

Original Article

Monitoring of Two Allergens, Bet v I and Profilin, in Dry and Rehydrated Birch Pollen by Immunogold Electron Microscopy and Immunoblotting¹

MONIKA GROTE,² SUSANNE VRTALA, and RUDOLF VALENTA

Institute of Medical Physics and Biophysics, Münster University, Münster, Germany (MG), and Institute of General and Experimental Pathology, AKH, University of Vienna, Vienna, Austria (SV,RV).

Received for publication August 7, 1992 and in revised form November 12, 1992; accepted November 21, 1992 (2A2780).

Dry and rehydrated birch pollen grains were anhydrously fixed and double immunogold-labeled for the presence of two allergens, Bet v I major allergen (17 KD) and profilin (14 KD). In dry pollen grains, both allergens are found exclusively inside the cytoplasm. In pollen grains rehydrated for 1 min, the cytoplasm is partially devoid of the two allergens, whereas the pollen wall and the germination aperture are specifically labeled. Pollen grains rehydrated for 5 min are largely free of the two allergens. In immunoblot experiments, both allergens could be detected in the aqueous supernatants of rehydrated pollen samples within 5 min. The

results obtained by both methods show the high solubility of both proteins. This makes them readily available to the immune system and characterizes them as potent allergens. Moreover, the solubilization of profilin might indicate a dissociation of the profilin-actin complex at the very first stage of pollen germination, which could favor formation of the cytoskeleton and pollen tube growth. (*J Histochem Cytochem* 41:745-750, 1993)

KEY WORDS: Birch pollen; Allergens; Bet v I; Profilin; Double labeling; Immunogold electron microscopy; Immunoblotting.

Introduction

Localization of pollen antigens and allergens by immunoelectron microscopic methods has been the aim of several research groups during the last 10 or 12 years. In addition to grass pollen (Staff et al., 1990; Vithanage et al., 1980,1982), the pollen of the birch tree has been an object of interest (Grote, 1989,1991; Grote and Fromme, 1984,1986). To overcome the difficult problem posed in the immunocytochemistry of pollen allergens by the extremely high water solubility of the molecules in question, different techniques have been applied, among which anhydrous fixation of the pollen grains proved to be an absolute prerequisite (Grote, 1991,1992; Staff et al., 1990).

The application of highly specific antibodies and the development of appropriate preparation techniques now allow the almost routine in situ detection of pollen proteins or glycoproteins which, because of their potency as allergens, may be of interest not only to the botanist but also to the allergist.

In the case of birch pollen, the localization and distribution pattern of the major allergen Bet v I, a protein of 17 KD (Ipsen and Löwenstein, 1983) was successfully demonstrated (Grote, 1991).

In the present study, a simultaneous detection of two birch pollen allergens was tried: Bet v I, the major allergen, and profilin, another low molecular weight allergen (14 KD) in birch pollen (Valenta et al., 1991a,b,1992).

Since it has long been known that pollen allergens rapidly leach from the grain after contact with water (e.g., Howlett et al., 1973; Knox and Heslop-Harrison, 1970), we wanted to analyze changes in the cell distribution pattern of the two allergens during rehydration of the pollen grain. From these experiments we hoped to obtain information about the pathway by which the allergens leave the grain after moistening. Ultrastructural details of the migration pathway of allergens in pollen grains have not been published.

For a confirmation of the immunocytochemical data, we checked the aqueous supernatants of rehydrated pollen samples by immunoblot experiments for the presence of the two allergens.

Materials and Methods

Immunoelectron Microscopy

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

Fixation and Embedding

Dry pollen grains were dusted onto glass slides and anhydrously fixed in

¹ Supported in part by grants 3/8603 of the "Fonds zur Förderung der gewerblichen Wirtschaft" and S6002Bio of the "Fonds zur Förderung der wissenschaftlichen Forschung," Vienna, Austria.

² Correspondence to: Dr. Monika Grote, Institut für Medizinische Physik und Biophysik, Robert-Koch-Str. 31, D-4400 Münster, Germany.

p-formaldehyde vapor for 24 hr at room temperature. Then they were treated with dimethoxypropane to remove residual water molecules and embedded in Lowicryl K4M resin at -35°C . For details of the fixation, embedding, and sectioning methods see Grote (1992).

Rehydration of Pollen Grains

Dry pollen grains were soaked for 1, 5, or 10 min in Beem capsules filled with distilled water. During rehydration the capsules were continuously shaken. Afterwards, the pollen grain suspension was briefly centrifuged and the supernatant was discarded. The pellet containing the pollen grains was put on glass slides with a spatula and distributed as a very thin layer to expose all pollen grains directly to the fixative vapor. Further treatment was as above.

Immunocytochemistry

Immunochemicals. The following primary antibodies were used: The monoclonal anti-Bet v I antibody, Bip 1 (Jarolim et al., 1989); the rabbit anti-recombinant birch profilin antibody (Valenta et al., submitted for publication); the normal rabbit serum and the control mouse monoclonal antibody Mup 1 (Bohle et al., 1992) were affinity-purified on protein G (MaAb trap; Pharmacia, Uppsala, Sweden). All affinity-purified antibodies contained comparable concentrations of immunoglobulins as determined by absorbance at 260 nm. The following secondary antibodies were used: goat anti-mouse IgG coupled to 10-nm colloidal gold particles (GAM IgG 10) and goat anti-rabbit IgG coupled to 20-nm colloidal gold particles (GAR IgG 20). Both antibodies were obtained from BioCell (Planz, Marburg, Germany).

Double-labeling Protocol. For dilution of antibodies and wash steps, two buffers were used: PBS at pH 7.4 and Tris at pH 8.2. Tris buffer was supplemented with 0.1% BSA. Sections were incubated at room temperature in a moist chamber on drops of the following solutions:

1. 5% (w/v) BSA (Serva No 11923; Heidelberg, Germany) in PBS for blocking of unspecific binding, 15 min.
2. Monoclonal antibody against Bet v I, Bip 1, undiluted, 2 hr.
3. Wash: PBS, 10 min; Tris-BSA, 10 min (twice).
4. GAM IgG 10, diluted 1:20 in Tris-BSA, 1 hr.
5. Wash: Tris-BSA, 10 min; PBS, 10 min (twice).
6. Rabbit anti-profilin antibody, diluted 1:5 in PBS, 2 hr.
7. Wash: PBS, 10 min; Tris-BSA, 10 min (twice).
8. GAR IgG 20, diluted 1:100 in Tris-BSA, 1 hr.
9. Wash: Tris-BSA, 10 min; distilled water, 10 min (twice).

Controls

1. The specific primary monoclonal antibody was replaced by a monoclonal control antibody of different specificity.
2. The specific rabbit antibody was replaced by a pre-immune rabbit antibody.
3. Sections were incubated with the gold-labeled antibodies alone.
4. Single-labeling experiments were done for each of the two allergens.
5. Double labeling was carried out in reverse order.

Staining and Electron Microscopy

For details see Grote (1992).

Biochemistry

Birch pollen (*Betula verrucosa*), 95% pure by light microscopy, was obtained from Allergon (Välinge, Sweden).

Preparation of Birch Pollen Proteins. Fifty-mg portions of birch pollen in Eppendorf tubes were rehydrated for 0.5 min, 1 min, 10 min, 30 min and 1 hr with 1 ml of double-distilled water by shaking end-over-end at room temperature. After hydration, pollen was centrifuged for 20 sec. Nine hundred μl of the supernatant were transferred to a new tube; 100 μl close to the pellet were discarded because they might have been contaminated with pollen grains during the pipetting procedures. The supernatants were examined by light microscopy and almost no pollen grains could be detected. The supernatants and pellets were immediately frozen in liquid nitrogen until use. All samples were analyzed in duplicate. The pellets containing the pollen grains were dissolved in 1 ml sample buffer (Laemmli, 1970).

Immunochemicals. The primary antibodies are discussed above.

Electrophoresis and Immunoblotting. Eighty μl of the supernatants containing the eluted birch pollen proteins and 20 μl of the pellet fraction were subjected to denaturing gel electrophoresis (Laemmli, 1970), and blotted to nitrocellulose (Towbin et al., 1979). Identical blots were probed either with a 1:1000 diluted rabbit anti-recombinant birch profilin antibody or the respective rabbit pre-immune serum, a 1:100 diluted mouse monoclonal anti-Bet v I antibody, Bip 1, or with a mouse monoclonal anti-mugwort pollen allergen antibody, Mup 1, without reactivity to birch pollen proteins (negative control).

Bound rabbit antibodies were detected with a ^{125}I -labeled donkey anti-rabbit antibody (Amersham; Poole, UK). Bound mouse antibodies were detected with a ^{125}I -labeled sheep anti-mouse antibody (Amersham). Blots were autoradiographed at -70°C using Kodak Xomat films and intensifying screens (Kodak; Heidelberg, Germany).

Results

Double immunolabeling of dry birch pollen grains for the simultaneous detection of Bet v I and profilin allergens showed that both allergens were confined to the cytoplasmic compartment of the pollen grain. There was no labeling of the exine and intine part of the wall (Figure 1A). Similarly, there was no labeling of the germination apertures (not shown).

Within the cytoplasm (Figures 1A and 1B), Bet v I (10-nm gold particles) and profilin (20-nm gold particles) often occurred in portions of the cytoplasmic matrix between the various cell organelles and vesicles. However, some labeling of vesicle-like structures was also seen.

In the core of the pollen grain, which is characterized by starch and lipid accumulations, both allergens were confined to the cytoplasmic strands between these materials (Figure 1C). Control experiments in which the specific antibodies were replaced by control antibodies gave very little background labeling (Figure 1D). This was also the case with the other controls.

In rehydrated pollen grains the localization of Bet v I and profilin was remarkably different from that observed in the dry grain. After only 1 min of rehydration gold particles were observed in the pollen wall. Bet v I and profilin could be found in the microchannels and in the bacular cavities, but sometimes no such visible "outlet" could be recognized. Aggregates of gold particles were found inside the pollen wall or on the surface of the grain (Figure 2A).

Inside the cytoplasm, the rather uniform distribution of 10-nm and 20-nm gold particles found in the dry pollen grain had changed. Large areas of the peripheral cytoplasm were completely free of gold particles, whereas others showed accumulations of allergens. Gold particles of both sizes were also observed in the intine part of the pollen wall adjacent to the cytoplasm (Figure 2B).

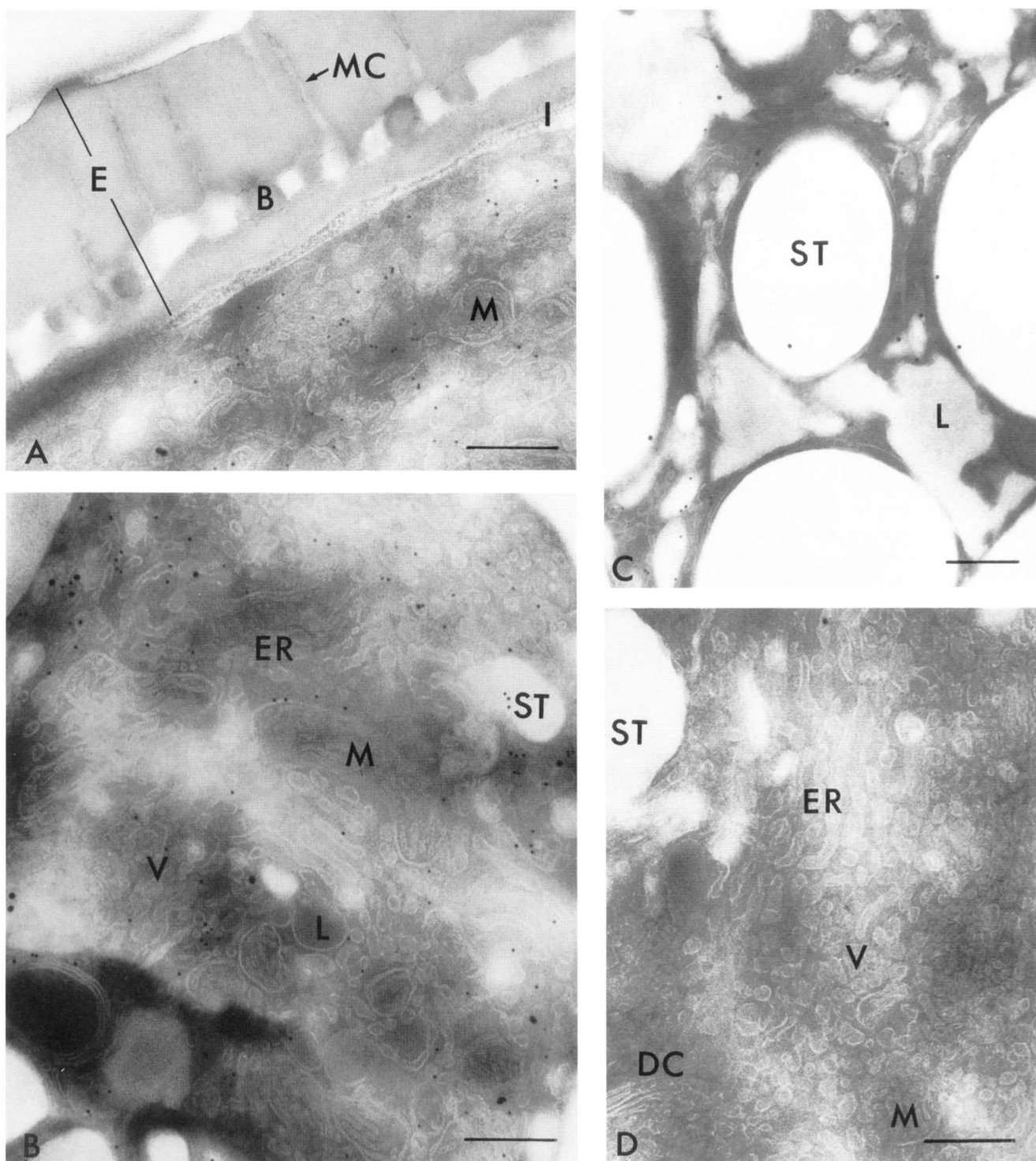


Figure 1. Ultrathin sections of dry birch pollen grains after anhydrous fixation and post-embedding immunogold double labeling. (A) Peripheral part of pollen grain. There are no gold particles in the pollen wall (exine and intine). The cytoplasm is double labeled by 10-nm gold particles (Bet v I) and 20-nm gold particles (profilin). (B) Peripheral pollen grain cytoplasm. Preferably cytoplasmic matrix sites but also vesicle-like structures are double labeled by 10-nm gold particles (Bet v I) and 20-nm gold particles (profilin). (C) Detail from the central part of the pollen grain cytoplasm. Starch and lipid are unlabeled, whereas cytoplasmic strands are double labeled by 10-nm gold particles (Bet v I) and 20-nm gold particles (profilin). (D) Control experiment. After incubation with the control antibodies and gold-labeled secondary antibodies a very low degree of background staining is visible. B, bacula; DC, dictyosome; E, exine; ER, endoplasmic reticulum; I, intine; M, mitochondrion; MC, microchannel; L, lipid; ST, starch; V, vesicle. Original magnifications: A $\times 60,000$; B, D $\times 59,500$; C $\times 45,500$. Bars: A, B, D = 0.25 μm ; C = 0.5 μm .

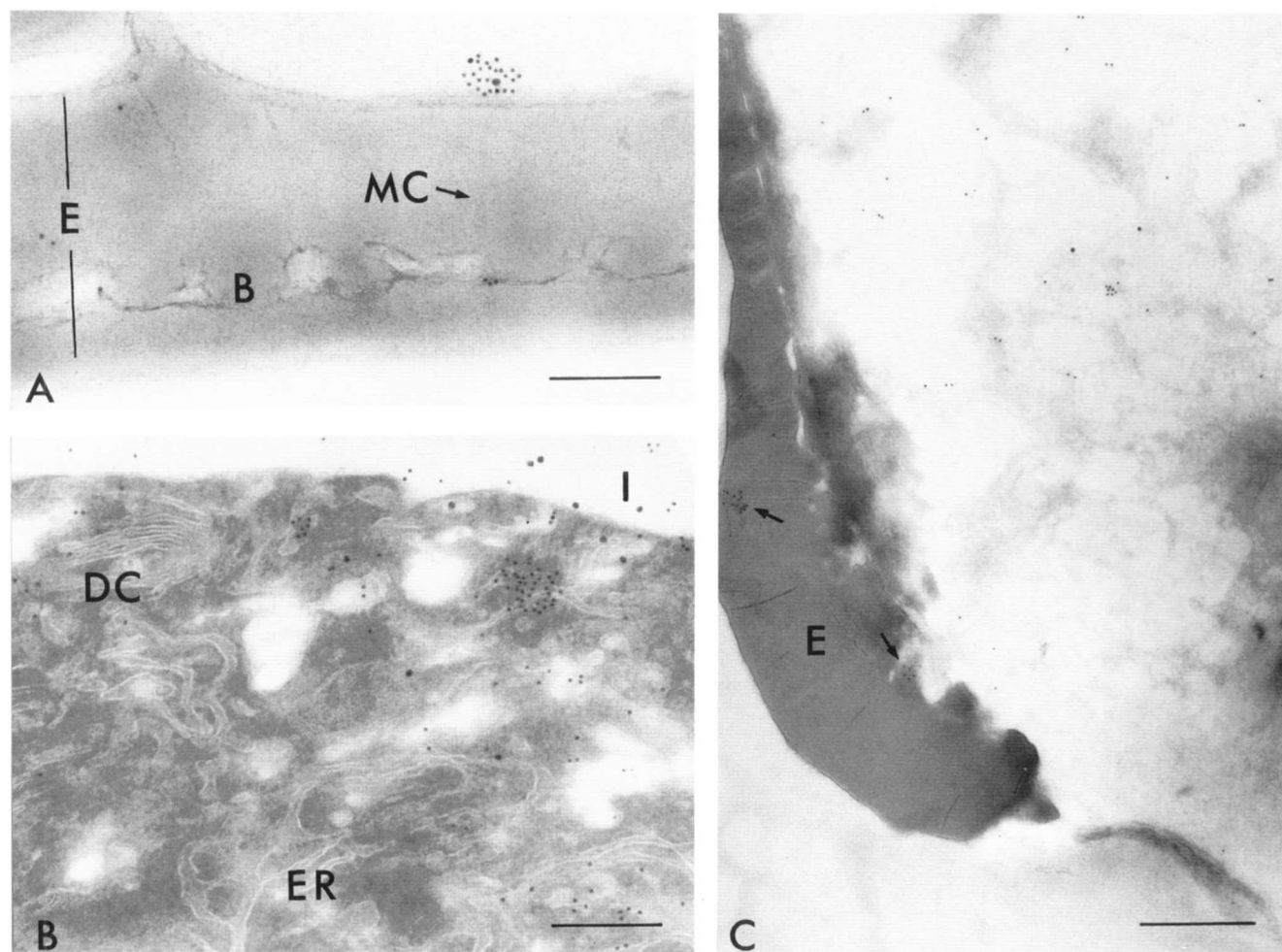


Figure 2. Ultra-thin sections of birch pollen grains rehydrated for 1 min. Anhydrous fixation and post-embedding immunogold double labeling. (A) Exine part of pollen wall showing single 10-nm and 20-nm gold particles (Bet v I and profilin, respectively) and an aggregation of 10-nm gold particles together with two 20-nm particles located at the opening of a microchannel at the pollen grain surface (arrow). (B) Peripheral part of pollen grain cytoplasm. Some areas do not show gold particles, whereas others are characterized by accumulations of allergens (10-nm gold particles, Bet v I; 20-nm gold particles, profilin). There are also gold particles in the intine part of the pollen wall adjacent to the cytoplasm. (C) Part of a germination aperture. There are single 10-nm gold particles (Bet v I) and accumulations of these particles in the exine collar around and in the interior of the pore (arrows). A few 20-nm gold particles are inside the pore (profilin). B, bacula; DC, dictyosome; E, exine; ER, endoplasmic reticulum; I, intine; M, mitochondrion; MC, microchannel; L, lipid; V, vesicle. Original magnifications: A $\times$ 69,500; B $\times$ 60,000; C $\times$ 32,500. Bars: A, B = 0.25 μ m; C = 0.5 μ m.

When the germination pores of the rehydrated pollen were inspected both allergens could be found in the aperture. Here, too, 10-nm gold particles could form aggregates (Figure 2C). After 5 min of rehydration, most pollen grains showed a considerably reduced number of gold particles inside the cytoplasm. After 10 min of rehydration almost all pollen grains were free of gold particles, indicating the disappearance of the two allergens in question (not shown).

Detection of profilin (Figure 3A) and Bet v I (Figure 3B) in the aqueous supernatants showed that both allergens could be found in the supernatants within 5 min. A parallel decrease of the allergen content in the pollen pellet fractions was observed in comparable immunoblot experiments (data not shown), indicating that large amounts of both allergens migrate into the aqueous phase within

a short time. An identical set of blots was probed with the control antibodies (rabbit pre-immune serum and Mup 1) and exhibited no staining (not shown). Coomassie-stained gels showed a constant increase of the amount of eluted proteins in the aqueous supernatants (data not shown).

Discussion

By double immunogold labeling, the distribution pattern of two allergens, Bet v I and profilin, could be simultaneously demonstrated within the birch pollen grain after strictly anhydrous preparation for electron microscopy. Whereas reports from various other pollen species, mostly grass or herb pollen, showed localization of allergens (at least partially) in wall sites of the pollen grain (Staff

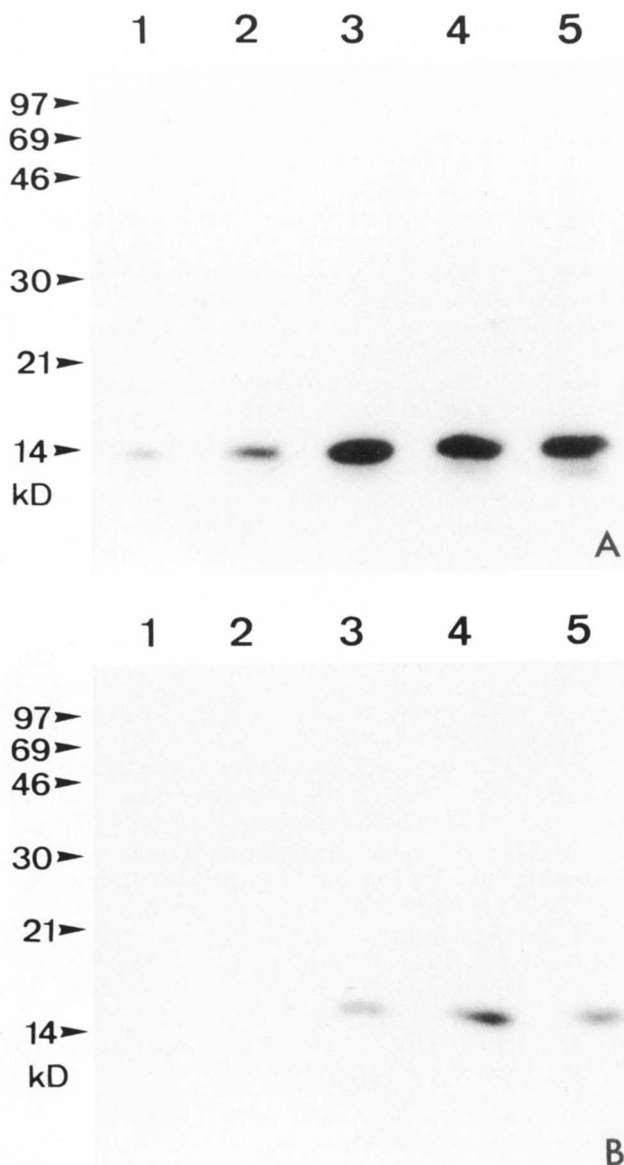


Figure 3. Immunoblot monitoring of eluted birch pollen allergens Bet v I and profilin. Rehydration of birch pollen for 30 sec (Lane 1), 60 sec (Lane 2), 10 min (Lane 3), 30 min (Lane 4), and 1 hr (Lane 5). In each lane equal amounts of aqueous supernatant were applied to the gel. (A) Rabbit anti-birch profilin antibody. (B) Mouse monoclonal anti-Bet v I antibody, Bip 1.

et al., 1990; Vithanage et al., 1980,1982; Howlett et al., 1973), no such wall-bound localization pattern was found in birch pollen.

It is not easy to explain this discrepancy of reports. Since a considerable number of different allergens are present in a birch pollen grain (as in most other pollen grains), the possibility cannot be excluded that some of these other allergens are actually localized in the pollen wall. Moreover, birch pollen (dicotyledons) might well behave differently from that of distantly related plant species such as grasses (monocotyledons). In any case, fixation of herb and grass pollen by the techniques presently used for birch

pollen (which is in progress) would probably supply some useful information concerning this problem.

In the resting pollen grain, not only Bet v I (Grote, 1991) but also profilin is often found in the cytoplasmic matrix. This would very probably mean an association with sites of ribosome accumulation (for a detailed discussion of this point see Grote, 1991,1992).

Gold particles that are located very close to or within a vesicle or any other membrane-bound cell organelle may actually label an antigenic site inside this structure. However, because of the comparatively bad lateral resolution of several ten nanometers found in the routine antibody-colloidal gold techniques (Hainfield, 1989), the labeled allergen might as well be located just outside the structure. An improvement of lateral resolution [by use of, e.g., F(ab) fragments or undecagold] would surely allow a still more precise localization of allergens on the subcellular level. These techniques, however, have not yet been adapted to the present investigation, so that definitive statements about the very high-resolution localization of both allergens cannot be made. In the rehydrated pollen grain, in addition to labeling of wall sites and germination pores, the formation of clusters and aggregates of the Bet v I allergen is most conspicuous. Clustering of allergens was observed in each pollen grain analyzed (approximately 100) as soon as distinct morphological signs of allergen migration had become obvious (appearance of gold particles in pollen wall and pore). Repetition of hydration experiments led to the same results. This means that clustering must not be regarded as artifactual. If it is caused by real aggregation of protein molecules by, e.g., charging, this must be questioned. A more plausible explanation would be a concentration of allergens within pre-formed tunnels. In the exine such tunnels can in many cases be morphologically recognized (cf. Figure 2A, microchannel). However, in the cytoplasm of the shortly rehydrated pollen grain the existence of pre-formed channels of allergen flow should also be postulated if clustering is brought about by their effect. Their morphological basis, however, remains speculative.

By showing a distinct labeling of wall and germination pores in the rehydrated pollen grain, the present experiments positively demonstrate that the allergens are released from the entire surface of the pollen grain. This confirms the early findings of Howlett et al. (1973) who, in pollen print experiments using ragweed pollen, found that very short exposure of the pollen grains to the moist agarose film led to antigen E release from the spinules of the exine surface, while longer exposure times gave rise to a massive leaching of the allergen out of the germination pores.

Our experiments were also designed to determine the rough temporal limits in which allergen release in birch pollen takes place. Both immunoelectron microscopy and immunoblotting showed that within minutes the two allergens are mobilized from the pollen grain and diffuse out into the aqueous surrounding. By means of this easy solubility of the two proteins, their availability to the immune-competent cells of the body is enormously increased, a circumstance which, together with their aerogenic presentation, makes them predestined for their role as allergens.

Not only the allergist but also the botanist and plant physiologist might find interesting details in the present results. In a separate study (Valenta et al., submitted for publication) we could demonstrate by biochemical and immunocytochemical methods that

in the resting tobacco pollen grain profilin and actin form complexes. In the germinating tobacco pollen (pollen tube formed), however, these complexes can rarely be found. Profilin labeling decreases, while actin labeling becomes predominant. The fact that profilin migrates out of the birch pollen grain during rehydration, as shown in the present study, may be taken as a confirmation of our opinion that in the rehydrating pollen grain a dissociation of the actin-profilin complex takes place, which mobilizes stored actin and probably initiates outgrowth of the pollen tube.

Acknowledgments

We wish to thank Ms Ursula Malkus, Institute of Medical Physics and Biophysics, Münster University, for her excellent technical assistance in the electron microscopy work and Ms Barbara Bohle, Institute of General and Experimental Pathology, University of Vienna, for skillful technical assistance in keeping the monoclonal antibody hybridomas.

Literature Cited

- Bohle B, Siemann U, Ferreira F, Kraft D, Rumpold H (1992): Characterization of mugwort pollen allergens by a monoclonal antibody (MUP-1). *Allergy* 47(suppl):93.
- Grote M (1992): Ultrastructural morphology and allergen detection in birch pollen after aqueous, anhydrous-liquid, and vapor fixation techniques. *Microsc Res Tech* 21:242
- Grote M (1991): Immunogold electron microscopy of soluble proteins: localization of Bet v I major allergen in ultra-thin sections of birch pollen after anhydrous fixation techniques. *J Histochem Cytochem* 37:181
- Grote M (1989): Techniques to preserve water-soluble surface components in birch pollen wall: a scanning and transmission electron microscopic study. *J Histochem Cytochem* 39:1395
- Grote M, Fromme HG (1986): Visualization of birch pollen allergens using IgE-containing sera from human atopic individuals in immunogold labeling experiments. *Histochem J* 18:24
- Grote M, Fromme HG (1984): Immunoelectron-microscopic localization of diffusible birch pollen antigens in ultrathin sections using the protein A/gold technique. *Histochemistry* 81:489
- Hainfield JF (1989): Undecagold-antibody method. In Hayat MA, ed. *Colloidal gold. Principles, methods and applications*. Vol 2, New York, Academic Press, 413
- Howlett BJ, Knox RB, Heslop-Harrison J (1973): Pollen wall proteins: release of the allergen antigen E from intine and exine sites in the pollen grains of ragweed and *Cosmos*. *J Cell Sci* 13:603
- Ipsen H, Löwenstein H (1983): Isolation and immunochemical characterization of the major allergen of birch pollen (*Betula verrucosa*). *J Allerg Clin Immunol* 72:150
- Jarolim E, Tejkl M, Rohac M, Schlerka G, Scheiner O, Kraft D, Breitenbach M, Rumpold H (1989): Monoclonal antibodies against birch pollen allergens: characterization by immunoblotting and use for single step purification of the major allergen Bet v I. *Int Arch Allerg Appl Immunol* 90:54
- Knox RB, Heslop-Harrison J (1970): Pollen-wall proteins: localization and enzymic activity. *J Cell Sci* 6:1
- Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680
- Staff JA, Taylor PE, Smith P, Singh MB, Knox RB (1990): Cellular localization of water-soluble, allergenic proteins in rye-grass (*Lolium perenne*) pollen using monoclonal antibodies with immunogold probes. *Histochem J* 22:276
- Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350
- Valenta R, Duchene M, Breitenbach M, Pettenburger K, Koller L, Rumpold H, Scheiner O, Kraft D (1991a): A low molecular weight allergen of white birch (*Betula verrucosa*) is highly homologous to human profilin. *Int Arch Allerg Appl Immunol* 488:368
- Valenta R, Duchene M, Ebner C, Valent P, Sillaber C, Deviller P, Ferreira F, Tejkl M, Edelmann H, Kraft D, Scheiner O (1992): Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 175:377
- Valenta R, Duchene M, Pettenburger K, Sillaber C, Valent P (1991b): Identification of profilin as a novel pollen allergen: IgE autoreactivity in sensitized individuals. *Science* 252:557
- Vithanage HIMV, Howlett BJ, Jobson S, Knox RB (1982): Immunocytochemical localization of water-soluble glycoproteins, including Group 1 allergen, in pollen of ryegrass, *Lolium perenne*, using ferritin-labelled antibody. *Histochem J* 14:949
- Vithanage HIMV, Howlett BJ, Knox RB (1980): Localization of grass pollen allergen by immunocytochemistry. *Micron* 11:411