

Visualization of birch pollen allergens using IgE-containing sera from human atopic individuals in immunogold labelling experiments

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Summary

Pollen from birch trees (*Betula pendula*) was fixed in paraformaldehyde with or without the addition of 0.5% cetylpyridinium chloride, dehydrated and embedded in Lowicryl K4M in the cold. Ultrathin sections were incubated using the following sequence of antibodies and antisera: IgE-containing serum from an atopic human individual allergic to birch pollen allergens, rabbit anti-human IgE antibodies, and colloidal gold-labelled goat anti-rabbit antibodies. Controls were performed by replacing the specific human antiserum by serum from an atopic person with a similar level of IgE antibodies directed against allergens other than birch pollen allergens, or by omitting the human antiserum or the anti-IgE antibody or both. In test experiments, there was a dense specific labelling of the exine and the cytoplasmic matrix of the pollen grain. There was moderate labelling of the apertural regions (poral plugs). There was no labelling of the intine. In pollen grains fixed with the addition of cetylpyridinium chloride, an electron-dense surface coat was precipitated on the outside of the pollen wall. This surface material also remained completely unlabelled.

Introduction

Pollen allergens are present in the pollen grains of various plant species. On contact with the wet human mucosa, they are rapidly released from the grains and, in susceptible human individuals, lead to the formation of specific reaginic antibody IgE.

In attempts to localize the morphological sites of these allergens within the pollen grain, basically two approaches are open to the histochemist or cytochemist. Firstly, the specific IgE antibodies contained in the blood serum of a pollen allergic human individual might be used for detecting allergenic sites in the corresponding pollen grain. Secondly, substances which have been extracted from the pollen grain, and examined for their allergenicity by clinical and biochemical tests (skin test, radio-allergo-sorbent test), may be used to raise specific antibodies of the IgG type in an experimental animal. These IgG antibodies are in their turn used for detecting specific allergens in the corresponding pollen grain.

The first approach was tried as early as 1961 by Campbell & Sussdorf who after indirect fluorescence immunolabelling of *Ambrosia* pollen using human sera found significant differences between serum from atopic and non-atopic individuals. Ten years later, Belin & Rowley (1971) were unsuccessful in their experiments to localize birch pollen allergens with the help of human

antisera. Very recently, however, a paper was published reporting the successful employment of human antisera in an attempt to localize birch pollen allergens by electron microscopical immunoautoradiography (Fromme *et al.*, 1985).

The second approach, i.e. raising specific antibodies in animals, was followed in a number of attempts to localize the whole of or individual allergens in pollen grains of various plants, among them ragweed, birch, and ryegrass (for a detailed review up to 1980, see Knox *et al.*, 1980). In the following years, two more publications appeared on the fluorescent (Howlett *et al.*, 1981) and immunoferritin (Vithanage *et al.*, 1982) localization of ryegrass pollen allergens. The present paper falls into the first category of experimental approach in that the authors have used serum from a patient allergic to birch pollen allergens for the specific detection of these allergens in the birch pollen grain. In this sense, it is a continuation of the study published previously (Fromme *et al.*, 1985, see above). Apart from this, however, a different immunocytochemical technique, the indirect immunogold labelling, was applied. The results of both experiments, the autoradiographic method formerly used and the immunogold labelling technique now employed will have to be compared with respect to their morphological and methodical values. Any

differences, if they arise, need to be evaluated. Moreover, a comparison will be drawn between the findings of specific IgG labelling of birch pollen (Grote & Fromme, 1984) and the IgE labelling results of this study, in the course of which special attention will be paid to the reactivity of pollen surface coat substances.

Materials and methods

Pollen fixation, embedding and sectioning

Pollen grains freshly shed from the flowering catkins of birch trees (*Betula pendula*) were used in the following experiments.

Samples of the pollen were placed in 0.071 M cacodylate-buffered, freshly prepared 4% paraformaldehyde, pH 7.4, with or without the addition of 0.5% cetylpyridinium chloride (Serva, Heidelberg, West Germany). The pollen was fixed overnight at 277 K and then washed for one hour at 277 K in the cacodylate buffer, pH 7.4. The following dehydration and embedding into the water-soluble resin Lowicryl K4M (Carlemalm *et al.*, 1982) was carried out in a cooling chamber of our own design which operates in the temperature range between 273 K and 223 K.

In detail, dehydration and embedding were performed according to the following scheme: 30% ethanol for 60 min at 273 K; 50%, 70%, 96% and twice 100% ethanol for 60 min each at 253 K. There were three intermediate ethanol–Lowicryl mixtures (2:1; 1:1; 1:2) in each of which the samples were kept for 6 h at 253 K. After that the pollen was successively infiltrated with three portions of degassed pure resin, each infiltration step lasting for 24 h at 253 K. Polymerization was achieved by ultraviolet radiation (two lamps each of 15 W; distance 25 cm; maximum radiation at 360 nm) for 48 h at 253 K. During the impregnation and subsequent polymerization procedures the polymerization inhibiting oxygen was excluded by flooding the cooling chamber with dry nitrogen gas. The polymerized blocks were stored in a desiccator until sectioning. Ultrathin sections were cut on a Reichert Om-U3 ultramicrotome and placed on pioloform-coated nickel grids.

Immunolabelling

First, the sections were pre-incubated for 10 min at room temperature with 5% bovine serum albumin (BSA; Serva, Heidelberg) for blocking unspecific binding sites. Then a three-step immunological labelling procedure was carried out (each step for 2 h at room temperature in a moist chamber, sections floating face-down on drops of the respective reagent):

1. Undiluted serum from a patient highly allergic to birch pollen allergens (radio-allergo-sorbent test class 4, clinical history of birch pollen allergy, total IgE of 526 U/ml as measured by a paper-radio-immuno-sorbent test). After washing for three times, 5 min each, in an aqueous solution of 0.1 M NaCl and 0.015 M Na₂N₃, pH 7.5, the grids were transferred to
2. Rabbit anti-human IgE (ϵ -chains; Dakopatts, Hamburg, West Germany), diluted 1:50 in the washing solution indicated above. After another washing [once for 5 min in the first washing solution, twice for 5 min each in Tris-BSA (20 mM Tris + 0.9% NaCl + 0.1% BSA, pH 8.2)], the grids were incubated with
3. Goat anti-rabbit IgG labelled with 15 nm gold particles (GAR G 15, Janssen Pharmaceutica, Beerse, Belgium), diluted 1:50 with the Tris-BSA buffer indicated above.

The three-step labelling procedure was terminated by thoroughly rinsing the sections, first with Tris-BSA (twice for 10 min each), then with deionized water (once for 10 min).

The following controls were run:

1. Replacement of the first antiserum by a serum from a patient not allergic to birch pollen (radio-allergo-sorbent test class 0), but with a similar level of IgE antibodies directed against other allergens (paper-radio-immuno-sorbent test value: 446 U/ml).
2. Omission of the second antibody (anti-IgE).
3. Omission of the first and second antibody, i.e. incubation with GAR G 15 alone.

After the final washing, the sections were stained with uranyl acetate (10 min) and lead citrate (10 s).

Microscopy and documentation

The sections were examined in a Hitachi H 500 transmission electron microscope operated at 75 kV. Micrographs were taken using Agfa-Gevaert 23 D 56 films.

Results

The outer part of a birch pollen grain fixed in paraformaldehyde without the addition of cetylpyridinium chloride is shown in Fig. 1. After the three-step immunolabelling for IgE binding sites gold particles are found within the outer layer of the pollen wall, the exine, and within the highly condensed cytoplasm. The inner layer of the pollen wall, the intine, is only very rarely labelled. Within the cytoplasm gold markers are distributed on the matrix material whereas reserve materials (starch, lipid) except for their peripheral (presumably membranous) part remain unlabelled. Within the exine, gold particles are preferably localized beneath the bacula region (Fig. 1), but labelling of the bacula layer itself (cf. Fig. 3) and of the tectum (cf. Fig. 3 and Fig. 5) also occurs.

The control experiments (Fig. 2) in which the human antiserum was replaced by serum from an individual not allergic to birch pollen allergens, show very few gold particles throughout the pollen grain (background labelling), as did the other types of control (micrographs not shown).

After fixation of birch pollen grains in aldehyde–cetylpyridinium chloride, patches of electron dense material are precipitated on the surface of many pollen grains (cf. Grote *et al.*, 1983). As is clearly shown in Fig. 3, the cetylpyridinium-precipitated surface material does not react with the IgE containing human serum since no gold particles are localized in it.

A detail from the central part of a birch pollen grain, closely packed with voluminous starch grains and lipid droplets is shown in Fig. 4. Here gold particles are located only in the highly condensed cytoplasmic matrix which only rarely allows the identification of special cell organelles such as rough endoplasmic reticulum or mitochondria. Pollen grain nuclei, however, are always

clearly recognizable. They also react with the human antiserum.

Aperture regions as shown on Fig. 5, exhibit a moderate degree of specific labelling. Within the interior of the poral plug, gold particles are preferably arranged along the electron dense lines of the reticular structures. The thickened exine collar of the pore is heavily labelled. In the control experiment using serum from a person not allergic to birch pollen (Fig. 6), moderate background labelling in the central part of the poral plug is to be seen whereas all other structures remain almost completely unlabelled.

Discussion

The present study has for the first time localized on the ultrastructural level specific birch pollen allergens using serum from a human atopic individual in combination with a three-step immunogold technique. The labelling pattern obtained in this study is not confined to the one atopic patient whose serum was used in the course of the immunocytochemical procedure. Sera from two other persons equally sensitive to birch pollen allergens gave similar results so that the findings of the present study may well be generalized.

In spite of a weak labelling of the control specimens, there is no doubt that the specificity of the immunological reactions for the gold marker density on the test specimens is many times higher than that of the controls. The assumption that the positive labelling of the test specimens could be due to specific binding of IgE or anti-IgE antibodies to endogeneous pollen lectins or lectin-like components (Klemawesch & Buckley, 1984) has to be rejected as in this case the specimens incubated with the control sera should also have shown positive labelling

results. On the other hand it cannot be excluded that the weak background label is indeed caused by such lectin binding.

Concerning the distribution pattern of allergens within the birch pollen grain there is basic agreement with results published by other authors (see below) and with the immunautoradiographical results published by Fromme *et al.* (1985). There are allergens within the exine and the cytoplasm of the grains. With respect to the intine the immunogold labelling results agree with those of immunautoradiography whereas they do not coincide with observations in other pollen species (e.g. Knox & Heslop-Harrison, 1971; Howlett *et al.*, 1973; Knox *et al.*, 1980; Howlett *et al.*, 1981; Vithanage *et al.*, 1980, 1982) which state that the intine also showed allergenic activity. For a detailed discussion of this problem see Grote & Fromme (1984) and Fromme *et al.* (1985). With regard to another pollen compartment, the germination pore, there is a good agreement between our results and those cited above in which (besides the intine), it was especially the aperture that was found to hold immunoreactive and/or allergenic diffusible proteins. The fact that labelling of the germination pore even under identical immuno-experimental conditions could not be detected in our immunautoradiographical study (Fromme *et al.*, 1985) may be explained by the much lower sensitivity of this method (see below).

Comparing the distribution patterns of human anti-birch IgE and rabbit anti-birch IgG binding sites (Grote & Fromme, 1984) it is found that rabbit anti-birch IgG binds to the exine and the cytoplasm, whereas it does not bind to the intine and the aperture region. There is, however, heavy labelling of the cetylpyridinium chloride precipitated pollen surface material. Thus it becomes obvious that there are two morphological compartments of the birch

Figs. 1–6. Ultrathin sections of birch pollen grains after postembedding labelling.

Fig. 1. Part of a birch pollen grain after incubation with IgE-containing antiserum from an atopic human individual, rabbit anti-IgE and goat anti-rabbit IgG–gold. There is labelling of the exine (e) and the cytoplasm (cy). The intine (i) and reserve materials (st, starch) are unlabelled. b, bacula region; mc, microchannel. $\times 36\ 000$.

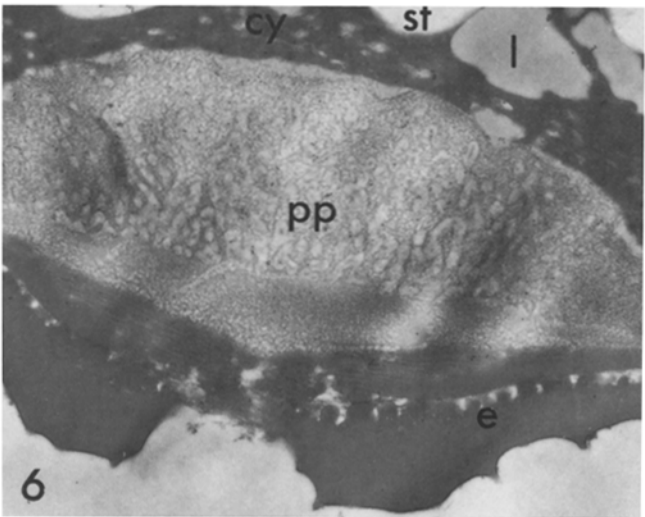
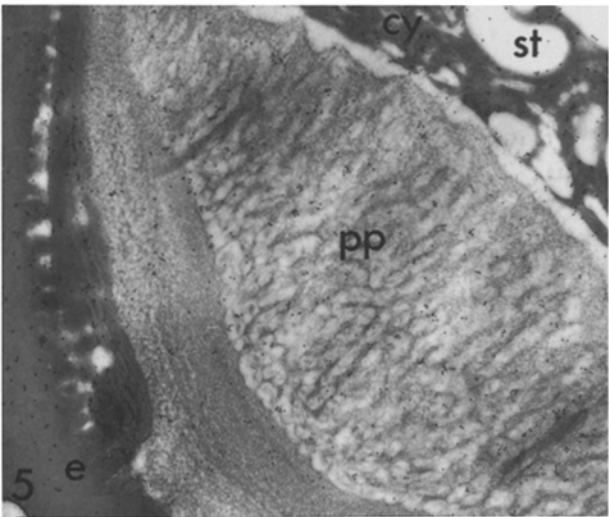
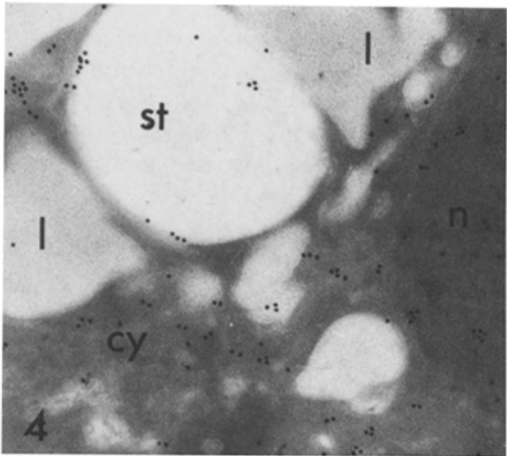
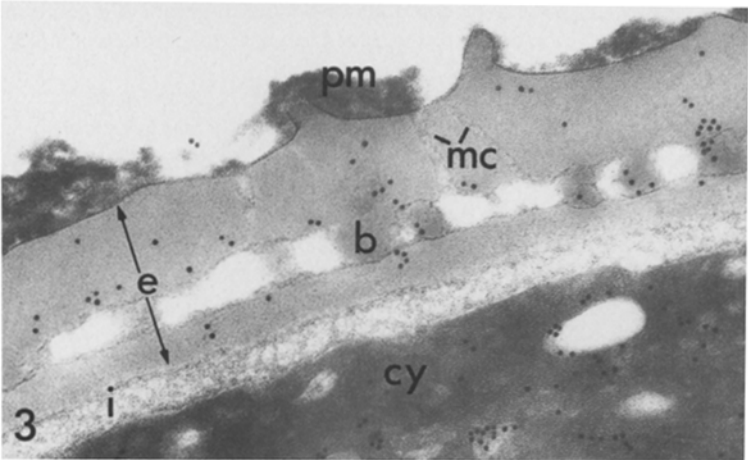
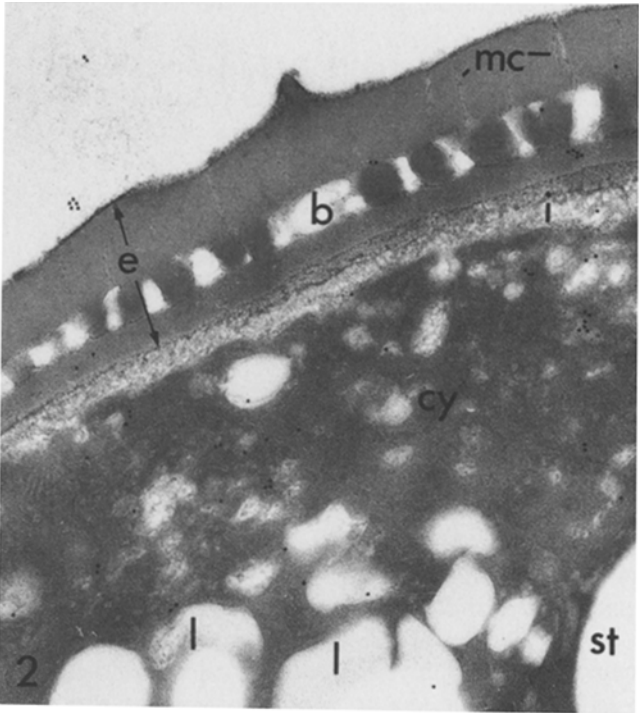
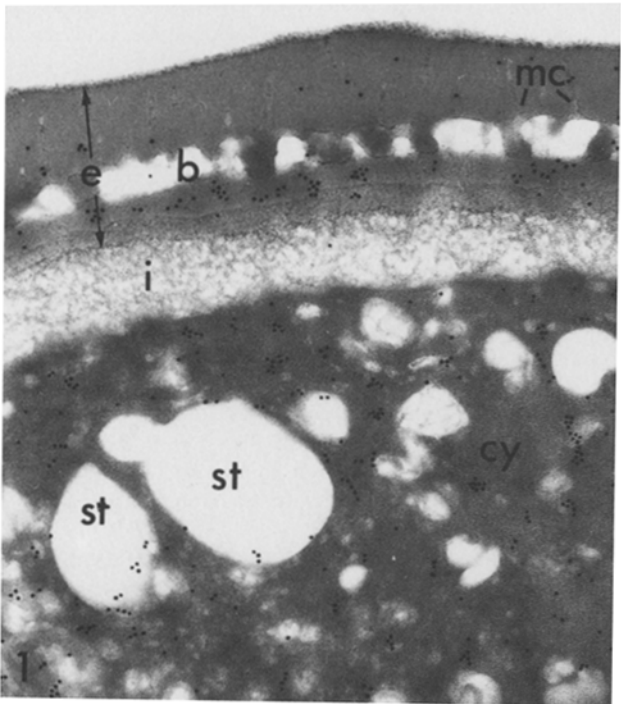
Fig. 2. Control experiment to Fig. 1. Human antiserum replaced by serum from an atopic person not allergic to birch pollen allergens. There is a very low degree of background labelling. Abbreviations as in Fig. 1. $\times 36\ 000$.

Fig. 3. Detail from the peripheral region of a birch pollen grain after incubation as in Fig. 1. The exine (e) is covered with patches of electron-dense material precipitated by cetylpyridinium chloride (pm). No gold particles are found within this surface coat and within the intine (i). The exine is distinctly labelled. The cytoplasm (cy) shows numerous gold markers. The higher electron transparency of the exine as compared to Figs. 1, 2, 5 and 6 must not be attributed to the influence of the detergent cetylpyridinium chloride but may probably be explained by random variations in the preparation and sectioning conditions. b, bacula region; mc, microchannel. $\times 54\ 000$.

Fig. 4. Detail from the cytoplasm of a birch pollen grain after incubation as in Fig. 1. Cytoplasm (cy) and nucleus (n) are labelled with gold particles. Starch grains (st) and lipid droplets (l) as reserve materials are unlabelled. $\times 42\ 000$.

Fig. 5. Part from the germination aperture of a birch pollen grain after incubation as in Fig. 1. The thickened exine collar (e) of the aperture is heavily labelled. Gold particles inside the poral plug (pp) are preferably arranged along the electron-dense lines of the reticular structures. cy, cytoplasm; st, starch grain. $\times 18\ 000$.

Fig. 6. Control experiment to Fig. 5 by incubating the section as in Fig. 2. There is a moderate degree of unspecific binding of gold particles inside the poral plug (pp), while the exine collar (e) and the cytoplasm (cy) is almost free from gold labelling. l, lipid droplet; st, starch grain. $\times 15\ 000$.



pollen grain that show a different reactivity towards human IgE and rabbit IgG antibodies: the poral plug and the surface coat.

This different reaction pattern of the human and animal antisera may be explained by the fact that antisera obtained by the experimental immunization of an animal with pollen extracts may well differ from those antisera produced in atopic human individuals by natural exposition to airborne pollen grains. More simple and evident, however, is the assumption that there are pollen specific materials which in rabbits show 'immunogenic signals' whereas the same substances in human individuals exhibit 'tolerogenic' qualities and vice versa. Such a behaviour of individual immune systems is already well known (c.f. Kölsch, 1984).

In spite of the fact that the GAR G 15 antibodies tended to form clusters and aggregates under our experimental conditions and that therefore a statistical evaluation by counting the gold particles in order to determine the number of actual IgE binding sites becomes very difficult, it is quite evident that the immunogold technique revealed much more allergens within the pollen grain than did our immunautoradiographic approach. As calculations show, this latter technique, due to limiting parameters such as radiation geometry, exposure time, specific sensitivity of the nuclear track emulsion (Fertuck & Salpeter, 1974), resulted in an efficiency of the detection of IgE binding sites in the order of only 5%. Thus, concerning the antigen localization, the immunogold labelling technique must be considered superior to immunautoradiography.

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