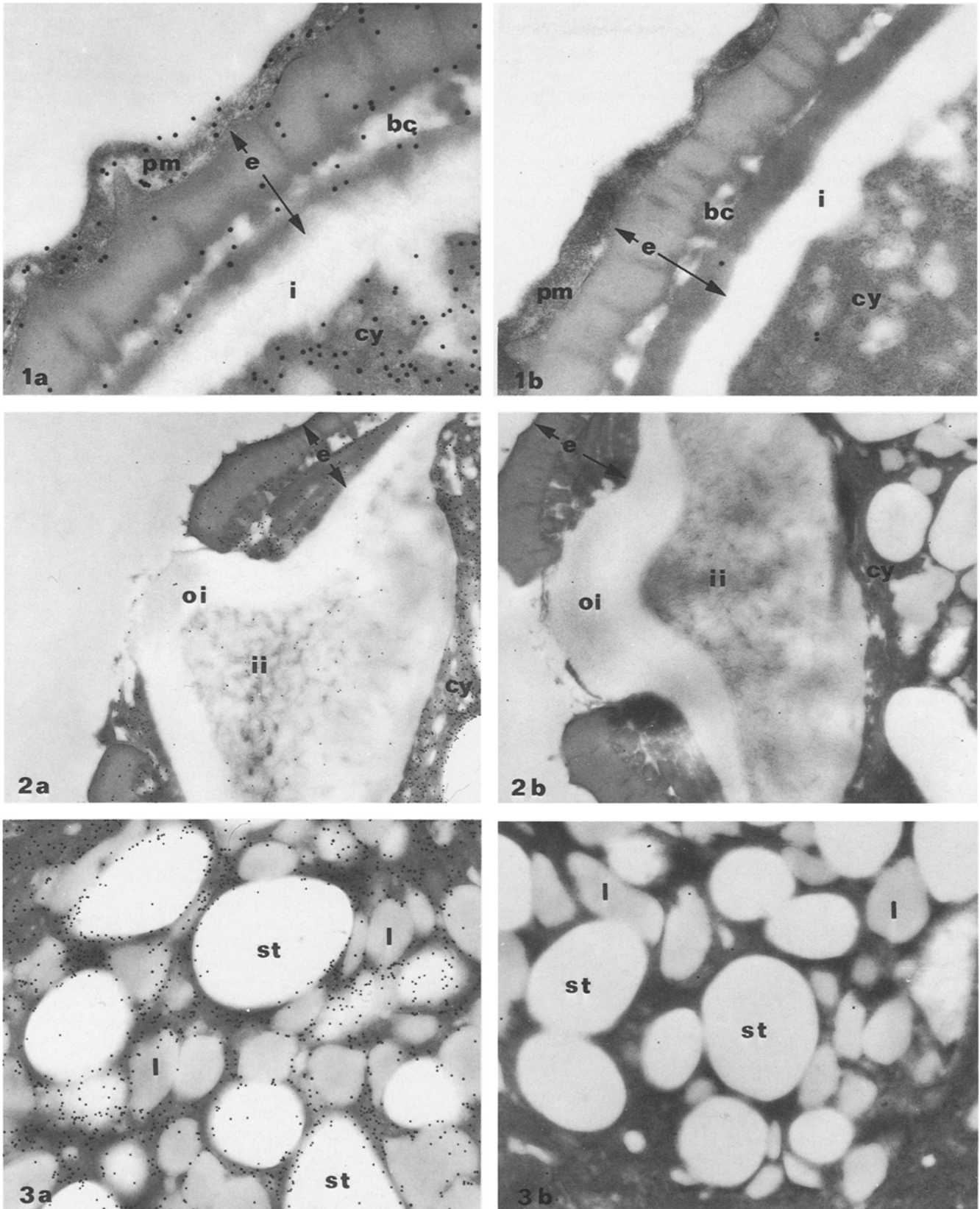


Fig. 3a, b. Detail from the cytoplasm after fixation with p-formaldehyde/CPC showing starch grains (*st*) and lipidic droplets (*l*). **a** After incubation with specific antibodies, heavy antibody binding occurs on the cytoplasm. Most starch grains and lipidic droplets show very few gold granules. $\times 24,000$. **b** After incubation with normal rabbit IgG, a very low degree of background staining is visible. $\times 24,000$

a danger of losing antigens. Furthermore, post-embedding immunostaining of ultrathin sections is the only way to expose all of the intracellular compartments to the labelling agent. In this context, it may be stated that the Lowicryl K4M embedding seems to be superior to conventional Epon embedding with regard to the preservation of antigenicity (M. Grote, unpublished results).

With regard to pollen immunocytochemistry, some findings of the present study are in good agreement with previous reports, while others are not. Early investigations using antibodies coupled with fluorescent dyes found that exine or intine or both wall layers contained the antigens. Thus, in birch-pollen, Belin and Rowley (1971) observed specific fluorescence surrounding the pollen grains. More detailed information has been given by Knox and Heslop-Harrison (1971) who localized an 'Antigen E' in *Ambrosia* spp. within the intine of the pollen grain. Howlett et al. (1973) detected diffusible immunoreactive proteins within the exine and intine of *Ambrosia* (localization of 'Antigen E') and *Cosmos*, especially at the germinal apertures. Knox and Heslop-Harrison (1971) have suggested that the intine is the main source of antigens in the pollen grains of *Phalaris tuberosa* L., and they found the main concentration of antigens in the thickened zone underlying the germination pore. Knox et al. (1980), Howlett et al. (1981) and Vithanage et al. (1980, 1982) localized 'Antigen A' and 'Group 1 allergen' in *Lolium perenne* pollen in the exine and intine layers, but also within some cytoplasmic sites of the pollen grain.

The results of the present study on birch-pollen grains confirm the presence of antigenic proteins both on and in the exine, especially in the bacular cavities, and in the cytoplasm; however, the almost total absence of labelling within the intine and the region of the germinal aperture is surprising. If one supposes that the method of fixation (CPC/p-formaldehyde) preserves the diffusible proteins in the birch-pollen grain and arrests them at their native sites, there would appear to be at least three explanations for the observed discrepancy:

1. There are – in contrast to other pollen species – no antigens within the intine of mature birch-pollen grains, and therefore, all of the antigens are released from the exine and cytoplasmic sites.

2. Assuming that there is no difference between birch-pollen and the pollen grains of other plant species, it might be concluded that the positive labelling of the intine was due to antigens from the cytoplasm which, after being moistened, are mobilized and (on their way out of the grain) then pass the intine at some time. This would mean that all previously reported results present a 'snapshot' of the dynamic process of antigen migration. Thus, most previous descriptions of antigens within the intine of the pollen grain or within both the cytoplasm and intine, could – perhaps due to a different preparation from that used in this study – show already mobilized antigens on their way from the cytoplasm through the intine and exine to the surface of the grain.

3. Last, but not least, one has to take into account that, as a result of differences in the composition of pollen extracts which, in turn, are due to different extraction conditions (extraction medium, extraction time, etc.), proteins

of different origins within the pollen grains have been used for immunization; this means that the antibodies which have been used in various studies are directed against antigens from different cellular sites. This may explain the variety of localization sites which have previously been reported.

Therefore, a standardization of pollen extracts seems to be important in all cases where instead of separated, chromatographically identified and isolated antigens the total protein fraction of the pollen extract is used for immunization.

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