

Cellular localization of water soluble, allergenic proteins in rye-grass (*Lolium perenne*) pollen using monoclonal and specific IgE antibodies with immunogold probes

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Summary

A postembedding method has been developed for localizing water soluble allergens in rye-grass pollen. This uses dry fixation in glutaraldehyde vapour, followed by 2,2-dimethoxypropane, prior to a 100% ethanol series leading into embedment in LR Gold. This has allowed the attachment of specific monoclonal antibodies to the allergen, which are themselves probed with specific immunogold labels to the antibodies. Wall and cytoplasmic sites have been identified, representing an improvement of fixation and localization of allergens over previous studies employing polyclonal, broad spectrum antibodies.

Rye-grass allergens are labelled in mature pollen grains in the exine (tectum, nexine and central chamber), and in the electron opaque areas of the cytoplasm, especially mitochondria. The allergens are absent from the intine, polysaccharide (P) particles, amyloplasts, Golgi bodies and endoplasmic reticulum. IgE antibodies derived from humans allergic to rye-grass pollen, bind to similar sites in the cytoplasm but only to the outer surface of the pollen grain wall. This method now provides a valuable tool for further developmental studies on the pollen grains, in order to establish the site/s of synthesis of the allergens.

Introduction

Rye-grass pollen is tricellular, comprising a vegetative cell and two sperm cells. It is important as a human allergenic agent, causing catarrh, hay fever and asthma in susceptible individuals, and is especially important in southeastern Australia (Hill *et al.*, 1979). The allergens are rapidly absorbed when pollen comes into contact with the mucosa of susceptible patients via the nasal or oral passages, or the eyes.

Active rye-grass allergens were first purified and characterized by Johnson and Marsh (1965a, b), and Marsh (1975), as glycoproteins ranging between 11–50 kDa. The four major groups have been named as *Lol p I*, *Lol p II*, *Lol p III*, and *Lol p IV* (Marsh *et al.*, 1986).

The soluble allergens are removed in water from pollen within 2 min at room temperature. This causes great difficulties in localizing allergens in the pollen grain without migration from their original sites, when aqueous staining or fixation procedures have been employed. Also, it is difficult to obtain good infiltration of pollen grains with embedding resins

used for electron microscopy. Published works on localization studies have shown results only of the pollen wall and not the cytoplasm, as structural preservation of organelles in the cytoplasm was poor or absent.

To overcome the problem of water solubility of the allergens, Knox *et al.* (1970) and Knox & Heslop-Harrison (1971a) used freeze sectioning; Howlett *et al.* (1979) and Vithanage *et al.* (1980, 1982) attempted immunocytochemical labelling of rye-grass pollen after killing in 100% methanol or by freeze drying. In these studies, the probes employed were FITC and immunoferritin labels, and antisera raised in rabbits were used as a source of polyclonal antibodies. The specificity of the antiserum was tested by immunodiffusion and immunoelectrophoresis (Howlett *et al.*, 1979). However, when tested recently on western blots of rye-grass proteins, the same antibodies not only bound to *Lol p I*, but also to a large number of other protein bands (Singh, unpublished observations). This observed cross-reactivity can be due to antibodies in the serum, directed to carbohydrate

epitopes on *Lol p I*. These glycan epitopes may be distributed on various rye-grass pollen proteins. Such antiserum showing non-specific cross-reactivity cannot now be considered as a valid reagent for localization of *Lol p I*.

Like the grasses, birch pollen allergy is a major threat to public health in the northern hemisphere (Breiteneder *et al.*, 1989). Grote *et al.* (1983, 1988) and Grote & Fromme (1984a, b) attempted to localize the allergens in birch pollen by precipitation with cetylpyridinium chloride in glutaraldehyde. However, the fixation procedure was aqueous and movement of water soluble allergens was likely before precipitation, although these workers claim to have prevented diffusion of proteins highly soluble in water, and glycoproteins from pollen grains.

Fromme *et al.* (1985) and Grote and Fromme (1986) used human IgE antibodies from strongly birch pollen-allergic patients on similar specimens, to confirm the localization of the allergens in the exine with gold-labelled antibodies. Völker *et al.* (1986) attempted to localize the hazelnut pollen allergen by precipitation with cuprolinic blue in phosphate-buffered saline, followed by immunogold labelling. As the only gold labelling occurred outside the exine, it is doubtful that this technique has fixed the allergen *in situ*. Also, poor structural preservation was evident in the pollen. Abadie *et al.* (1988) sought to localize the pollen allergens of the grass *Dactylis glomerata* with human antisera and probes linked to concanavalin A. Their most successful fixation technique was using 100% methanol. They found no concanavalin A binding to allergens in controls, and concluded that gold immunolabelling of allergens occurred only in the inner part of the exine. Any labelling of the cytoplasm was considered an artefact.

In this study, pollen grains of *Lolium perenne* (rye-grass) were not exposed to aqueous solutions when attempting localization of water soluble allergens. Instead, they were fixed in glutaraldehyde vapour, followed by 2,2-dimethoxypropane (DMP) and then the addition of chemicals only in anhydrous conditions prior to embedment. The DMP was used because it converts any residual water to acetone and methanol (Kaesler, 1989; Muller & Jacks, 1975), and the allergens are insoluble in acetone, methanol and ethanol. Thus, minimal extraction is expected with this technique. The aim is to identify the site of synthesis or accumulation of pollen specific allergens, as well as the subsequent movement of these substances to their final sites in the pollen grain at maturity. The technique of localization of allergen in mature pollen is described below.

Materials and Methods

Mature anthers (3.5–4.0 mm long) of *L. perenne* were

collected from flowering spikes from the grounds of Melbourne University and Pheasant Creek, Victoria, between December, 1988 and April, 1989.

FIXATION FOR ULTRASTRUCTURAL STUDIES

Anthers of *L. perenne* were fixed aqueously in 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, or in Karnovsky's (1965) fixative followed by 1% OsO₄. They were then dehydrated in a graded series of ethanol or acetone, following a step with 2% aqueous uranyl acetate, and infiltrated and embedded in LR White or Spurr's (1969) resins respectively. Ultrathin sections, 80 nm thick, on copper grids, were stained in both uranyl acetate and lead citrate before observation in a Siemens 101 transmission electron microscope at 60 kV.

Alternatively, anthers were fixed as above, dehydrated in ethanol and critical point-dried. The pollen was released onto stubs coated with semi-dried carbon dag and sputter coated with platinum/palladium, prior to observation in a Jeol JSM-840 scanning electron microscope.

FIXATION FOR ALLERGEN LOCALIZATION

For allergen localization, where water soluble glycoproteins were to be kept in place in the pollen grains, mature anthers were fixed in glutaraldehyde vapour for 24 h, followed by immersion in cold DMP for 24 h, and then taken through a graded series of DMP–100% ethanol over 2 days. They were then infiltrated with LR Gold resin over 2 days, and polymerization was effected at room temperature, so that the hardening process took at least 12 h, thus avoiding overheating of the specimen. This was achieved by polymerization of LR Gold resin with 3% benzoyl peroxide paste in sealed gelatin capsules. Ultrathin sections, 80 nm thick, of the anthers were mounted on picroform coated gold grids.

Controls were employed in which the DMP step was omitted from the above treatment. Also, results were compared with pollen fixed only with aqueous glutaraldehyde in phosphate buffer, followed by ethanol dehydration and embedment in LR Gold resin (see Table 1).

SPECIFICITY OF MONOCLONAL ANTIBODY (MAB) PROBE

A mixture of three different monoclonal antibodies was used in this study. MAb 40.1 was kindly supplied by Dr I. J. Smart (Smart *et al.*, 1983); MAb 21.3 and MAb 3.2 were made available to us by courtesy of Dr D. G. Marsh (Kahn & Marsh, 1986). Both MAb 40.1 and MAb 21.3 seem to recognize similar epitopes, while MAb 3.2 binds to a different epitope on the allergen molecule (Knox *et al.*, 1989). In the present study, the specificity of all three antibodies to *Lol p I* was reevaluated by immunoblotting analysis; 150 µg of rye grass pollen proteins were used in each lane of SDS-PAGE gel, and the antibodies were diluted 1:1000 in PBS (phosphate-buffered saline = 3.3 mM monobasic sodium phosphate, 6.7 mM dibasic sodium phosphate, 149 mM sodium chloride, pH 6.8) with 1% BSA (bovine serum albumen, from Sigma Chemical Co., St Louis, MO, USA) before use. The methods of allergen extract preparation, SDS-PAGE and immunoblotting were the same as described by Singh & Knox (1985).

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Preparation of Lol p I specific affinity purified IgE antibodies

The allergen specific IgE antibodies were purified from the sera of rye-grass allergic patients by adapting the procedure described by Olmsted (1988). Briefly, multiple lanes of rye-grass pollen extract were separated on SDS-PAGE, and then electroblotted onto a nitrocellulose membrane (BA 85 S&S), 0.45 µm in thickness. The position of Lol p I was located by Ponceau S staining, and the long strip of nitrocellulose containing that region was cut out. This was followed by destaining in water, and then blocking the excess protein binding capacity of nitrocellulose; the strips were incubated overnight at room temperature in pollen-allergic sera (1 part serum: 1 part 0.5% BSA in PBS). After one washing with PBS containing 0.1% Tween-20, and then three washes in PBS alone, the antibodies bound to Lol p I were eluted by treating the blots with 0.1 M glycine-HCl in 1% BSA at pH 2.6 for 5 min. The pH of the eluant was then adjusted to 7.5 with a predetermined quantity of 1 M Tris. Such eluted antibodies were tested for their Lol p I specificity by immunoblotting, using ¹²⁵I-anti Human IgE as a detection reagent (Sutton *et al.*, 1982). Following verification of the specificity of IgE antibodies to Lol p I, the eluant was used as such for immunolocalization experiments.

In a control experiment, sera from patients with no evidence of rye-grass atopy (allergy) was used and carried through the whole affinity purification procedure, as described above.

DETECTION OF ALLERGEN BY MONOCLONAL ANTIBODIES (MAbs)

Standard treatment

This treatment was adapted from the MAb labelling of sporopollenin by Southworth *et al.* (1988). Gold grids with ultrathin sections were washed with 1% BSA in PBS for 20 min. This was followed by the mouse anti-rye-grass allergen antibodies (the mixture of three monoclonal antibodies described above) further diluted to 1:200 in PBS with 1% BSA and incubated for 1 h. Grids were washed in PBS for 15 min, and then treated with a gold-goat-anti-mouse IgG auroprobe (AuGAM), of 15 nm particle size (Janssen Life Sciences Products, Belgium) diluted 1:10 in PBS-BSA, for 1 h. They were then washed in PBS for 15 min, followed by rinsing in double distilled water, fixed in 1% glutaraldehyde and post stained in uranyl acetate and lead citrate.

Control treatments. Various controls were performed as suggested by Poole *et al.* (1983), to determine the specificity of the immunocytochemical localization. A method control was carried out by omitting the MAb step. An antibody specificity control was used, in which sections were treated with Lol p I protein, obtained by affinity purification. These were preincubated for 1.5 h with the MAb in order to reduce the level of receptor mediated recognition of allergenic sites on the section, before probing with the AuGAM. Fixation controls were performed by either omitting DMP during processing of the rye-grass anthers, or by utilizing an aqueous, rather than anhydrous, fixation (see Table 1).

DETECTION OF ALLERGEN BY HUMAN IgE ANTIBODIES (IgEAH)

Standard treatment

Grids with sections of rye-grass pollen were washed with PBS-BSA for 20 min; 200 µl samples of IgE antibodies from rye-grass allergic patients (IgEAH), affinity purified by binding to Lol p I, were diluted in PBS-BSA and incubated on the sections for 1 h.

Grids were washed in PBS for 15 min, and then treated with a rabbit-anti-human IgE antibody (DAKO Immunoglobulins) in PBS-BSA for 1 h. After washing in PBS for 15 min, a gold-goat-anti-rabbit IgG auroprobe of 15 nm particle size, diluted 1:10 in PBS-BSA, was added for 1 h. Grids were then washed in PBS for 15 min, followed by rinsing in double distilled water and stained for TEM as previously described.

Control treatments. Antibodies from non-allergic patients (IgENAH) were used in place of antibodies from allergic patients (IgEAH), as a sera specificity control. Method controls were performed in which either the IgEAH or IgERAH step, or both these steps, were omitted (see Table 2).

Results

ULTRASTRUCTURE

Pollen grains of *L. perenne* are spheroidal and uniporate, with sculpturing comprised of papillae, each with a variable number of excrescences (Figs 1–3). The pollen wall has an exine and an intine, as viewed by normally fixed and stained ultrathin sections (Figs 3, 4 and 6). The exine is comprised of: (i) an outer layer – the *tectum*, 0.3–0.7 µm in width, which includes the excrescences on papillae, over (ii) a central chamber called the *interbacular cavity*, 0.6–0.9 µm in width, which is traversed by anticlinal columellae or baculae and whose contents are usually removed by aqueous fixation procedures; (iii) an inner layer – the *nexine*, which is usually narrower than the tectum, being 0.2–0.3 µm wide (Figs 3, 4 and 6). All three layers are traversed by microchannels or micropores 0.04 µm wide (Figs 4 and 6). These microchannels are numerous, as illustrated by a tangential section of the exine (Fig. 5), are very often cylindrical but occasionally branched (Figs 4 and 6).

The intine, a wall layer immediately internal to the exine, is thinner than the exine, being usually 0.3–0.4 µm in thickness. This wall layer may be stained in such a way that it shows electron-opaque areas which are microchannels joining the plasmalemma with the exine (Figs 6 and 7). There is also evidence of another electron-dense staining reaction (LP) in the intine by lead citrate, with or without osmium postfixation, but in the absence of *en bloc* staining with uranyl acetate prior to, or during, dehydration (Figs 4 and 6).

Sections through the germinal pore area reveal an extra electron-opaque wall layer normally called the Z-layer (Heslop-Harrison & Heslop-Harrison, 1980;

Table 1. Results of monoclonal antibody treatments and controls applied to *Lolium perenne* pollen grain sections.

Pollen part	Standard treatment*	Method control (omit MAb)	Antibody specificity control (premix Lol p I with MAb)	Fixation controls (no DMP)	(aqueous fixation)
Exine	+	–	–	–	–
Intine	–	–	–	–	–
E.O. cyt.	+	–	+	+	+
P part.	–	–	–	–	–
Amyl.	–	–	–	–	–
Mit.	+	–	–	+	+
Nucleus	+	–	–	–	+

+: gold label above background level; –: no significant label; *Standard treatment = (GV, DMP, EtOH, LR gold), MAb, AuGAM; GV: Dry fixation with glutaraldehyde vapour; DMP: 2,2-dimethoxypropane; MAb: Lol p I monoclonal antibody; AuGAM: Gold-goat-anti-mouse probe; IgE AH: IgE purified from serum from humans allergic to rye-grass, IgE NAH: IgE purified from serum from humans not allergic to rye-grass; IgERAH: Rabbit-anti-human IgE antibody; AuGAR: Gold-goat-anti-rabbit probe; Lol p I: Rye-grass affinity purified allergen; GAQ: Aqueous fixation with glutaraldehyde. Amyl. = amyloplast; Mit = mitochondrion; E.O. cyt. = electron opaque cytoplasm; P part. = P particle.

Table 2. Results of IgE antibody treatments and controls applied to *Lolium perenne* pollen grain sections.

Pollen part	Standard treatment*	Sera specificity control (replace IgEAH with IgENAH)	Method controls (omit IgERAH)	(omit IgEAH)	(omit IgERAH & IgEAH)
Exine	+	–	–	–	–
Intine	–	–	–	–	–
E.O. cyt.	+	–	–	–	–
P part.	–	–	–	–	–
Amyl.	–	–	–	–	–
Mit.	+	–	–	–	–
Nucleus	+	–	–	–	+

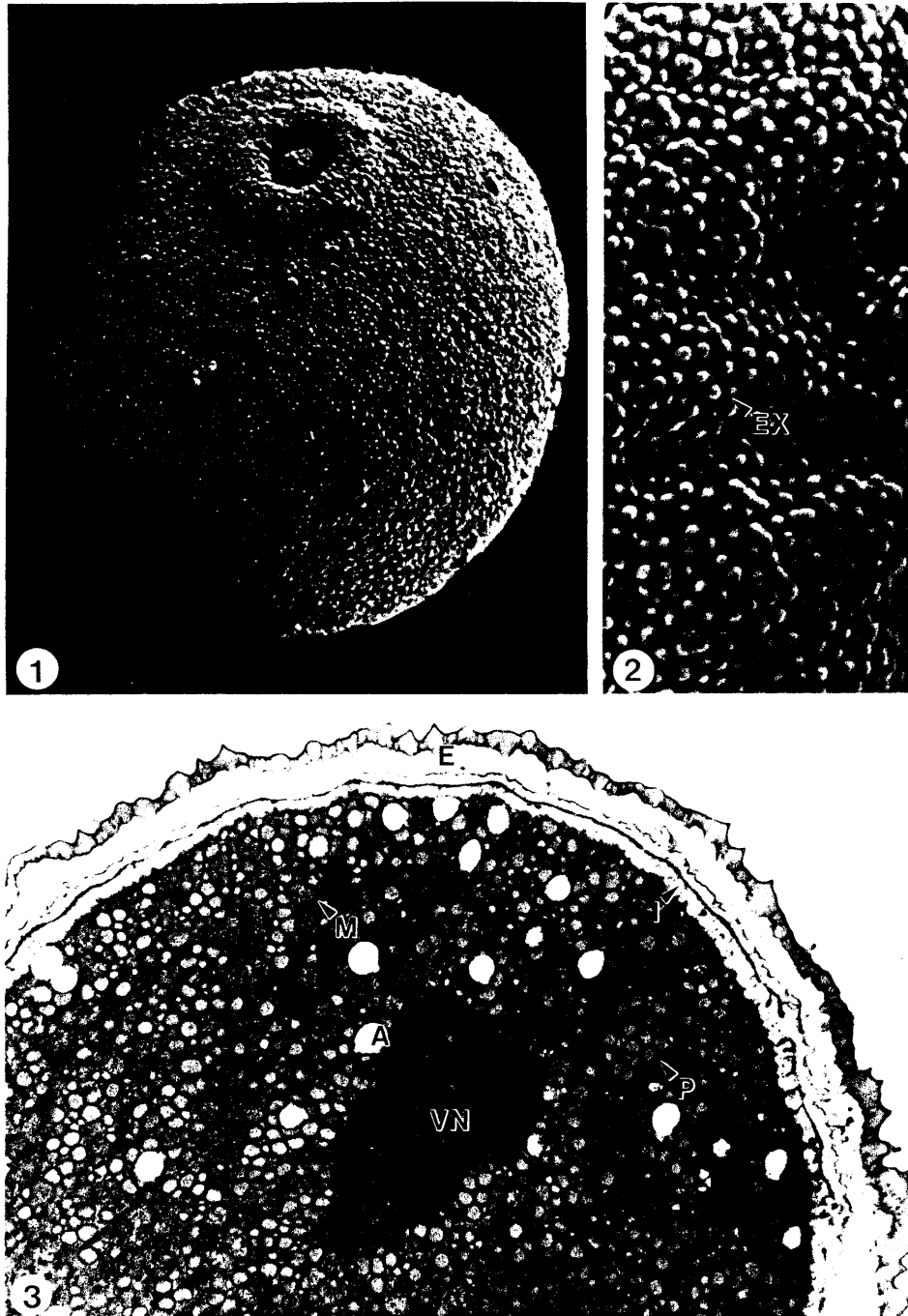
+: gold label above background level; –: no significant label; *Standard treatment = (GV, DMP, EtOH, LR gold), IgEAH, IgERAH, AuGAR; abbreviations as in Table 1.

Abbreviations used: A, amyloplast; B, bacula; D, dictyosome; E, exine; EX, excrescences; ER, endoplasmic reticulum; I, intine; IC, interbacular cavity; M, mitochondria; MC, microchannels; N, nexine; P, P particles; SN, sperm nucleus; LP, lead staining particles; T, tectum; VN, vegetative nucleus; Z, Z-layer.

Fig. 1. Scanning electron micrograph of whole mature pollen of *L. perenne*, with apertural pore. ×4750.

Fig. 2. Scanning electron micrograph of whole mature pollen of *L. perenne*. The sculptured pollen surface consists of excrescences (EX) on papillae. ×15000.

Fig. 3. *L. perenne* sections from anthers fixed in glutaraldehyde and osmium, and embedded in Spurr's resin. The contents of the pollen cytoplasm include an evaginated vegetative nucleus, spheroid P particles, mitochondria, and electron lucent amyloplasts enveloped by the intine and a sculptured exine. TEM, ×9000. E = exine; I = intine; P = P particles; VN = vegetative nucleus; A = amyloplast; M = mitochondria.



Knox, 1984). This forms an intermediate and an outer layer of the intine, and extends beyond the pore area into the wall of the rest of the pollen grain, tapering markedly (Fig. 8). The intine also has a greater electron opacity under the pore than in the rest of the pollen wall. Scanning electron microscopy reveals a central raised platform in the pore, also showing sculpturing similar to that seen on the rest of the grain (Fig. 1). The bacula situated near the rim of the pore shows great irregularity, appearing to be branched reticulately (Fig. 8).

The cytoplasm has peripherally, rounded, membrane-bound organelles known as P particles (Figs 3, 4, 6, 8, 10, 11 and 16). These have been shown in pollen of other species to be polysaccharides and are believed to be secreted into the pollen walls (Heslop-Harrison, 1979a). Endoplasmic reticulum, ribosomes and dictyosomes are present (Fig. 7), as are mitochondria (Figs 3, 4, 7, 10, 15 and 17). Numerous amyloplasts are also present, and these can be found adjacent to the plasmalemma but they extend to greater depths in the pollen grain (Figs 3, 4). However, each grain has a discrete region of cytoplasm mostly free of amyloplasts, in which the male germ unit is located. There is an evaginated vegetative nucleus (Figs 3, 13 and 19), and two smaller sperm cells which are recognizable by the electron-lucent periplasmic space around them (Figs 14 and 18).

MAB-gold probes

The three MABs used in this study bind specifically to the *Lol p* 1 28–35 kDa protein component in rye-grass pollen (Fig. 9).

The mix of MABs used here has identified rye-grass pollen allergens in their cellular sites, despite their water soluble properties. Figures 10–15 and 19 allow a comparison between gold labelling and a control in a representative pollen grain section. In Fig. 10, the gold particles are concentrated over the exine and electron opaque areas in the cytoplasm, and are absent from the intine, except for background label. Gold particles have also labelled starch grains (Figs 10 and 11), and are not significantly present over P particles (Figs 10, 11 and 12). The only organelles with clear labelling of

concentrated numbers of gold particles are the mitochondria (Fig. 10) and vegetative nuclei (Fig. 13). Sperm nuclei also show limited gold label (Fig. 14). Endoplasmic reticulum and Golgi bodies were rarely found, and were not labelled with significant numbers of gold particles. The exine was shown to have retained electron-dense contents in its internal chambers, and in the microchannels in which labelling with gold probes occurred. The control in which MAB was omitted gave no gold label above background (Fig. 19).

Comparison with pollen fixed traditionally with glutaraldehyde in phosphate buffer, followed by osmic acid, allows us to confirm the identities of the organelles seen in the dry-fixed materials used for immunogold labelling (Figs 3, 4, 6 and 7).

Table 1 shows a summary of results of the immune reactions on controls as well as test specimens. The control using glutaraldehyde vapour fixation, with the omission of subsequent treatment of DMP before infiltration and embedment, showed a weak localization of allergen in the electron-opaque portion of the cytoplasm (Fig. 21). The preservation of organelles was less clear than in the standard treatment using DMP (cf Figs 11, 13–18 and 24). The control using aqueous glutaraldehyde fixation also displayed a weak localization of allergen in the electron opaque portion of the cytoplasm (Figs 22 and 23), as did the control using *Lol p* 1 protein preincubated with the MAB (Fig. 24). The use of aqueous fixation or ethanol in place of DMP resulted in little label being detected on the wall and outer regions of the cytoplasm (Figs 21–23).

IgE-gold probes

Affinity purified *Lol p* 1 specific IgE antibodies were prepared from pooled sera of allergic patients using a protocol detailed in 'Materials and methods' and confirmed in Fig. 9. Experiments reported here indicate the binding of allergens of rye-grass pollen with the use of human IgE antibodies derived from rye-grass allergic patients, followed by auroprobes. The major sites of localization were in the cytoplasm, mitochondria, the sperm nucleus, and on the surface of the exine (Figs 16–18). Controls using comparable

Fig. 4. The exine is comprised of an outer layer, the tectum, over a central chamber, the interbacular cavity which is traversed by baculae; and an inner layer, the nexine. Immediately internal to the exine is the intine which shows up a stain precipitate. TEM, $\times 22000$. T = tectum; IC = interbacular cavity; LP = lead staining particles; M = mitochondria; A = amyloplast; N = nexine; B = bacula; I = intine; P = P particles.

Fig. 5. Tangential section of the exine, showing numerous microchannels. TEM, $\times 50000$.

Fig. 6. Cross section of pollen grain showing both branched and unbranched microchannels in the exine. Detail of the intine, showing lead staining particles and microchannels, the intine microchannels and exine microchannels are coincident at the intine-exine interface (arrows). TEM, $\times 48000$. MC = microchannels; LP = lead staining particles.

Fig. 7. Numerous ribosomes occur individually and in association with endoplasmic reticulum. Mitochondria and dictyosomes are also present in the cytoplasm. TEM, $\times 24000$. M = mitochondria; D = dictyosome; ER = endoplasmic reticulum.

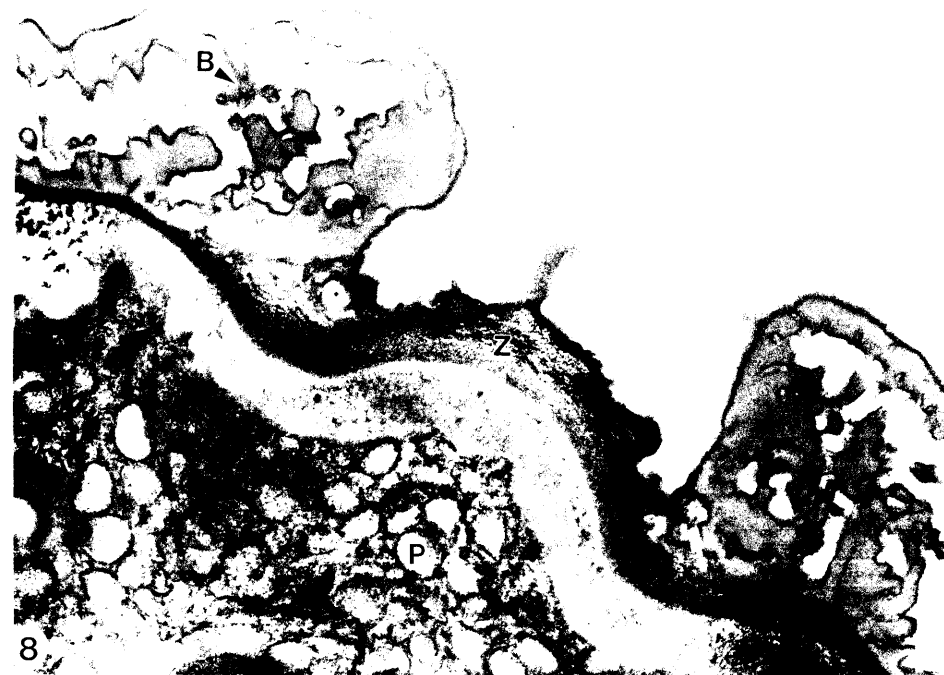
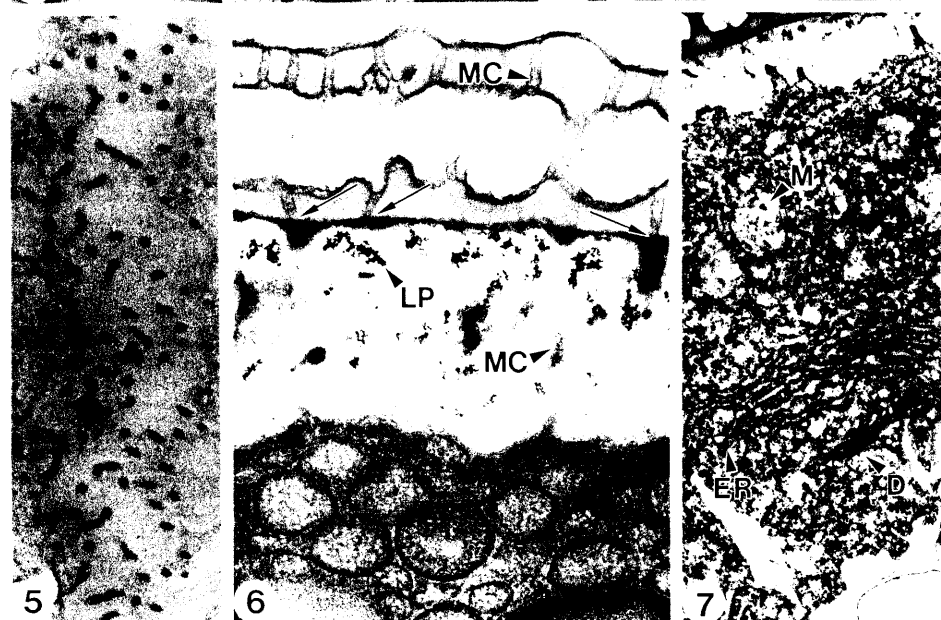
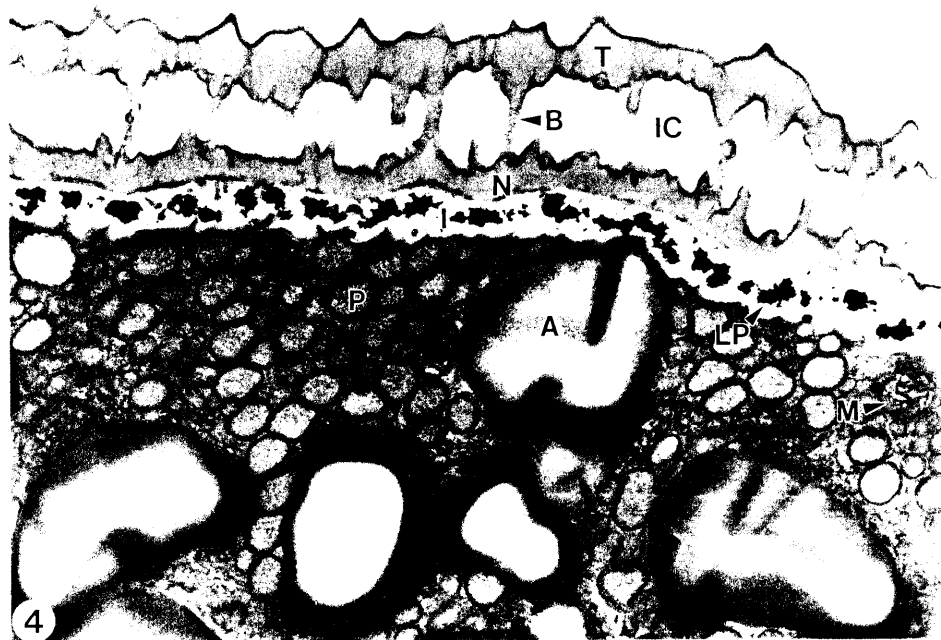


Fig. 8. Section through the aperture pore shows the Z-layer, an electron dense wall layer of the outer intine, and a centrally raised platform in the pore. Irregularly branched baculae are located about the rim of the pore. TEM, $\times 23\,000$. P = P particles; Z = Z-layer; B = bacula.

antibodies from non-allergic patients showed no significant localization of allergens (Fig. 20). Omission of the various steps in the standard treatment for IgE gave negative results in all cases, with the exception of a nuclear accumulation after omission of IgERAH and IgEAH (Table 2).

Throughout the various labelling treatments employed here, there was no significant label detected on the cell of the anther wall.

Discussion

ULTRASTRUCTURE

Previous ultrastructural studies of *L. perenne* mature pollen grains have described the surface structure of exine, the nature of exine, intine and the complement of organelles (Knox, 1979). This study provides new information about the exine of this species from pollen fixed with aqueous fixative. The microchannels of both tectum and nexine, which are mostly cylindrical, have limited branching. This property has been demonstrated by Abadie *et al.* (1988) in another grass, *Dactylis*. Also, the microchannels do not enter the

papillae of the pollen surface wall, as shown in the diagram of Knox (1979), and their length is much greater. The papillae are shown to be aggregated into clumps, with excrescences ranging between 2–7 in number, not as described by Peltre *et al.* (1987). However, these workers viewed the pollen after acetolysis, a treatment that may have altered the pollen wall somewhat. The central chamber of the exine, the interbacular cavity, is traversed by vertical columellae (baculae) in which microchannels are not usually evident. However, most of these observations have been made when the central chamber is empty, after aldehyde fixation (Heslop-Harrison, 1979a; see Figs 3, 4 and 6). Such treatment allows the grains to hydrate and expand. It is likely that expansion of the chamber during fixation has caused stretching of the columellae (see Fig. 4), and may have masked their visualization (see Fig. 19).

The intine has been shown to consist of lead staining particles, suggesting the presence of clumped glycoproteins (Hayat, 1981). Microchannels have also been observed in this layer, in some instances, opposite the microchannels of the nexine (see Fig. 6). Knox & Heslop-Harrison (1971b) used 2-naphthyl-thiol phos-

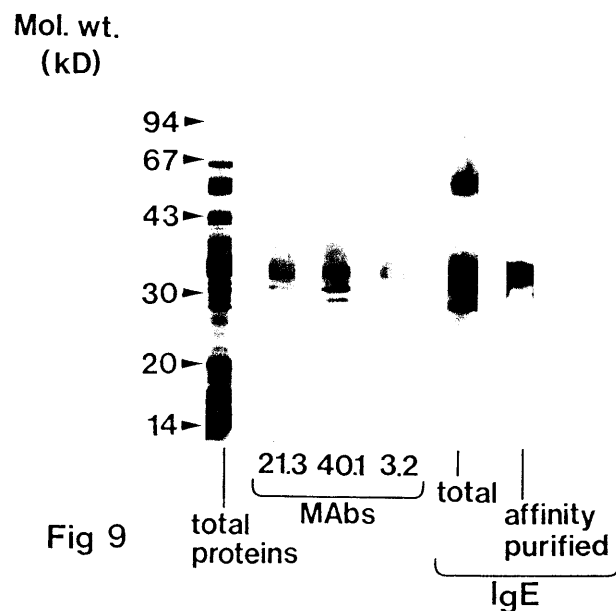


Fig. 9. Immunoblotting analysis of antibodies using SDS-PAGE Gels. The three MAbs (3.2, 21.3 & 40.1) bind specifically to the *Lol p* 128–35 kDa protein components in rye-grass pollen. Pooled IgE and affinity-purified *Lol p* 1 specific IgE antibodies also bind to the 28–35 kDa region.

phate, followed by Fast Blue BBN and osmication, to localize the enzyme acid phosphatase in similar channel-like evaginations in the intine of *Crocus* pollen grains.

MAb-gold probes

The pollen grains of terrestrial angiosperms are partially dehydrated at the time of dispersal from the anther (Heslop-Harrison, 1979b). When the water

content of pollen is reduced below 20%, membranes no longer adopt a lipid bilayer conformation (Simon, 1974). Rehydration is an essential prelude to germination, and when pollen grains are placed in contact with water, soluble cell constituents leak out for up to several minutes, until membrane integrity is reestablished (Simon, 1974). To minimize the exudation of allergenic proteins, it was necessary to fix pollen anhydrously prior to probing with antibodies.

Fig. 10. Section of *L. perenne* after MAb labelling, showing sites of *Lol p* 1 localization. The allergen has labelled heavily in the electron opaque regions of the cytoplasm and the mitochondria and to a lesser extent in the exine but it is absent from the intine, P particles and amyloplasts. TEM, $\times 26\,000$. E = exine; I = intine; M = mitochondria; P = P particle; A = amyloplast.

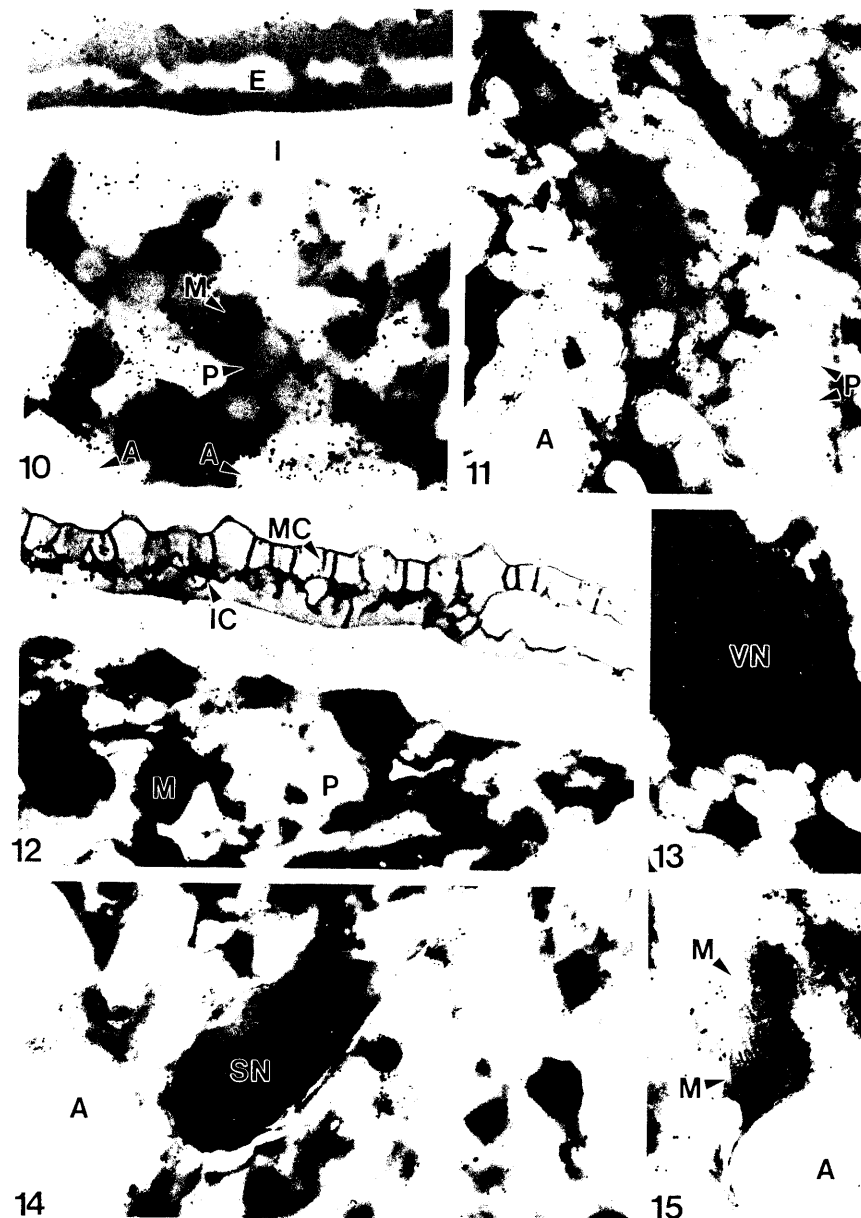
Fig. 11. *Lol p* 1 localization. The allergen labelling is most dense about the periphery of the tightly packed P particles (P) and amyloplasts (A). TEM, $\times 23\,000$.

Fig. 12. The standard labelling procedure also produced a binding of the gold probe in the interbacular cavity, the contents of which have been preserved in this section, and also to the microchannels of the exine. TEM, $\times 23\,000$. MC = microchannel; IC = interbacular cavity; P = P particle; M = mitochondria.

Fig. 13. The vegetative nucleus (VN) showed heavy labelling. TEM, $\times 23\,000$.

Fig. 14. The sperm cell nucleus (SN) attracted a light but significant labelling. TEM, $\times 23\,000$. A = amyloplast.

Fig. 15. Detail of the cytoplasm showing some labelling in the intact mitochondria (M). TEM, $\times 34\,000$. A = amyloplast.



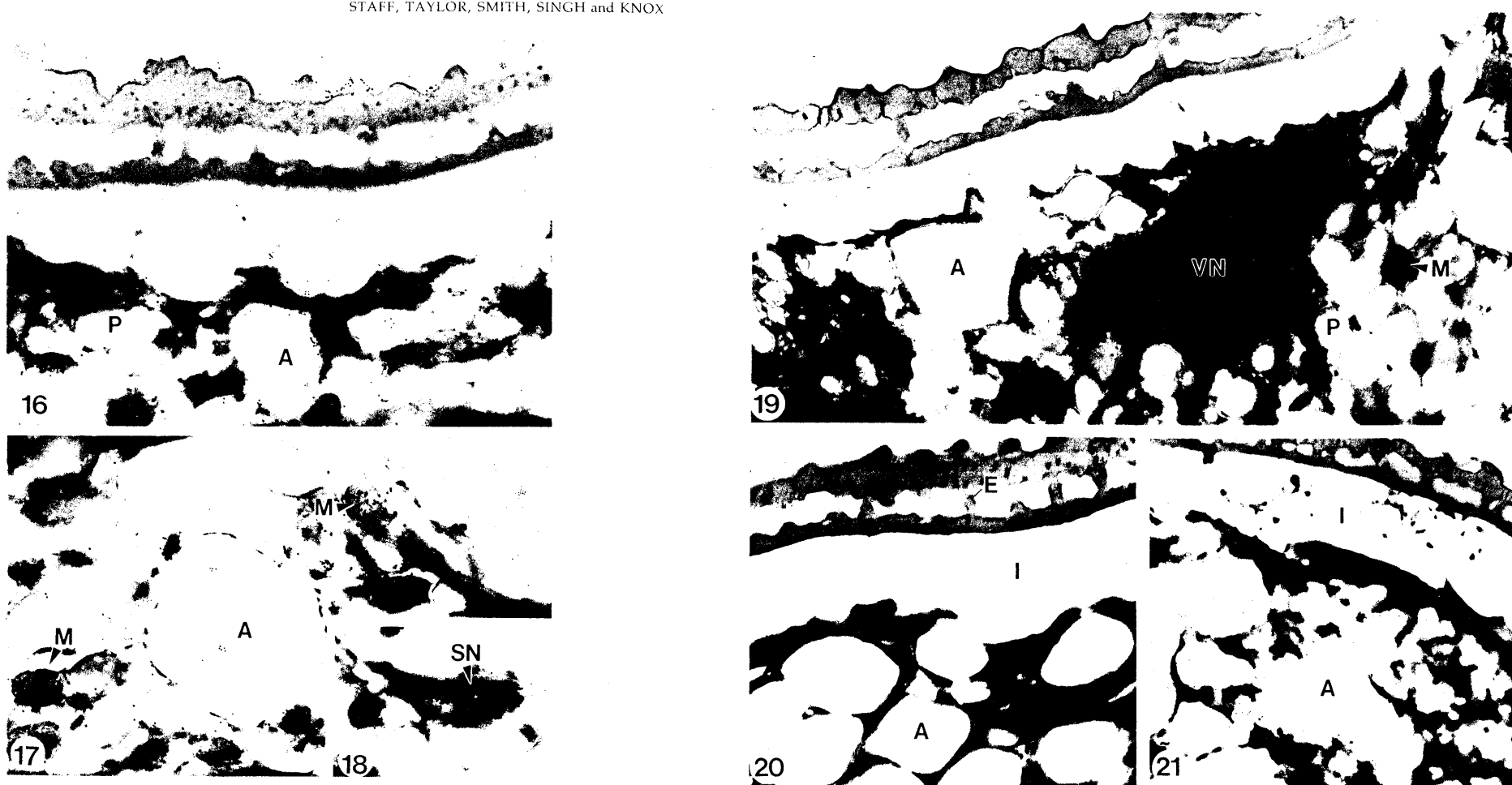


Fig. 16. *L. perenne* sections after IgEAH labelling. The label has localized on the exine surface and electron opaque regions of the cytoplasm but is not significant in the intine, P particles (P) and amyloplasts (A). TEM, $\times 23\,000$.

Fig. 17. The label is also clearly present on mitochondria (M). TEM, $\times 23\,000$. A = amyloplast.

Fig. 18. The sperm cell nucleus (SN) attracted a heavy labelling. TEM, $\times 23\,000$.

To enhance staining sensitivity, a combination of three MABs was utilized (Lin *et al.*, 1983). The gold-labelled probes bound specifically to the surface of the exine wall of the grains, and to cytoplasmic sites. The occurrence of heavy gold-labelling following specific MAB treatment is confined to the electron-opaque cytoplasm of the pollen grain, and coincides with the distribution of large numbers of ribosomes and ground cytoplasm, as found from the ultrastructural

observations. Preliminary observations on labelling of developing pollen with the MABs indicates a similar pattern of localization, as found in the mature pollen.

Previous studies of protein synthesis and storage in a different system, cereal endosperm, reveal that proteins are synthesized at the ribosome area of rough endoplasmic reticulum. There are two distinct pathways – in prolamine synthesis in maize (Khoo & Wolf, 1970; Larkins & Hurkman, 1978), rice (Oparka &

Harris, 1982; Krishnan *et al.*, 1986) and sorghum (Taylor *et al.*, 1985); these proteins are then deposited in the lumen of the endoplasmic reticulum. In the case of glutelin synthesis in rice (Krishnan *et al.*, 1986), transfer from ribosomes of the rough endoplasmic reticulum is via the Golgi complex, especially Golgi vesicles, and then to the protein bodies (Kim *et al.*, 1988). Proteins for secretion are synthesized on the membranes of the rough endoplasmic reticulum and transported to the cell surface by secretory vesicles formed at the Golgi apparatus (Jones & Robinson, 1989).

The absence of significant labelling of our probe in the endoplasmic reticulum and Golgi apparatus may indicate a novel mechanism of protein synthesis in the production of the *Lol p I* allergen. The rye-grass allergenic proteins are products of haploid gene expression. A cDNA library prepared by using mRNA, isolated from mature pollen grains, contains the clones having allergen encoding nucleotide sequences. Northern hybridization results have shown that allergenic mRNA is expressed only in pollen (Knox *et al.*, 1989).

The absence of a significant label in the intine and inner exine layers of the pollen grain, and the overall low level of labelling when anthers were fixed aqueously or after replacement of DMP with ethanol, is most likely the result of leaching of the allergens during processing. The allergen is water soluble, readily passing through the plasma membrane upon contact of the pollen with an aqueous solution. Localization of the allergens in the exine and its central chamber would appear to be a result of secretion of the allergen from its cytoplasmic site of synthesis during pollen development. The finding of heavy labelling in the mitochondria is difficult to interpret, although it may be an accumulation related to the ability of the allergens to cross membranes of partially dehydrated pollen, or blocking of the mitochondrial active ion

pump during fixation and subsequent inflow of water to the cristae (Yoshida & Sato, 1968). The mitochondria also possess nucleoids, and nuclear material has been shown here to attract the MAB-gold probe. Labelling of the nuclei in the pollen grain may be non-specific, as found with gold probes and monoclonal antibodies to photosynthetic fructose-1,6-bisphosphatase in pea leaf tissue (Hermoso *et al.*, 1989), leghemoglobin in the cytoplasm of nitrogen-fixing root nodules of pea (Robertson *et al.*, 1984), and abscisic acid in tissues of *Chenopodium polyspermum* (Sossountzov *et al.*, 1986).

IgE-gold probes

The preliminary reports of localization of allergens with IgE were performed using human sera without purification (Fromme *et al.*, 1985; Grote & Fromme, 1986). This sera may not be monospecific, but it probably cross reacts with a number of other proteins.

Localization of the allergen reported here, using human IgE antibodies derived from grass pollen-allergic patients, was similar in pattern to the results obtained with monoclonal antibodies to *Lol p I* raised in mice. These results have demonstrated that human IgE antibodies, produced as a result of contact with allergenic proteins, recognize the same specific allergenic molecules in the rye-grass pollen. This is the first report of the use of affinity purified IgE, specific to *Lol p I*, to localize a pollen allergen.

It will be interesting to use this new technique that we have developed, for detecting allergens during pollen development. This will enable the sites of synthesis, subsequent accumulation and specialized function of this novel family of proteins to be established.

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Localization of water soluble, allergenic proteins in *L. perenne*

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Fig. 19. *L. perenne* sections after various control treatments. Here, the labelling procedure was performed with the omission of the MAB step. No significant label was evident on the section. TEM, $\times 23000$. VN = vegetative nucleus; P = P particle; M = mitochondria; A = amyloplast.

Fig. 20. When IgEAH was replaced with IgE sera from non allergic humans, no significant labelling occurred. TEM, $\times 23000$. E = exine; I = intine; A = amyloplast.

Fig. 21. The absence of DMP during fixation resulted in poor preservation of cytoplasmic organelles. However, a light labelling of the cytoplasm was evident after the standard MAB treatment. No label was present in the peripheral regions of the pollen grain. TEM, $\times 17000$. I = intine; A = amyloplast.

Fig. 22. When the anthers were fixed aqueously in glutaraldehyde and the standard MAB treatment was applied, a light labelling of the mitochondria and electron-opaque cytoplasm occurred, but no significant label was present on the exine, intine, endoplasmic reticulum, P particles or amyloplasts. TEM, $\times 20000$.

Fig. 23. The vegetative nucleus (VN) was also lightly labelled after the same treatment as in Fig. 22. TEM, $\times 17000$.

Fig. 24. When the MAB was incubated with *Lol p I* prior to labelling, little significant binding of probe occurred on the section. TEM, $\times 17000$. E = exine; I = intine; A = amyloplast; M = mitochondria; P = P particle.

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Post embedding immunoelectron microscopy of human breast cancer: a comparison of three acrylic resins

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Summary

The suitability of three acrylic resins for the immunoelectron microscopical localization of cell surface and cytoskeletal antigens in surgically excised, immersion fixed human breast cancer, using an immunogold system, has been assessed.

Good localization of milk fat globule membrane was achieved with LR White, LR Gold and Lowicryl K11M, although the embedding schedule for LR White had to be modified. The best results were achieved with Lowicryl K11M. Only scanty labelling of actin and cytokeratin was seen in LR White embedded tissue, whereas there was clear localization in LR Gold and Lowicryl K11M embedded samples. Tubulin and α -actinin was detected at low level in tissues in the low temperature embedding resins, but not in LR White embedded samples. The morphology of the latter was poorer, and there was greater variability in ultrastructure and labelling.

Of the two low temperature embedding resins, Lowicryl K11M gave slightly better results. However, the advantages could be outweighed by the problem incurred in achieving the low temperatures, and by poorer handling properties than LR Gold.

Introduction

Postembedding immunoelectron microscopy is a more widely applicable approach for the ultrastructural localization of antigens, than the various pre-embedding methods (Newman & Jasani, 1984), made easier by the advent of acrylic resins as embedding agents. To achieve optimal results, there must be sufficient retention of antigenicity balanced with a high standard of ultrastructure. This could be more difficult to achieve with surgically excised tissue which requires immersion fixation, and which may have a dense stroma such as in human breast tissue.

The present evaluation was undertaken as a preliminary to a more detailed immunoelectron microscopical study of cell surface (milk fat globule membrane) and cytoskeletal antigens in surgically-excised human breast cancer. These cytoplasmic components may exhibit differences in retention of antigenicity, and the aim was to find a method suitable for allowing correlation of their localization in serial sections.

LR White has been used successfully by some workers, particularly for the localization of stored hormones (Newman *et al.*, 1983; Maldonado & Aoki, 1986). However, the resin is infiltrated at room temperature and cured at 50°C. These temperatures, together with the fixation schedules recommended for preservation of antigenicity, may result in denaturation and extraction of components by dehydrating

agents and resin monomer (Newman & Hobat 1987). McPhail *et al.* (1987) found that LR Gold, and aromatic crosslinked acrylic resin which can be used at low temperatures, gave good preservation of ultrastructures and antigenicity for a wide variety of tissues and markers. The lowest usable temperature is –25°C, which may limit the preservation of some antigens. Lowicryl K4M, the aliphatic crosslinked acrylic resin, can be cured by ultraviolet radiation at temperatures down to –35°C (Carlmalin *et al.*, 1982), with good preservation of fine cellular structure and greater specificity of labelling (Roth *et al.*, 1981; Roth, 1986). Lowicryl K4M has been successfully used for the ultrastructural labelling of secretory proteins (Roth *et al.*, 1981), mitochondrial proteins (Bendayan & Shore, 1982) and cytoskeleton (Armbruster *et al.*, 1983). A new hydrophilic Lowicryl resin, K11M, has been developed with which even lower temperatures can be used for embedding, possibly further improving preservation of antigenicity (Acetarin *et al.*, 1986).

This study compares LR White, LR Gold and Lowicryl K11M, with optimization of conditions for their suitability for the immunoelectron microscopical localization of cell surface and cytoskeleton antigens of surgically excised human breast cancer. The effect of different fixation regimes has also been considered.