

Original Article

Immunogold Electron Microscopy of Soluble Proteins: Localization of Bet v I Major Allergen in Ultra-thin Sections of Birch Pollen After Anhydrous Fixation Techniques

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To localize the highly water-soluble major allergen Bet v I in ultra-thin sections of birch pollen, pollen grains were cracked, air-dried, and processed for electron microscopy using one of the following preparation techniques: fixation in aqueous *p*-formaldehyde + cetylpyridinium chloride; fixation in *p*-formaldehyde vapor; fixation in benzoquinone vapor; inert dehydration; or no fixation. Afterwards the pollen grains were embedded in Lowicryl K4M resin at low temperature. Ultra-thin sections were cut and incubated with a monoclonal antibody against Bet v I, followed by a gold-labeled secondary antibody. In some experiments, commercial rabbit IgG antibodies against birch pollen allergens were also used, followed by incubation with the protein A-gold com-

plex. Bet v I could be localized only after vapor fixation and in the inert dehydrated specimens. Best preservation of ultrastructure and antigenicity was obtained after *p*-formaldehyde vapor fixation. Bet v I antibody binding sites were detected only in the cytoplasmic matrix of the pollen grain, never in the pollen wall. Commercial rabbit antibodies bound to cytoplasm and wall of all prepared specimens, even after aqueous fixation. This might be explained by the assumption that these antibodies recognize a variety of antigenic and allergenic structures, not all of which are so highly soluble as Bet v I. (*J Histochem Cytochem* 39:1395-1401, 1991)
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Introduction

Light and electron microscopists working in the field of pollen research noticed very early that routine fixation techniques using aqueous solutions of aldehydes or osmium do not prevent partial or complete loss of a variety of soluble pollen proteins. As the result of such experience, other strategies have been followed for the fixation of these specimens. (a) Aqueous fixation was employed as usual, but it was carried out with the addition of some substance that was thought to precipitate and thus retain the pollen diffusates. For this purpose, cetylpyridinium chloride (CPC) was used by Grote and Fromme (1984) and Grote et al. (1983). Völker et al. (1986) added cuproline blue (CB) to the fixative solution. (b) The second strategy to keep water-soluble pollen material in place for morphological and analytical studies was the technique of non-aqueous fixation, which was tried by a variety of authors. Thus, e.g., fixation in 90% acetone with the addition of glutaraldehyde or osmium and alcian blue was adopted by Dunbar (1981). Treatment with osmium vapor was applied by Heslop-Harrison (1979). Fixa-

tion in formaldehyde gas, methanol-cyanuric chloride, freeze-sectioning, and freeze-substitution were used by Howlett et al. (1973). Fixation in absolute methanol and/or freeze-drying was carried out by Howlett et al. (1981), Knox et al. (1980), and Vithanage et al. (1980,1982). In a very recent report which appeared after the completion of the experimental part of the present study, Staff et al. (1990) employed fixation in glutaraldehyde vapor, 2,2-dimethoxypropane, and ethanol.

In contrast to the results obtained after aqueous fixation techniques, in most cases the morphology of pollen grains has thus far been rather inadequately preserved after non-aqueous fixation. However, if only differentiation between pollen wall and cytoplasm was required for the experiment or if the main interest was focused on the pollen wall, which was easier to preserve, these methods gave quite satisfactory results.

Very often, the special fixation techniques outlined above were used for the immunocytochemical localization of antigens and allergens. Therefore, the need for sufficient preservation of ultrastructural morphology had to be combined with the demand for good preservation of antigenicity of the antigen/allergen molecules under investigation.

Recent reports of good immunocytochemical results obtained with benzoquinone vapor and *p*-formaldehyde vapor as fixatives

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for the soluble progesterone receptor (Pekki and Tuohimaa, 1989) led us to reconsider the problem of keeping in situ soluble birch pollen proteins for an immunocytochemical study of allergen distribution in the birch pollen grain.

A new monoclonal antibody (MAb) directed against the major allergen of birch pollen, Bet v I, was developed by Jarolim et al. (1989) and kindly provided to us for testing in an immunogold electron microscopic study of its localization in the pollen grain.

We wished to determine if we could localize Bet v I in pollen sections after aqueous fixation with the addition of precipitating agents. Furthermore, we wanted to test two vapor fixatives, benzoquinone and *p*-formaldehyde, for their ability to keep Bet v I in situ and at the same time preserve the antigenicity of this allergen. Last but not least, the "old" technique of "inert dehydration" (Pease, 1966), in which there is no fixation but dehydration of the tissue by the "inert" medium, and the investigation of totally native pollen grains were to be included. In all cases, common sense led us to look for a possibility to open the pollen grain right at the beginning of the experiments to allow fixatives and embedding medium to penetrate better into the pollen grain. If the thick and durable shell around the cytoplasmic core, the pollen wall, could be cracked, so we argued, fixation and embedding results might be better than with the intact and rather impermeable grain.

Materials and Methods

Pollen Material

Pollen from *Betula verrucosa* (L) was collected from the flowering catkins and stored at -18°C until use.

At the beginning of the experiment, pollen grains were taken out of the refrigerator and dusted onto glass microscope slides to obtain pollen "monolayers." The pollen grains were cracked on the glass slides by stamping them gently with the polished end of a copper rod. An inspection of the slides by light microscopy showed that approximately 75% of the grains had more or less been opened. The glass slides with the adhering pollen grains were air-dried for 3 days at room temperature.

Preparation Procedures

Aqueous Fixation in *p*-Formaldehyde + Cetylpyridinium Chloride (CPC). This fixation was carried out as described earlier (Grote and Fromme, 1984).

Fixation in *p*-Formaldehyde Vapor. *p*-Formaldehyde powder was equilibrated for 24 hr with an atmosphere showing a relative humidity of 60% [obtained with H_2SO_4 according to the procedure described by Er  nk   (1964, 1967)]. The glass slides with the cracked pollen grains were put for 24 hr into the *p*-formaldehyde atmosphere and kept at 30°C . Then the pollen grains were collected by scratching them with a spatula from the glass slides into Eppendorf capsules filled with Lowicryl K4M resin. They stayed in pure Lowicryl resin for 24 hr at -35°C , with an exchange of the resin after 12 hr. After 24 hr the pollen grains were put into fresh Lowicryl resin and polymerized as described earlier (Grote and Fromme, 1984).

Fixation in Benzoquinone Vapor. For fixation in benzoquinone vapor according to Pearse and Polak (1974), a solution of 3% 1,4-*p*-benzoquinone (Serva; Heidelberg, FRG) in toluene was prepared. Microscope slides with the adhering cracked pollen grains were put into the vapor for 24 hr at 30°C . Further treatment was as described above.

Inert Dehydration. Inert dehydration was carried out in pure ethylene glycol (Serva). After cracking and drying, the pollen grains were scratched into Eppendorf capsules filled with pure ethylene glycol. They stayed in this medium for 3 days, with an exchange of the medium after every 24 hr. Embedding in Lowicryl was as described above.

No Fixation. For the embedment of native, unfixed pollen grains in Lowicryl K4M resin, the grains were cracked and dried as above and then, without any treatment, transferred from the glass slides into the pure resin, where they stayed for 24 hr. After this, they were put into fresh resin and polymerized as described above.

Ultramicrotomy. Ultra-thin sections (silver color) were cut using a Reichert OM U3 ultramicrotome. They were collected on nickel grids and used for immunocytochemistry.

Immunocytochemistry

Immunochemicals. A monoclonal antibody against birch pollen allergen was obtained from Dr. Dietrich Kraft (Institute of General and Experimental Pathology, University of Vienna, Austria). In immunoblotting experiments, this MAb was shown to react with a 17 kD protein considered to represent the major birch pollen allergen, Bet v I, which was also identified by human IgE antibodies. In double immunodiffusion tests, the MAb was found to be of IgG₁ subclass. Likewise, for control purposes, we received another MAb of irrelevant specificity, also of subclass IgG₁. Goat anti-mouse IgG antibodies coupled to 10-nm gold particles were obtained from BioCell (Plano; Marburg, FRG).

Rabbit IgG antibodies against birch pollen allergens were purchased from ALK (Copenhagen, Denmark). Normal rabbit IgG for control purposes was obtained from Dakopatts (Hamburg, FRG). Protein A conjugated to 15-nm gold particles was prepared in our own laboratory according to the method described by Roth (1982).

Labeling Protocol. Sections were incubated at room temperature in a moist chamber on drops of the following solutions:

1. 5% BSA in PBS buffer, pH 7.4, 15 min.
2. Monoclonal antibody against Bet v I (culture supernatant), undiluted, 2 hr. Alternatively: rabbit antibodies against birch pollen allergens (ALK) diluted 1:60 in PBS buffer, pH 7.4, 2 hr.
3. After monoclonal antibody: PBS buffer, pH 7.4, 10 min, Tris-BSA buffer, pH 8.2, 2×10 min. Goat anti-mouse IgG coupled to 10-nm gold particles (GAM IgG10) diluted 1:20 in Tris-BSA buffer, pH 8.2, 2 hr. After rabbit IgG antibodies: PBS buffer, pH 7.4, 3×10 min, protein A-gold complex diluted 1:18 in PBS buffer, pH 7.4, 1 hr.
4. After GAM IgG: Tris-BSA buffer, pH 8.2, 1×10 min, distilled water, 2×10 min. After protein A-gold: PBS buffer, pH 7.4, 1×10 min, distilled water, 2×10 min.

Controls. The following control experiments were carried out: (a) Replacement of the specific monoclonal antibody by another MAb of irrelevant specificity or, in the case of specific rabbit antibodies, by normal rabbit IgG antibodies; and (b) incubation of the sections with BSA and the GAM IgG 10-nm antibodies or protein A-gold complex alone.

Staining

All sections were double stained with uranyl acetate (3 min) and lead citrate (5 sec).

Microscopy and Documentation

Sections were analyzed in a Hitachi H500 transmission electron microscope operated at 75 kV and photographed using Agfa-Gevaert-23D56 films.

Results

As already experienced by other authors working in the field of anhydrous fixation of desiccated biological tissues, such as seeds or spores, these preparation procedures lead to specimens which are difficult to section (Hallam, 1976). In the case of the pollen grain, which is also characterized by a low water content in its native air-borne state, it proved impossible in the present experiments to cut ultra-thin sections from pollen grains that had not been cracked before non-aqueous fixation and embedding. Only sections of the exine part of the pollen wall could be obtained, whereas the cytoplasmic core had either been lost from the section or its remnants remained on the grid in the form of very electron-dense, structureless patches. Accordingly, the immunocytochemical binding behavior of the cytoplasm could not be studied.

Pollen grains that had been opened before processing for electron microscopy could be cut much better, depending on the pretreatment of the grains. However, the quality of "normal" specimens processed by routine methods for electron microscopy (aqueous fixation in aldehyde/osmium, graded dehydration and embedding) could not be reached, one of the main problems being the formation of chatter within the sections. In all cases, however, it proved possible to obtain at least areas of the cytoplasm that were large enough to allow inspection for the quality of morphological preservation and the antibody binding behavior.

The following ultrastructural and immunological observations were made after the different preparation procedures outlined above.

Fixation in a Buffered Solution of p-Formaldehyde + Cetylpyridinium Chloride (CPC)

Birch pollen grains fixed in aqueous *p*-formaldehyde with the addition of CPC showed a morphology and a rabbit antibody binding pattern which were described in an earlier publication (Grote and Fromme, 1984) (Figure 1). After incubation with the MAb against Bet v I, no gold particles were bound to the sections (Figure 2). The electron-dense surface coat precipitated by CPC (Grote, 1989) is not shown in both micrographs. It was labeled after incubation with the rabbit antibodies, but unlabeled like the rest of the pollen grain after incubation with the MAb.

Fixation in p-Formaldehyde Vapor

After fixation in gaseous *p*-formaldehyde, pollen grains showed a satisfactory ultrastructural preservation. Cell organelles, such as mitochondria, endoplasmic reticulum, dictyosomes, or nucleus with nuclear envelope, could easily be recognized. In addition to these organelles, many small vesicles filled the cytoplasmic matrix. Starch grains and lipid droplets could also be identified.

After incubation with the MAb against Bet v I (Figures 3 and 4), binding sites seemed to be preferentially located within the cytoplasmic matrix. Pollen grain nuclei, too, were distinctly labeled (Figure 3). There was no antibody binding in the pollen wall or on the surface of the pollen grain (Figure 5).

After incubation with rabbit antibodies against birch pollen allergens, gold particles were also bound predominantly throughout the cytoplasmic matrix (Figure 6). In contrast to Bet v I localiza-

tion, the pollen wall was also distinctly labeled. However, only the exine part of the wall exhibited a positive reaction (Figure 7).

Fixation in Benzoquinone Vapor

In comparison with *p*-formaldehyde vapor, benzoquinone vapor preserved ultrastructural details of the pollen grain much worse. Some membranous components (mitochondria, endoplasmic reticulum) were faintly visible in the cytoplasm. As regards Bet v I antibody binding sites, very few of them were located within the cytoplasm (Figure 8) and the nucleus. There were no gold particles in the pollen wall.

Inert Dehydration

After inert dehydration, pollen grains were easier to cut than after the two vapor fixation techniques. The preservation of ultrastructural morphology, however, was like that obtained after fixation in benzoquinone vapor, rather unsatisfactory. The pollen cytoplasm consisted of a rather structureless substance in which only some membranes and the outlines of the nucleus could be distinguished. After incubation with MAb against Bet v I, some labeling by gold particles could be found within the cytoplasm (Figure 9) and the nucleus. Again, the pollen wall did not show antibody binding sites.

No Fixation

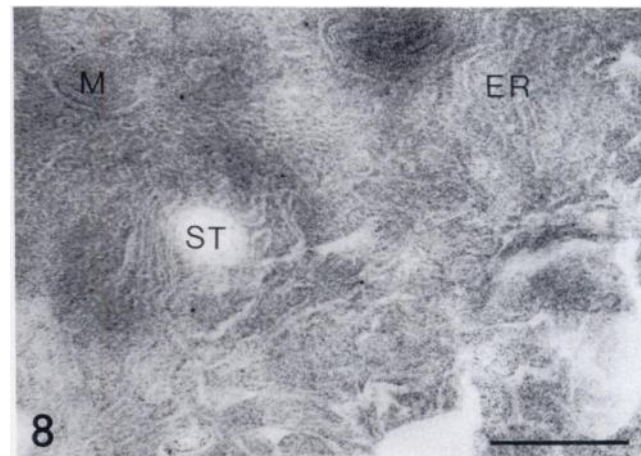
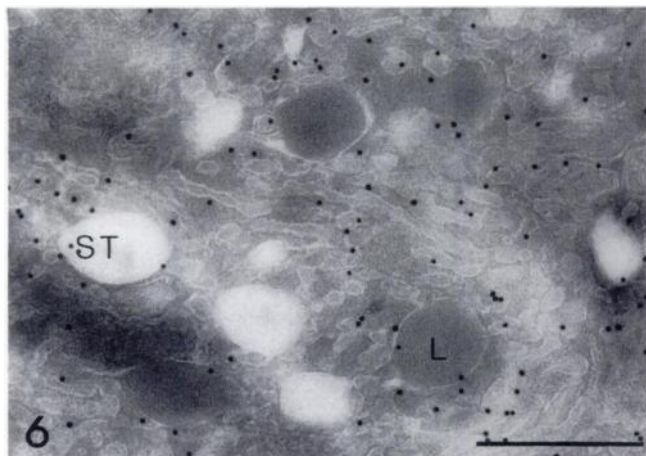
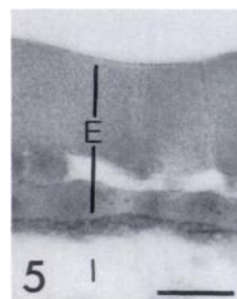
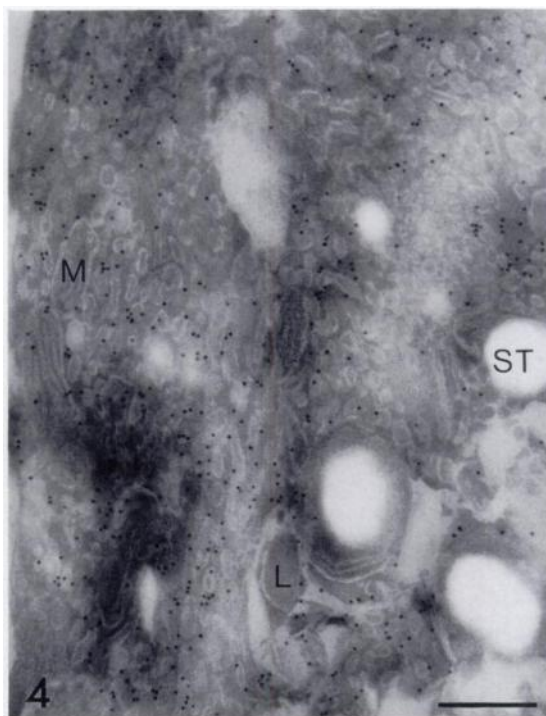
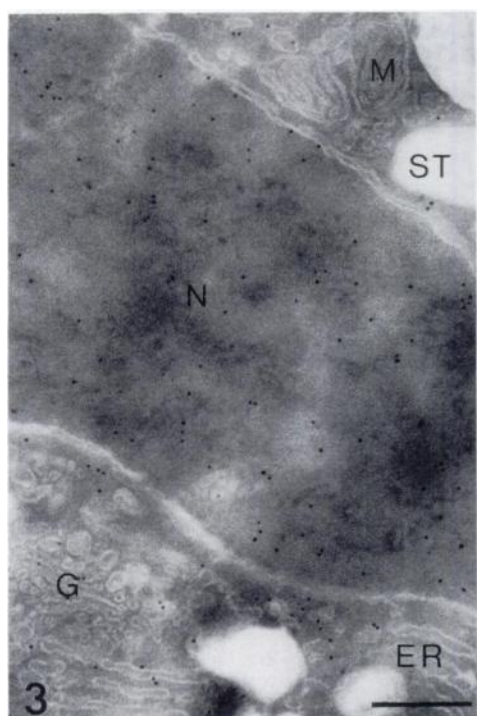
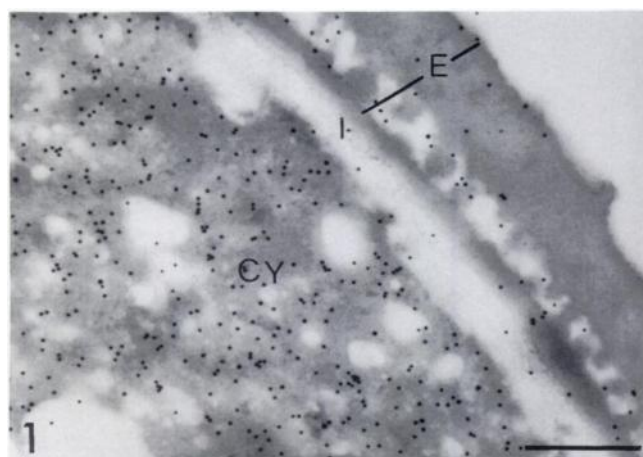
Pollen grains cracked, dried, and directly embedded in Lowicryl K4M resin were very difficult to cut. Cell organelles could not be distinguished except for some lamellar structures and the outlines of the nucleus. Starch grains and lipid droplets were visible. After labeling with the MAb against Bet v I, no gold particles were bound to the section (Figure 10). However, rabbit antibodies against birch pollen allergens strongly bound to the cytoplasm (Figure 11) and the nucleus. The pollen wall (exine part) was also labeled.

Controls

Incubation of birch pollen grains with an MAb of irrelevant specificity instead of the specific antibody, or incubation of the sections with normal rabbit IgG instead of specific rabbit antibodies, in all experiments showed few non-specifically bound gold particles. This is demonstrated in Figure 12 for pollen fixed in *p*-formaldehyde vapor and incubated with the control MAb. Not only these immunological but also the methodological control experiments (incubation of the pollen sections with the goat anti-mouse-gold secondary antibody or with the protein A-gold complex alone) showed a very low degree of background staining.

Discussion

Like other soluble antigens, the major allergen of birch pollen (Bet v I) is a protein molecule which is extremely difficult to keep in situ for morphological and/or analytical investigations. In the present study, this was demonstrated by two experiments. First, birch pollen fixed in aqueous *p*-formaldehyde + CPC shows no binding of antibodies against Bet v I. Therefore, the addition of this



precipitating agent could not retain the allergen molecule as originally hoped by Grote and Fromme (1984) and Grote et al. (1983). Second, native pollen grains likewise showed no binding of antibodies against Bet v I. It is assumed that the "unfixed" allergen diffused from the pollen grain as soon as the section touched the water surface of the knife trough or, later on, during incubation with the different aqueous antibody solutions. Thus, Bet v I antibody binding could be demonstrated only after non-aqueous fixation and preparation conditions.

The anhydrous techniques tested in this study preserve ultrastructural morphology and antigenicity of the birch pollen grain to a very different extent. Fixation with gaseous *p*-formaldehyde gave the best results of all the fixation techniques tried, with regard to both morphology and preservation of antigenicity. Fixation in benzoquinone vapor showed results far inferior to those obtained after fixation in *p*-formaldehyde vapor. This does not confirm the report published by Pekki and Tuohimaa (1989), whose results were vice versa: soluble progesterone receptor was best preserved after fixation in benzoquinone vapor. However, it seems questionable whether both results can be compared: soft animal tissue may well respond in a different way to the influence of fixative agents than do desiccated plant cells.

The results obtained after the "inert dehydration" of the pollen grains were rather disappointing, because preservation of both morphology and antigenicity had been hoped to be favorably influenced by this technique. However, ethylene glycol as a polar medium might well show some sort of interaction with soluble proteins (mobilization?). In addition, it should not be forgotten that the original protocol proposed by Pease (1966) is based on a stepwise dehydration of the tissue by the "inert medium." This was, of course, not possible in the case of pollen grains, since it would again have meant contact with water.

An analysis of the subcellular distribution of Bet v I in the pollen grain allows the following conclusion to be drawn: Bet v I allergen is not located within the pollen wall or on the wall surface, but exclusively within the cytoplasm of the birch pollen grain. This find-

ing is rather surprising for two reasons. First, in other investigations that located the binding sites of allergens in various pollen species, these molecules were always shown to be bound to the pollen wall and, if the morphological preservation was good enough to allow such a statement, perhaps additionally to cytoplasmic sites (Staff et al., 1990; Vithanage et al., 1980, 1982; Howlett et al., 1981; Howlett et al., 1973). Second, the occurrence of Bet v I antibody binding structures exclusively within the cytoplasm of the pollen grain raises the question of how the allergen can diffuse from the pollen grain into the surrounding aqueous medium within a few seconds. This rapid diffusion would be more reasonable if allergen molecules were located, in addition to their original cytoplasmic sites, in the pollen wall and/or the germination pores of the grain. However, investigations in dry plant cells and pollen grains have revealed an altered configuration of the phospholipid bilayers in these cells which enables soluble cell components to diffuse from the cells for some minutes on rehydration until the normal membrane architecture is reestablished (Shivanna and Heslop-Harrison, 1981; Simon, 1974).

As far as the ultrastructural cytoplasmic location of the Bet v I allergen is concerned, the micrographs thus far obtained show that this molecule is predominantly located in the cytoplasmic matrix, since no accumulation of the gold label was observed in cell organelles such as the endoplasmic reticulum, the Golgi apparatus, and membrane-bound vesicles. This subcellular distribution partially coincides with that reported by Staff et al. (1990), who localized specific gold labeling in "electron-opaque" areas of the cytoplasm, which these authors interpret as accumulation of ribosomes in mitochondria, starch grains, and the (vegetative) nucleus. In our experiments, preference of the gold label for matrix sites and for the nucleus was likewise observed. As a comparison with *p*-formaldehyde vapor-fixed but not immunocytochemically treated specimens showed, these matrix sites represent accumulations of ribosomes.

Staff et al. (1990) concluded from their results that synthesis and transport of Lol p I allergen in rye grass pollen takes a route

Figure 1. Transmission electron micrograph of birch pollen grain showing wall (E, exine; I, intine) and cytoplasm (CY). Aqueous fixation in *p*-formaldehyde + cetylpyridinium chloride. Incubation with rabbit polyclonal antibodies against birch pollen allergens and protein A-gold 15-nm complex. Bar = 0.5 μ m.

Figure 2. Transmission electron micrograph of birch pollen grain showing wall (E, exine; I, intine) and cytoplasm (CY). Aqueous fixation in *p*-formaldehyde + cetylpyridinium chloride. Incubation with MAb against Bet v I major allergen followed by goat anti-mouse Ig-gold 10-nm complex. ST, starch grain. Bar = 0.5 μ m.

Figure 3. Detail of birch pollen grain showing nucleus (N) and surrounding cytoplasm in the transmission electron microscope. Fixation in *p*-formaldehyde vapor. Incubation with MAb against Bet v I major allergen and goat anti-mouse Ig-gold 10-nm complex. ER, endoplasmic reticulum; G, Golgi apparatus; M, mitochondrion; ST, starch grain. Bar = 0.25 μ m.

Figure 4. Detail from the cytoplasm of birch pollen grain in the transmission electron microscope. Fixation and incubation as in Figure 3. L, lipid; M, mitochondrion; ST, starch grain. Bar = 0.25 μ m.

Figure 5. Transmission electron micrograph of birch pollen wall showing exine (E) and intine (I). Fixation and incubation as in Figure 3. Bar = 0.25 μ m.

Figure 6. Detail from the cytoplasm of birch pollen grain in the transmission electron microscope. Fixation as in Figure 3 and incubation with rabbit polyclonal antibodies against birch allergens and protein A-gold 15-nm complex. L, lipid; ST, starch grain. Bar = 0.5 μ m.

Figure 7. Transmission electron micrograph of birch pollen wall showing exine (E) and intine (I). Fixation as in Figure 3. Incubation with polyclonal antibodies against birch pollen allergens and protein A-gold 15-nm complex. Bar = 0.25 μ m.

Figure 8. Detail from the cytoplasm of birch pollen grain in the transmission electron microscope. Fixation in benzoquinone vapor. Incubation with MAb against Bet v I major allergen and goat anti-mouse Ig-10-nm gold complex. ER, endoplasmic reticulum; M, mitochondrion; ST, starch grain. Bar = 0.25 μ m.

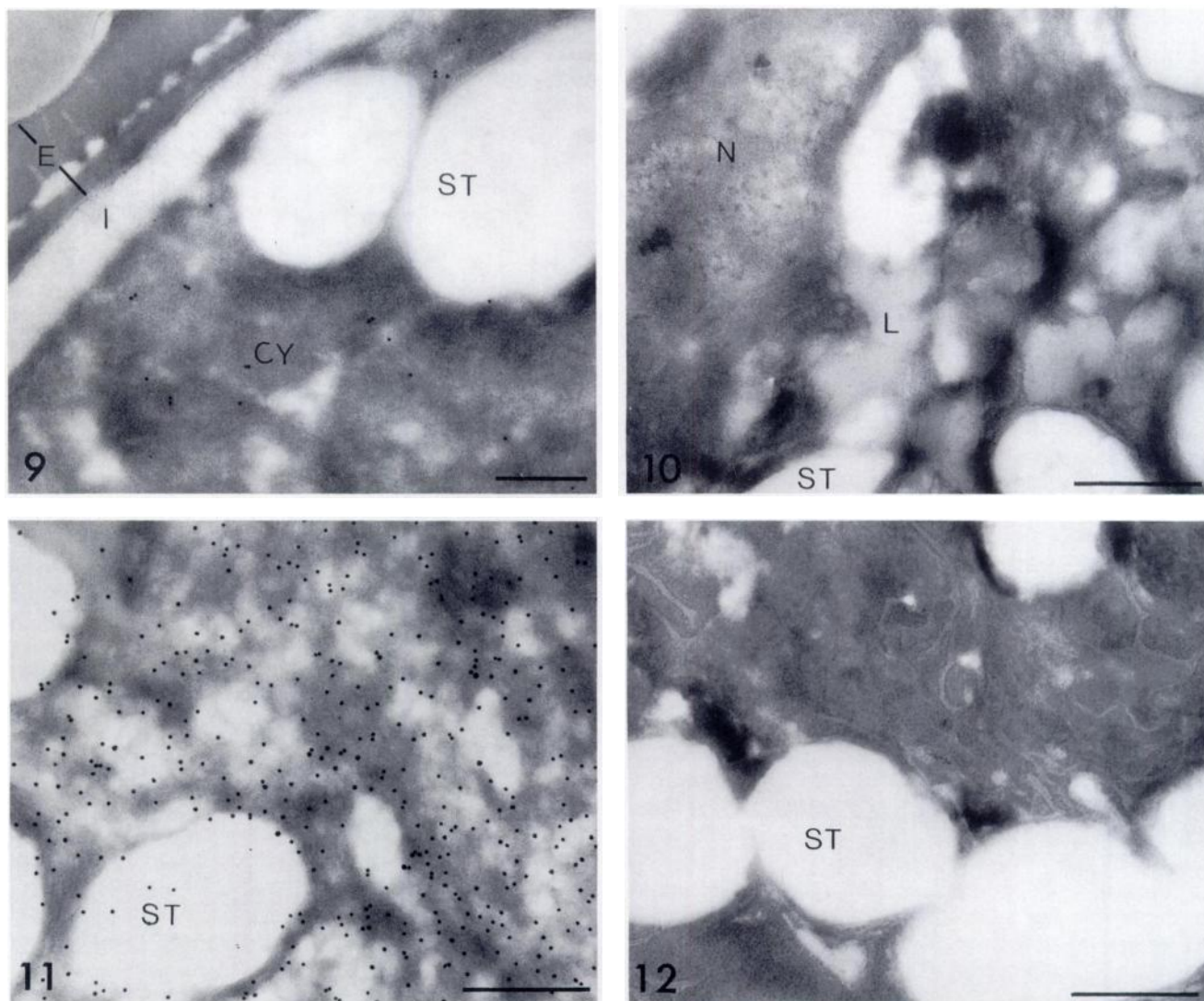


Figure 9. Transmission electron micrograph of birch pollen grain showing wall (E, exine; I, intine) and cytoplasm (CY). Inert dehydration. Incubation with MAb against Bet v I major allergen and goat anti-mouse Ig-gold 10-nm complex. ST, starch grain. Bar = 0.5 μ m.

Figure 10. Transmission electron micrograph of birch pollen grain showing part of the cytoplasm. No fixation. Incubation with MAb against Bet v I major allergen and goat anti-mouse Ig-gold 10-nm complex. L, lipid; N, nucleus; ST, starch grain. Bar = 0.5 μ m.

Figure 11. Transmission electron micrograph of birch pollen grain showing part of the cytoplasm. No fixation. Incubation with rabbit polyclonal antibodies against birch pollen allergens and protein A-gold 15-nm complex. ST, starch grain. Bar = 0.5 μ m.

Figure 12. Transmission electron micrograph of birch pollen grain showing part of the cytoplasm. Fixation in *p*-formaldehyde vapor. Incubation with MAb of irrelevant specificity followed by goat anti-mouse Ig-gold 10-nm complex. ST, starch grain. Bar = 0.5 μ m.

different from that of normal secretory cell proteins. At the present state of our investigations, such a conclusion might also suggest itself, but for verification further evidence should be collected, not only from cytochemistry, but also from, e.g., autoradiography or biochemistry.

Staff et al. (1990) explain their labeling of the nucleus as the result of the nonspecific attraction of the gold probe to nuclear material. However, if all adequate control experiments do not show

labeling of the nucleus, as was the case in the present study, another explanation, such as the free diffusion of the small allergen molecule (17 kD) into the nucleus via the nuclear pores (Gerace and Burke, 1988; Newport and Forbes, 1987), might well be taken into consideration.

In addition to the monoclonal antibodies against Bet v I, commercial rabbit IgG antibodies against birch pollen allergens were tested in some experiments. These antibodies still bind to the birch

pollen grain (cytoplasm *and* wall) even when there is no binding of Bet v I antibodies. This might indicate that they are directed against a more comprehensive "pool" of allergens and, perhaps, antigens not all of which are so extremely soluble as the major allergen Bet v I. As far as the ultrastructural localization of these rabbit antibody binding structures within the cytoplasm is concerned, the labeling pattern within the cytoplasm seems to be similar to that obtained with the monoclonal antibody.

Acknowledgments

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