

Immunoautoradiographic and protein-A/gold labelling experiments for localization of pollen allergens using antisera from atopic human individuals

H.G. Fromme¹*, M. Grote¹, N.J. Sinclair¹, and K. Kalveram²

¹ Institut für Medizinische Physik, Hüfferstrasse 68, D-4400 Münster, Federal Republic of Germany

² Abteilung für Allergologie der Hautklinik, Westfälische Wilhelms-Universität, D-4400 Münster, Federal Republic of Germany

Accepted December 21, 1984

Summary. Using serum from human atopic individuals with a sufficiently high titre of IgE and IgG antibodies to birch- or hazel-pollen allergens and antigens, the localization of IgE binding sites in birch- and hazel-pollen grains was determined by pre- and post-embedding electron microscopic immunoautoradiography with ¹²⁵I-anti-IgE, whereas the IgG binding sites were localized in ultrathin sections of birch-pollen grains by the protein-A/gold technique. Concerning the distribution patterns of both IgE/IgG binding sites within the pollen grains, no difference could be observed in the dormant pollen grain: Labelling was found in the exine part of the pollen wall and throughout the highly condensed cytoplasm except for starch grains and lipid droplets. The intine part and the germination pores were almost completely unlabelled. In pollen grains which had been soaked in a hypotonic buffer for 15 min, however, IgE binding sites were predominantly localized within the intine and the germination pores. The specificity of the labelling reactions and the observed differences in the localization patterns are discussed.

Introduction

In the past several studies have been made in order to localize the cytomorphological sources of the highly water-soluble allergenic and antigenic proteins or glycoproteins of different pollen species. The methods used for these purposes were mostly immunocytochemical procedures with specific IgG fractions raised in experimental animals. The IgG were labelled either with fluorescent dyes (FITC etc.) for light microscopy (Knox et al. 1970; Belin and Rowley 1971; Knox 1971; Knox and Heslop-Harrison 1971a, b; Heslop-Harrison et al. 1973; Knox 1973; Howlett et al. 1973, 1981) or with ferritin for immunoelectron microscopy (Knox et al. 1980; Vithanage et al. 1980, 1982). Recently birch pollen antigens were localized by immunoelectron microscopy using specific rabbit IgG and protein-A/gold conjugates (Grote and Fromme 1984c).

Since the antiserum from animals contains the total anti-pollen IgG, most of the immunocytochemical results show the localization of allergenic and antigenic proteins without distinguishing between these two fractions. In order to obtain a clear differentiation between the binding sites of allergens and antigens, some authors isolated those frac-

tions of the pollen extract which had proved positive for allergy in prick test or RAST, raised specific antibodies in animals against these compounds and thus succeeded in localizing individual allergens in several pollen grains (Belin and Rowley 1971; Knox et al. 1980; Vithanage et al. 1980; Howlett et al. 1981; Vithanage et al. 1982).

In the present paper the authors tried to localize the allergenic (IgE) and antigenic (IgG) binding sites of birch- and hazel-pollen, not by employing animal-produced specific IgG antibodies, but by using human specific IgE- and IgG-containing serum from atopic individuals showing the clinical findings of birch- or hazel-pollen allergy.

IgE binding sites can be visualized by immunoautoradiography using ¹²⁵I-anti-IgE antibodies. The distribution pattern of IgG binding sites can be demonstrated by protein-A/gold labelling. In the binding of protein-A to IgG antibodies, there is no danger of interference with the IgE of the antiserum since the binding of protein-A to IgE is negligibly low (Zola et al. 1978). Moreover, IgE is a thermolabile type of antibody which can be selectively inactivated in an antiserum by heating (Lindberg et al. 1982).

Both these procedures were performed by labelling ultrathin sections of resin-embedded material which has the advantage of exposing intracellular binding sites to the immunoelectron probe. Additionally, labelling experiments using whole pollen grains were carried out in order to estimate the possible loss of antigenicity caused by resin embedding. For this purpose the pollen grains were made permeable by soaking in a hypotonic fluid causing swelling of the grains and disruption of the membranes since neither antibodies nor protein-A can penetrate through the intact or fixed cell membrane. This procedure allowed a comparison between the pre- and post-embedding labelling results.

Materials and methods

Fixation and embedding procedures for post-embedding immunolabelling. Pollen grains were collected from the mature catkins of birch- and hazel-trees (*Betula pendula*; *Corylus avellana*) in spring 1984 and stored until use in small glass vials in the freezing compartment of a household refrigerator at 250 K. The frozen samples of pollen grains were fixed at room temperature overnight in 4% freshly prepared p-formaldehyde in 0.071 M cacodylate buffer (pH 7.4) containing 0.5% cetylpyridinium chloride (CPC). The following washing, dehydration and embedding into the water-soluble resin Lowicryl K4M (Carlemalm et al. 1982) at low temperatures was carried out as described by Grote and Fromme (1984c).

* To whom offprint requests should be sent

Ultrathin sections were cut on a Reichert-Om-U3 ultramicrotome and placed on Pioloform coated nickel grids.

Fixation and embedding procedures for pre-embedding immunolabelling. After the incubation with immunochemicals (see below) was finished, the pollen was centrifuged, the supernatant was discarded and the pollen pellet was resuspended in 1.5% glutaraldehyde in veronalacetate buffer, pH 7.4, for 1 h. Dehydration was carried out with a graded ethanol series (50%, 70%, 2 × 90%, and 2 × 100%). The pollen grains were conventionally embedded in Spurr's low viscosity resin (Spurr 1969).

Ultrathin sections were cut using a LKB Ultratome I and placed on Pioloform coated copper grids.

Immunocytochemical reagents. Antisera were obtained from patients with a clinical history of birch- or hazel-pollen allergy and serum RAST class 4 (RAST = Radio-Allergo-Sorbent-Test). The total IgE concentrations as determined by PRIST (PRIST = Paper-Radio-Immuno-Sorbent-Test) were 185 U/ml for antiserum I, 500 U/ml for antiserum II and 718 U/ml for antiserum III. No information was available concerning the concentrations of pollen specific IgG within these antisera.

Modified antisera were produced by heating portions of each antiserum at 329 K for 4 h, thus selectively inactivating the IgE part of the immunoglobulins.

Control sera were obtained from atopic individuals without the specific symptoms of a birch- or hazel-pollen allergy (RAST class 0). The sera total IgE concentrations (PRIST) were 229 U/ml for control serum I and 705 U/ml for control serum II. There was no information concerning the concentrations of pollen specific IgG.

Anti-human-IgE raised in rabbits and radioiodinated with ^{125}J was part of the RAST kit. Its specific radioactivity was about 13,300 Bq/ml. Reagents for the RAST and PRIST techniques were supplied by Deutsche Pharmacia, Freiburg, FRG.

Colloidal gold was prepared according to the method of Frens (1973) by reducing chloroauric acid with citrate. Chloroauric acid was obtained from Merck, Darmstadt, FRG. The mean diameter of the gold granules was 15 nm.

Protein-A was supplied by Sigma, München, FRG and coupled to colloidal gold according to Roth (1982).

Immunolabelling of ultrathin sections with anti-serum and ^{125}J -anti-IgE for immunoautoradiography. Sections were pre-incubated in 5% bovine serum albumine (BSA) in PBS, pH 7.4, supplied by Serva, Heidelberg, FRG, for 10 min at room temperature. Then the grids were transferred face down without washing onto drops of the undiluted anti-serum I where they stayed for 2 h at room

temperature in a moist chamber. After thoroughly washing with PBS buffer, pH 7.4, the sections were placed face down upon drops of the undiluted radioactive anti-IgE and incubated for another 2 h in a moist chamber at room temperature. Then the grids were thoroughly washed and kept for autoradiographical processing.

Immunolabelling of ultrathin sections with anti-serum and protein-A/gold. Sections were pre-incubated as above and labelled with anti-serum III, using dilutions from 1:1 to 1:500 in PBS, pH 7.4, for 2 h in a moist chamber. After thoroughly washing with PBS, pH 7.4, the grids were placed upon drops of the protein-A/gold solution in a moist chamber at room temperature for 1 h. The labelling procedure was terminated by thoroughly washing, first with PBS, then with distilled water. Sections were stained with uranyl acetate for 3 min.

Immunolabelling of whole pollen grains with antiserum and ^{125}J -anti-IgE. Small aliquots of pollen were suspended in 1.5 ml PBS, pH 7.4, and after 15 min 50 µl undiluted anti-serum II was added followed by the addition of 50 µl undiluted ^{125}J -anti-IgE. The incubation periods were 1–2 min for IgE and 3–4 min for anti-IgE. For fixation and embedding procedures see above.

Autoradiographical processing. The grids bearing the IgE/ ^{125}J -anti-IgE labelled sections as well as the sections from the material labelled prior to embedding were mounted on plastic screws and covered with melted (274 K) and 1:1 diluted L4 nuclear track emulsion (Ilford, Dietzenbach, FRG) using the glass loop method (Fromme 1969). The specimens were then stored at 277 K in a household refrigerator for 40 to 50 days. The photographic development was carried out at 292 K for 90 s using Gevaert G 201 developer. After washing, fixing in an aqueous solution of 25% $\text{Na}_2\text{S}_2\text{O}_3$ and 2.5% $\text{K}_2\text{S}_2\text{O}_8$ and final washing, the specimen were allowed to dry thoroughly in a dust free atmosphere. Before examination in the electron microscope the specimens were stained with lead citrate as usually.

Electron microscopy. All specimens were examined in a Hitachi-H-500 transmission electron microscope operated at 75 kV. Microphotographs were taken using Agfa-Gevaert-23D56 films.

Control experiments. The following controls were performed:

- For postembedding autoradiographical immunolabelling of IgE binding sites: 1. replacement of the anti-serum by a control serum (I or II, according to the total IgE content) using the same dilution as in the test experiment and 2. replacement of the antiserum by the modified (pre-heated) antiserum using the same dilutions as in the test incubation.

Fig. 1. Ultrathin section through a birch-pollen grain after post-embedding immunoautoradiography with antiserum and ^{125}J -anti-IgE. Silver grains are deposited in the cytoplasm (cy) except in starch (st) and lipid (l) inclusions and the exine (e) whereas the intine (i) and the germination pore (p) are nearly unlabelled. The circles surrounding the silver grains represent areas within which the radioactive source is located by a probability of 50%. × 3,600

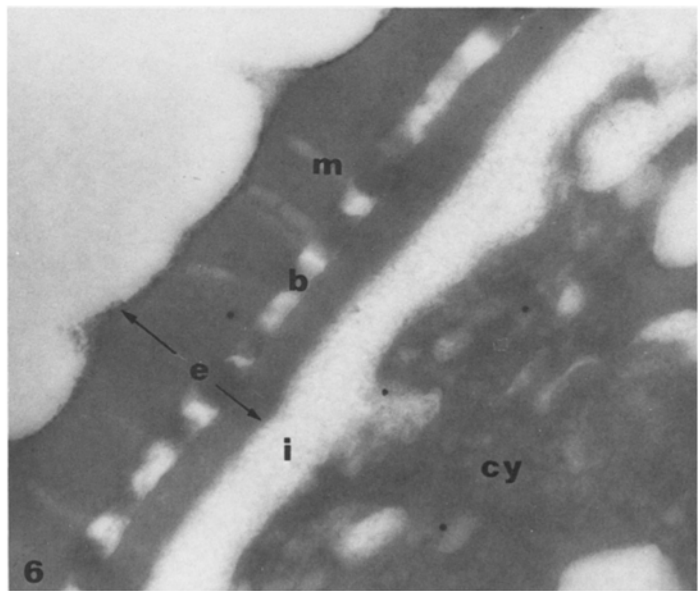
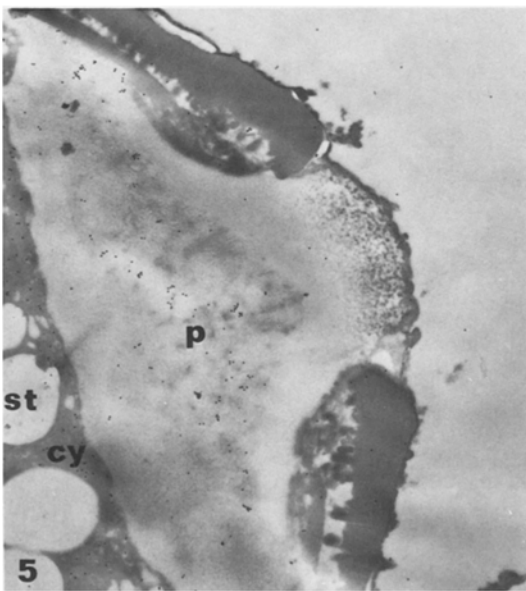
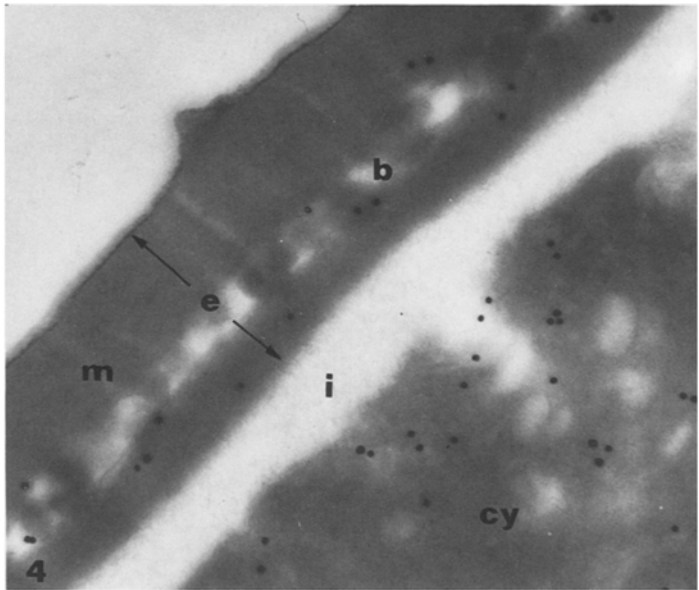
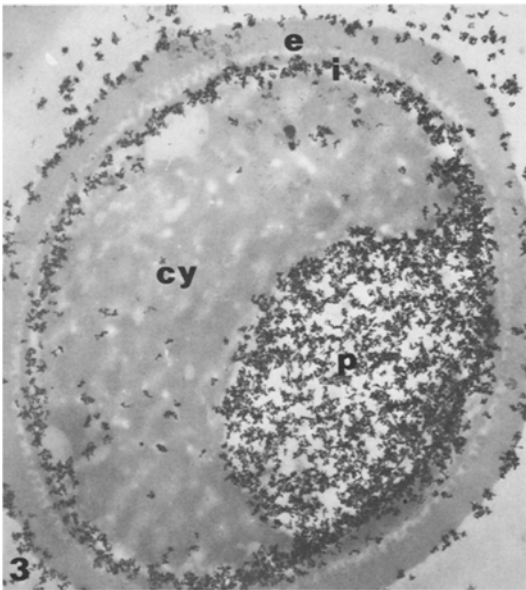
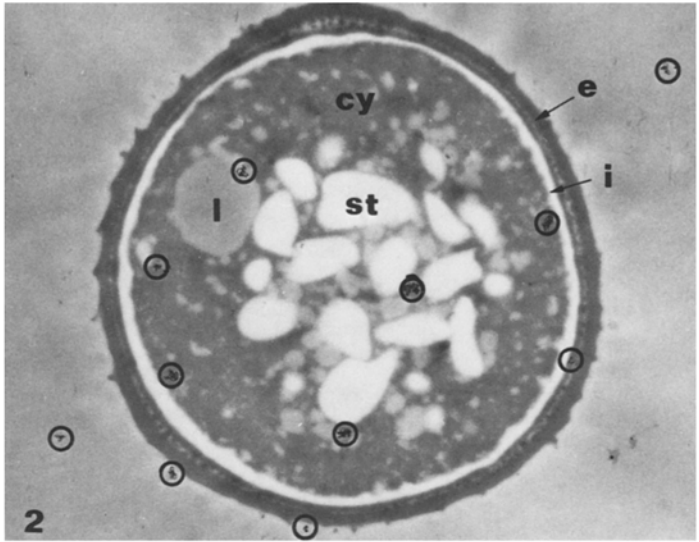
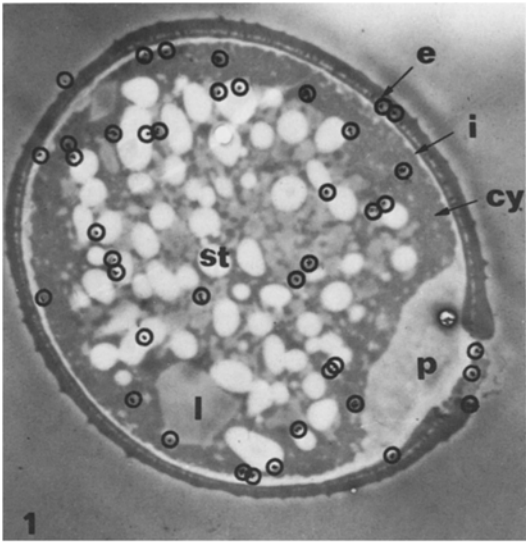
Fig. 2. Control specimen to Fig. 1. Incubation with a control serum (containing no birch-pollen specific IgE) and ^{125}J -anti-IgE. Few silver deposits are unspecifically distributed within the pollen grain. The circles surrounding the silver grains represent areas within which the radioactive source is located by a probability of 50%. × 5,300

Fig. 3. Ultrathin section through a hazel-pollen grain after pre-embedding labelling with antiserum and ^{125}J -anti-IgE. There is a dense deposition of silver grains within the intine (i) including the germination pore (p) and on or near the surface of the exine (e). The cytoplasm (cy) is weakly labelled. × 5,100

Fig. 4. High magnification of an ultrathin section through the peripheral region of a birch-pollen grain after post-embedding immunolabelling with antiserum and protein-A/gold. Gold granules are found within or near the bacula region (b) of the exine (e) and within the cytoplasm (cy). The intine layer (i) is nearly unlabelled. m = microchannel. × 59,800

Fig. 5. Aperture region of a birch-pollen grain treated as in Fig. 4. A few gold granules are found within the interior of the germination pore (p). The adjacent cytoplasm (cy) is distinctly labelled but there are almost no gold markers within starch grains (st). × 12,000

Fig. 6. Control specimen to Fig. 4. Incubation with a control serum (containing no birch-pollen specific IgG) and protein-A/gold. The micrograph exhibits a very low degree of background staining. cy = cytoplasm, e = exine, i = intine, b = bacula, m = microchannel. × 50,400



- For immunolabelling of IgG binding sites with protein-A/gold: 1. replacement of the antiserum by control serum II using the same dilution as in test experiment. 2. incubation with an anti-IgE-antibody between the anti-serum and the protein-A/gold steps. 3. incubation of the sections with the protein-A/gold complex alone.
- In the case of pre-embedding immunolabelling of IgE binding sites: omitting the incubation with antiserum II before adding the ^{125}J -anti-IgE.

Results

Immunoautoradiographical findings

Labelling of ultrathin sections with antiserum and ^{125}J -anti-IgE. The autoradiographic specimens show IgE binding sites throughout the cytoplasm except over starch grains and lipid droplets. Concerning the pollen wall, the inner wall layer (intine) including the aperture region is rarely labelled. The outer wall layer (exine), however, shows distinct IgE binding sites (Fig. 1). Control specimens which had been treated with the adequate control serum or with the modified (pre-heated) antiserum before incubation with anti-IgE, exhibit a moderate degree of unspecific binding (Fig. 2).

In an attempt of quantitation the numbers of silver grains per unit area of pollen cross section were determined and corrected for the numbers of silver grains per unit area of the surrounding tissue-free resin (background, autoradiographical fog). Between both control experiments there was no difference concerning the calculated values. In the test experiments, however, the silver grain density was 7.5 times that of the controls.

Labelling of whole pollen grains with antiserum and ^{125}J -anti-IgE. All sections through immunolabelled pollen material show a heavy precipitation of radioactive anti-IgE as visualized by a dense deposition of silver grains. Concerning the localization of ^{125}J -anti-IgE, there is a prominent labelling of three parts: the intine layer, the germination plug and the outer surface region of the pollen grain. The exine part of the wall including the bacula region is only weakly labelled as is also the case with the pollen cytoplasm (Fig. 3).

The controls showed a negligible degree of background staining throughout the pollen grains (micrographs not shown).

Protein-A/gold labelling

Of all the anti-serum concentrations tested, the 1:300 dilution in PBS gave the highest amount of specific labelling together with a comparatively low degree of unspecific background staining.

As shown on a section through the peripheral part of a birch-pollen grain (Fig. 4), gold particles are observed within the pollen wall and within the cytoplasm of the grain. As regards the wall, protein-A binding sites are found within the exine, preferably within or adjacent to the bacula layer. The intine is practically free from gold granules. Within the interior of the pollen, protein-A reactive antibodies are associated with the highly condensed cytoplasmic substance except for the voluminous reserve materials of the pollen grain (starch granules (see Fig. 5, upper left corner) and lipid droplets). The aperture region (Fig. 5) is

weakly labelled. Here the gold particles are concentrated within the interior of the poral plug.

Sections additionally incubated with the anti-IgE between the antiserum and protein-A/gold steps did not exhibit differences to the test experiment as regards number and localization of gold granules (micrographs not shown). Specimens incubated with the adequate control serum showed a very low degree of background staining (Fig. 6). Incubation with the protein-A/gold complex alone (micrographs not shown) resulted in even less unspecific adsorption of the complex to the section.

Discussion

Immunoautoradiography as an alternative to immunofluorescence in the detection of antibody binding sites has already been employed in some investigations (e.g. Moar et al. 1979; Gerdes et al. 1982; Moshakis et al. 1982; Das et al. 1984). Besides these light microscopic applications the combination of immunoautoradiography and electron microscopy was reported by Paiement and Leblond (1977) and Paiement and Kopriwa (1978).

In these electron microscopic studies, the localization of antigens was performed either by radioactive protein-A or by radioiodinated antibodies. But whereas Paiement and coworkers (see above) used autoradiography for quantitation of immunocytochemical results by silver grain counting, in the present study autoradiographic localization was chosen in order to achieve a clear differentiation between IgG and IgE binding sites.

The results of the present investigations for the first time show on the one hand that such an immunocytochemical differentiation is possible and on the other hand that human serum from atopic individuals can be successfully employed for these experiments.

To our knowledge there are two reports in the literature describing pollen grain labelling experiments using sera from human allergic individuals. Thus as early as 1961 Campbell and Sussdorf described surface labelling of whole pollen grains by 'fluorescein-conjugated antiglobulins' after incubation with human or rabbit antiserum. Prior to the labelling procedure, the pollen grains had been washed for 2-3 h in saline to 'remove surface material'. Our findings obtained from the pre-embedding immunolabelling experiments show IgE binding sites also on the surface of the pollen grains so that this result might confirm this early observation, although after such a long washing period most of the antigens and allergens should have been extracted from the pollen grain (see below). The second investigation reporting the use of human antisera was carried out by Belin and Rowley (1971). These authors tried to localize birch-pollen allergens on the surface of the grains using the incubation sequence: human antiserum/sheep-anti-human-IgE/rabbit-anti-sheep-IgG/goat-anti-rabbit-IgG-FITC. They were not able by this to demonstrate specific fluorescence on the grains whereas antisera against birch-pollen allergens raised in rabbits led to specific labelling. The authors explained their failure by a presumably too low concentration of IgE antibodies in the human serum.

A prerequisite in our method is, of course, the use of a serum that contains, besides a high titre of specific IgE, sufficient specific IgG antibodies as was the case with our antiserum III. The occasional occurrence of significant amounts of IgG antibodies in pollen allergic patients even

before specific immunotherapy has already been reported elsewhere (e.g. by Djurup and Østerballe 1984).

As to the specificity of labelling, the results of autoradiographic as well as of protein-A/gold immunomarking must be regarded as giving reliable pictures of the distribution of IgE and IgG binding sites, respectively, throughout the birch- or hazel-pollen grains.

In the case of IgE localization, there were negative results when using a control serum containing nearly the same amount of total IgE (PRIST value 229 U/ml) but with immunoglobulins directed against types of allergen other than birch or hazel. There was also no labelling when the IgE portion of the birch-pollen specific antiserum was selectively inactivated by heating.

As already mentioned, in the case of IgG localization, the reactivity of protein-A itself with human IgE must be regarded as negligibly low (Zola et al. 1978). Moreover, the comparatively high dilution of the antiserum which had to be used in order to achieve a low degree of background staining, made it rather improbable that a significant amount of IgE antibodies should still be present in the incubation serum. This theoretical consideration was practically confirmed by the results of the control experiment inserting an anti-IgE-antibody bridge between the antiserum and the protein-A/gold labelling steps which should have resulted in an increase in gold deposition because the anti-IgE-antibody belongs to the IgG-class of antibodies which readily react with protein-A. Since this experiment did not result in an increase in the number of gold granules as compared to the number of gold granules found after labelling with antiserum and protein-A/gold alone, we had to conclude that only IgG antibodies were involved in the observed gold labelling.

The immunological specificity of the localization of IgG-binding sites was proved by the results of the experiment using a serum from an individual without birch- and/or hazel-pollen allergy.

Last, but not least, unspecific labelling due to binding of antibodies by endogenous pollen lectins or lectin-like components (Klemawesch and Buckley 1984) can be excluded by our experiments since in this case control sera or modified antisera should also have shown positive labelling results.

Considering the localization of IgE and IgG binding sites within the pollen grain, there are no spatial differences between both types of antibody binding site in the quiescent pollen grain. As demonstrated by our post-embedding immunomarking results, allergens and antigens occur side by side throughout the cytoplasm and in the exine of the pollen wall. The reserve materials in the cytoplasm (starch grains and lipid droplets), however, are almost completely unlabelled. These findings are very similar to those obtained by use of rabbit IgG against birch-pollen extract (Grote and Fromme 1984c). However, since details of fine structure can hardly be observed in the highly condensed cytoplasm of dormant pollen grains, we must at present leave the question undecided if there are nevertheless differences in the localization of antigens and allergens which might become more obvious in physiologically active, e.g. germinating pollen grains.

The above observed pattern of the distribution of allergens and antigens, however, becomes strikingly different in those pollen grains which had been kept in hypotonic buffer for 15 min and thus been made permeable to anti-

bodies. Here many IgE binding sites are found within the intine of the pollen wall and in the intinal thickenings at the germination pores, together with a distinct labelling of the exine part of the wall as described above. The cytoplasm, on the contrary, is rather weakly labelled or, in some pollen grains, almost free from allergens.

Such or very similar are also the distribution patterns of antigens and/or allergens reported by other authors in their studies on various allergenic pollen species (Knox et al. 1970; Knox 1971; Knox and Heslop-Harrison 1971a, b; Knox et al. 1980; Vithanage et al. 1980, 1982).

The results of our present study confirmed us in the opinion preliminarily indicated in a former publication (Grote and Fromme 1984c) that distribution patterns like those reported in the literature or like that now observed in our pre-embedding immunolabelling experiment actually represent 'snapshots' taken from the dynamic process of allergen/antigen migration out of the pollen grain during moistening. This would necessarily lead to a gradual depletion of the cytoplasm concerning its content of allergens/antigens and – in the first instance – to a concentration of these substances in the intine and the germination pores. As the diffusion process out of the grain through the microchannels of the exine and the germination pores continues, a more or less complete depletion of the whole grain will presumably take place.

Our post-embedding labelling experiments, on the other hand, were carried out on pollen material fixed in a mixture of aldehyde and cetylpyridinium chloride (Grote et al. 1983) which precipitates carbohydrates (glycoproteins, among them probably antigens and/or allergens) in their original cytoplasmic or wall-associated sites (Grote and Fromme 1984a, b). Thus we are convinced that our post-embedding immunolabelling results show the original state of allergen/antigen distribution in the quiescent pollen grain.

Finally, our results are a good illustration for the decrease in the intensity of immunolabelling after fixation and embedding which has repeatedly been pointed out in the literature (e.g. Hemming et al. 1983). However, in the case of the very intricate problem of fixing and labelling the highly diffusible pollen proteins, we feel that informations from a variety of modern preparation techniques (whole pollen specimens, sections, chemical fixation with some precipitating substance or dye, cryofixation, pre- and post-embedding labelling etc.) must be gathered together in order to increase our knowledge about the nature and distribution of these allergy causing agents.

Thus, concerning, e.g., the question of 'allergen/antigen migration', the authors hope to obtain more information from a series of experiments using, e.g. different extraction periods of pollen grains combined with the successfully tested fixation (CPC) and the pre- or post-embedding labelling procedures outlined above.

Acknowledgements. The authors wish to acknowledge the excellent technical assistance of Miss Helga van Eys and Miss Ursula Malikus.

References

- Belin L, Rowley JR (1971) Demonstration of birch pollen allergen from isolated pollen grains using immunofluorescence and a single radial immunodiffusion technique. *Int Arch Allergy* 40: 754–769

- Campbell DH, Sussdorf DH (1961) The use of pollen grains for the detection of specific antibody. *J Allergy* 32:357–362
- Carlemalm E, Garavito RM, Villiger W (1982) Resin development for electron microscopy and an analysis of embedding at low temperature. *J Microsc* 126:123–143
- Das ND, Ulshafer RJ, Zam ZS, Leverenz VR, Shichi H (1984) Radioimmunocytochemical localization of retinal S-antigen with monoclonal antibodies. *J Histochem Cytochem* 32:834–838
- Djurup R, Østerballe O (1984) IgG Subclass antibody response in grass pollen-allergy patients undergoing specific immunotherapy. *Allergy* 39:433–441
- Frens G (1973) Controlled nucleation for the regulation of the particle size in monodispersive gold suspensions. *Nature (Phys Sci)* 241:20–22
- Fromme HG (1969) Die Anwendung der Autoradiographie in der Elektronenmikroskopie. *Acta Histochem* 34:86–125
- Gerdes JC, McNally I, Hileman L, Burks JS (1982) Autoradiographic detection of IgG and viral antigens. *J Immunol Methods* 54:191–202
- Grote M, Fromme HG, Sinclair NJ (1983) The use of cetylpyridinium chloride to preserve water-soluble surface material in pollen for electron microscopy. *Micron* 14:29–32
- Grote M, Fromme HG (1984a) Electron microscopic localization of Concanavalin A receptor sites in pollen surface material after fixation with glutaraldehyde-cetylpyridinium chloride. *J Histochem Cytochem* 32:869–871
- Grote M, Fromme HG (1984b) Ultrastructural demonstration of a glycoprotein surface coat in allergenic pollen grains by combined cetylpyridinium chloride precipitation and silver proteinate staining. *Histochemistry* 81:171–176
- Grote M, Fromme HG (1984c) Immunoelectron microscopic localization of diffusible Birch-pollen antigens in ultrathin sections using the protein-A/gold technique. *Histochemistry* 81:489–492
- Hemming FJ, Mesguich P, Morel G, Dubois PM (1983) Cryoultramicrotomy versus plastic embedding: comparative immunocytochemistry of rat anterior pituitary cells. *J Microsc* 131:25–34
- Heslop-Harrison J, Heslop-Harrison Y, Knox RB, Howlett B (1973) Pollen wall proteins: 'gametophytic' and 'sporophytic' fractions in the pollen walls of *Malvaceae*. *Ann Bot* 37:402–412
- Howlett BJ, Knox RB, Heslop-Harrison J (1973) Pollen wall proteins: Release of the allergen antigen E from intine and exine sites in pollen grains of ragweed and *Cosmos*. *J Cell Sci* 13:603–619
- Howlett BJ, Vithanage HIMV, Knox RB (1981) Immunofluorescent localization of two water-soluble glycoproteins including the major allergen from the pollen of ryegrass, *Lolium perenne*. *Histochem J* 43:461–480
- Klemawesch SJ, Buckley CE (1984) The lectin reactivity and lectin-like activity of allergenic pollen extracts. *J Allergy Clin Immunol* 73:619–627
- Knox RB (1971) Pollen wall proteins: localization, enzymic and antigenic activity during development in *Gladiolus (Iridaceae)*. *J Cell Sci* 9:209–237
- Knox RB (1973) Localization of proteins in plant cells by immunofluorescence. *Zeiss Information* 20:52–55
- Knox RB, Heslop-Harrison J (1971a) Pollen wall proteins: fate of intine-held antigens in compatible and incompatible pollinations of *Phalaris tuberosa* L. *J Cell Sci* 9:239–251
- Knox RB, Heslop-Harrison J (1971b) Pollen Wall Proteins: Localization of antigenic and allergenic proteins in the pollen grain walls of *Ambrosia* ssp. (ragweeds). *Cytobios* 4:49–54
- Knox RB, Heslop-Harrison J, Reed CE (1970) Localization of antigens associated with the pollen grain wall by immunofluorescence. *Nature* 225:1066–1068
- Knox RB, Vithanage HIMV, Howlett BJ (1980) Botanical immunocytochemistry: A review with special reference to pollen antigens and allergens. *Histochem J* 12:247–272
- Lindberg RE, Pinnas JL, Jones JF (1982) Pollen-induced chemiluminescence: inhibition by serum from allergic individuals. *J Allergy Clin Immunol* 69:388–396
- Moar MH, Rosén A, Klein G (1979) Detektion of virus and cellular-determined antigens in situ using [^{125} I] protein-A and autoradiography. *J Immunol Methods* 30:219–229
- Moshakis V, Ormerod MG, Westwood JH, Imrie S, Neville AM (1982) The site of binding of anti-CEA antibodies to tumor CEA in vivo: an immunocytochemical and autoradiographic approach. *Br J Cancer* 46:18–21
- Palement JM, Kopriwa BM (1978) Use of two different methods for the electron microscope radioautographic detection of labeled antibodies bound to thin sections. *J Histochem Cytochem* 26:765–771
- Palement J, Leblond CP (1977) Localization of thyroglobulin antigenicity in rat thyroid sections using antibodies labeled with peroxidase or ^{125} I-radioiodine. *J Cell Biol* 74:992–1015
- Roth J (1982) The protein A – gold (pAg) technique: A qualitative and quantitative approach for antigen localization on thin sections. In: Bullock GR, Petrusz P (eds) *Techniques in Immunocytochemistry*, vol 1. Academic Press, New York, pp 107–133
- Spurr AR (1969) A low viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* 26:31–43
- Vithanage HIMV, Howlett BJ, Knox RB (1980) Localization of grass pollen allergen by immunocytochemistry. *Micron* 11:411–412
- 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 antibodies. *Histochem J* 14:949–966
- Zola H, Garland LG, Cox HC, Adcock JJ (1978) Separation of IgE from IgG subclasses using staphylococcal protein-A. *Int Arch Allergy appl Immunol* 56:123–145