

Immunofluorescent localization of two water-soluble glycoproteins including the major allergen from the pollen of ryegrass, *Lolium perenne*

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Received 26 August 1980 and in revised form 2 December 1980

Summary

Two major glycoproteins have been localized in sectioned grains of ryegrass pollen by direct and indirect immunofluorescence methods using Fluorescein isothiocyanate (FITC)-labelled IgG fractions of antisera. These glycoproteins are the major allergen, Group 1 allergen, and a principal antigen, Antigen A. Four methods of fixation were employed: freeze-drying, methanol, 2.5% glutaraldehyde and 4% paraformaldehyde for 1 h at 4° C. The post-embedding staining technique of immunocytochemistry was used: anthers were embedded directly, or after dehydration, in JB-4 plastic resin and antibody reacted with sectioned pollen.

The effects of these fixatives on the antibody combining sites of the antigens were quantified by a solid phase radioimmunoassay using [¹²⁵I]protein A to measure antibody binding. Glutaraldehyde was the only fixative to significantly depress antibody binding of both Antigen A and Group 1 allergen to their homologous antisera. This radioimmunoassay was modified to reveal that FITC conjugation to either antibody did not impair antigen binding.

In mature pollen, these antigens were located in the cytoplasm and in the complex wall. In developing grains early in the maturation period, specific fluorescence was concentrated at the periphery of the cytoplasm.

Introduction

Airborne grass pollen is an important cause of allergic disease in temperate climates (Marsh, 1975). The allergens held in the pollen grains are defined by their effects in man and have no known role in the plant. They are proteins and glycoproteins, and four groups of allergens, named Groups I–IV (Johnson & Marsh, 1965; Marsh, 1975), have been described in ryegrass pollen. The major allergen, Group 1 allergen, is an acidic glycoprotein, mol. wt. 32 000, which exists in four multiple

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electrophoretic forms differing only slightly in amino acid composition. We have recently purified group 1 allergen by ion exchange and size chromatography (Howlett & Clarke, 1981). A second ryegrass pollen glycoprotein, Antigen A, with a mol. wt. of 68 000 has a low allergenic activity when compared with Group 1 allergen (Smart & Knox, 1980). It has been possible to isolate Antigen A by making use of its high isoelectric point (Howlett & Clarke, 1980). The purpose of this study has been to localize these antigens with mature and developing ryegrass pollen grains.

Using a freeze-sectioning technique, we have previously shown that the diffusible antigens of canary grass (*Phalaris tuberosa*) are laid down in the inner intine wall sites at the vacuolate period of development (Knox & Heslop-Harrison, 1971). This technique could not be applied successfully to mature pollen without hydrating it and consequently mobilizing the allergens to the grain surface. Mature grass pollen grains resemble a seed in being progressively dehydrated during the final stages of development until the water content is reduced to 10–30% (see review by Heslop-Harrison, 1979). In this study, the structure of the grains has been preserved by a freeze-drying fixation treatment in a non-aqueous environment with the intention of reducing the opportunity for the antigens to diffuse during processing.

However, because aldehyde fixation has been successfully employed in other immunocytochemical studies of plant antigens (Knox *et al.*, 1980), we have examined its effects in this study. We have found previously that the diffusible antigens could not be detected in the walls of other pollen types after aldehyde fixation (Knox *et al.*, 1975). In this investigation, we have used the post-embedding staining technique of immunocytochemistry, in which the antigens are infiltrated and embedded in the glycol methacrylate resin JB-4 at 4° C to prevent loss of antigenic activity by the prolonged processing by heat or u.v. treatment needed for polymerization of previous glycolmethacrylate resins (Feder & O'Brien, 1968; Ruddel, 1971). This method has the advantage that sectioned grains may be uniformly exposed to labelled antibody solutions, with a minimum loss of antigens by diffusion. The plastic sections produced are of suitable thickness for high resolution light microscopy, while the method may be readily adapted for electron microscopy.

In a recent review in this journal, we presented preliminary findings using anthers fixed in methanol (Knox *et al.*, 1980). The pollen grains in the plastic sections are spherical, and not slightly shrunk as might be expected from their dehydrated state, suggesting that this procedure may have involved a degree of hydration. In this paper, freeze-drying has been employed in an effort to avoid this problem.

Materials and methods

Formaldehyde was purchased from May and Baker, Dagenham, England. JB-4 resin and glutaraldehyde (70% v/v) were obtained from Polysciences, Inc., Warrington, PA, U.S.A. Protein A and protein A–Sepharose CL-4B were purchased from Pharmacia Ltd, Uppsala, Sweden. Iodogen was obtained from Pierce Chemical Company, IL, U.S.A. Biogel P2 and P6

were purchased from Biorad Laboratories, CA, U.S.A. Sodium iodide¹²⁵ was obtained from the Radiochemical Centre, Amersham, England. Microtitre trays were purchased from Cooke Laboratories, VA, U.S.A. Fluorescein isothiocyanate (FITC) and Auramine O were from Difco, Surrey, England. Toluidine Blue was purchased from Chroma-Gesellschaft, Schmid & Co., Stuttgart, Germany. Calcofluor White M2PS was from American Cyanamid Company, NJ, U.S.A. Bovine serum albumin and Coomassie Brilliant Blue R250 were purchased from Sigma, MI, U.S.A. FITC-conjugated goat anti-rabbit serum was from Behringwerke Ag, Marburg-Lahn, Germany. Eukitt non-fluorescent mounting medium was from Kindler, Freiburg, Germany.

POLLEN SOURCE

Anthers for cytochemical work were obtained from field grown plants in Melbourne. Flowering spikes were usually collected in the early afternoon prior to the principal flowering period of ryegrass in Melbourne, which occurs late in the afternoon (Smart & Knox, 1979). Mature anthers were collected by dissection from florets about to open; anthers 12–24 h prior to flowering were collected by holding the spikes as cut flowers in water until the afternoon's flowering was over (2–3 h after cutting), then dissecting out spikelets bearing freshly dehiscid anthers and selecting anthers from the oldest floret present. Anthers at earlier developmental periods were selected as described by Vithanage & Knox (1979).

FIXATION AND TISSUE PREPARATION

Anthers were fixed in anhydrous methanol or freshly prepared 2.5% glutaraldehyde or 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4, at 4° C for 1 h and dehydrated in an ethanol series. Alternatively, anthers were lyophilized by freezing them in a pre-cooled tube in dry ice or directly in liquid Freon held in liquid nitrogen and then placing them on the freeze-drier (Dynavac High Vacuum Pty Ltd., Burwood, Australia) for 16 h. After fixation the anthers were embedded in JB-4 resin using a slight modification of the technique described in the JB-4 embedding kit.

Anthers were infiltrated directly into the JB-4 catalyst A solution for 3 h at 4° C. To 2 ml of catalyst A, 50 µl of solution B was added and rapidly stirred for 5 min. This mixture was poured into cylindrical vial caps and the anthers were embedded in the mixture for 16 h at 4° C. Air was excluded during polymerization of resin by placing Parafilm across the top of the resin-filled vial. The JB-4 blocks were stored at 4° C until sectioning. Using a Cambridge rocker microtome, sections of 2–4 µm thickness were cut, mounted in water and stretched. Alternatively, sections were pressed dry onto pre-dried 1% gelatin-coated slides, or flattened onto clean slides in absolute methanol or polyethylene glycol, to prevent release of mobile antigens from the sections. Slides were stored at 4° C prior to use.

IMMUNOFLUORESCENCE METHODS

Antisera against purified Group 1 allergen and Antigen A (Howlett & Clarke, 1981) were prepared in rabbits following the schedule of Livingston (1974). The IgG population was isolated from the antiserum using the method of Hjelm *et al.* (1972) which depends on the specificity of binding of the Fc fragment of IgG to staphylococcal protein A. Antiserum (10 ml) was loaded onto a column containing 5 ml protein A-Sepharose CL-4B in 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS). The unbound material was washed through and discarded, while the bound material was eluted with 0.1 M acetic acid and immediately dialysed against several changes of bicarbonate buffer (0.05 M NaHCO₃, in 0.15 M NaCl, pH 9.5). The concentration of IgG was adjusted to within the range of 30–50 µg/ml. FITC-conjugation was carried out as described by Goldman (1968). FITC was added to the bicarbonate buffer (20 mg in 200 ml) and dialysis sacs containing the IgG were immersed over

night at 4° C. Unreacted FITC was removed from conjugated IgG by chromatography on a Biogel P2 column (10 × 1.8 cm). The FITC-IgG was eluted with water, frozen and lyophilized, and stored at -20° C. The average number of FITC molecules bound to each IgG molecule was determined by the method of Goldman (1968) whereby the absorbances of the conjugates at 495 nm and 280 nm were measured. For both FITC-anti-Group 1 allergen and FITC-anti-Antigen A the average number of FITC molecules per IgG molecule was 1.9.

IMMUNOLOGICAL TECHNIQUES

The FITC-IgG conjugates were examined by immunodiffusion (Ouchterlony, 1958) or by radioimmunoassay to determine whether the antigen binding sites on the IgG molecules were markedly impaired. The radioimmunoassay used was a modification of the technique of Marier *et al.* (1979) and was performed at room temperature in 96-well, flexible polyvinylchloride microtitre plates to which proteins and glycoproteins adsorb.

Antigen A, Group 1 allergen or *L. perenne* pollen extract (40 µl of 50 µg/ml) were added to each well. After 2 h, the unadsorbed antigens were removed by washing the wells three times with 1% bovine serum albumin (BSA) in PBS. The remaining potential antibody binding sites were saturated by the BSA. Dilutions of FITC-IgG or IgG (80 µl) in BSA-PBS were added to the plate and incubated for at least 2 h. The plate was washed (three times with BSA-PBS and eight times with water) and [¹²⁵I]protein A (80 µl containing 2 × 10⁴ ct/min) added to each well. After 2 h the plate was again extensively washed, the wells cut out and individually counted in a deep well gamma counter (Packard Instruments Company, IL, U.S.A.).

A modification of this technique was used to examine the effect of fixatives on the binding of the antigens to the antisera. Antigens were applied to the wells for 2 h then fixatives (80 µl) were added for varying times at 4° C. For the freeze-drying treatment, antigen-coated trays were placed at -20° C for 1 h then placed on the freeze-drier for 16 h. After all the fixation treatments, the wells were washed in BSA-PBS then a large excess of antibody added. [¹²⁵I]Protein A was then delivered as described above.

IODINATION OF PROTEIN A

Protein A was iodinated by a slight modification of the Iodogen technique described by Fraker & Speck (1978). Iodogen (4 mg) was dissolved in 1 ml methylene chloride and then diluted 1 in 20 with methylene chloride. The bottom of a glass tube was coated with 100 µl of this solution and dried by a stream of nitrogen. The tube was rinsed with 2 × 300 µl of phosphate buffer (0.6 M sodium phosphate, pH 7.5) to remove excess Iodogen flakes. Protein A (100 µl of 1 mg/ml in the phosphate buffer), Na[¹²⁵I] (5 µl containing 100 µCi) and the phosphate buffer (200 µl), were added to the tube which was shaken intermittently at 4° C for 5 min. The reaction was stopped by the addition of saturated tyrosine (100 µl) to the tube. The unbound iodide was removed by chromatography of the solution on a Biogel P6 column (5 × 1 cm). Fractions (200 µl) were collected and the radioactivity monitored on a gamma counter. The iodinated protein A was eluted in 5 × 200 µl fractions which were pooled and stored at 4° C. The specific activity of protein A was 1 × 10⁶ ct/min/µg.

IMMUNOFLUORESCENT STAINING

Direct method

Sections of ryegrass pollen were incubated in the FITC-IgG preparation from pre-immune rabbit serum (control) or anti-Group 1 allergen or anti-Antigen A sera. FITC-IgG (2-4 mg) was dissolved in 0.5 ml PBS and one drop placed over the sections for 40 min at room temperature in a moist Petri plate. After incubation, the sections were gently washed in several changes of PBS for 10 min, rinsed in distilled water, air-dried and mounted in Eukitt (a non-fluorescent

mounting medium) following the basic procedure developed for plant tissues by Knox (1971). This method enhances microscopical resolution, without increasing autofluorescence, in addition to giving a permanent preparation. Sections were viewed by incident fluorescence microscopy, using a Carl Zeiss IIRS incident illumination system with KP490 and KP500 exciter filters, and Rfl 510, and LP528 barrier filter. Photographs were taken with Kodak Tri-X panchromatic film, using a standard exposure of 5 s, so that test and control sections can be directly compared for fluorescence intensity.

Indirect method

Specific IgG or pre-immune rabbit IgG (40 μ l of 2–4 mg/ml) was placed over the section. After 40 min, the sections were gently washed in several changes of PBS during a 10 min period, before incubation in FITC–goat anti-rabbit IgG for 40 min at room temperature. After a further 10 min period of gentle washing in PBS, the sections were rinsed briefly in distilled water and air-dried, before being mounted in Eukitt. Alternatively, sections were mounted directly in glycerol without drying and were viewed by fluorescence microscopy and photographed as described above.

Cytochemical methods

A number of high resolution cytochemical methods were used to define the exine and intine layers of the pollen grain wall. The exine was visualized by its fluorescence after staining with Auramine O (Heslop-Harrison, 1976), by observation of autofluorescence and by Toluidine Blue staining (Southworth, 1974). The polysaccharides of the intine were detected by the periodic acid–Schiff (PAS) reaction. In addition, the fluorescent brightener Calcofluor M2R New, known to stain cell wall polysaccharides (Maede & Ishida, 1967) including the intine of *Secale* and *Populus* pollen (Heslop-Harrison, 1979; Ashford & Knox, 1980) was used (0.01% in water). The protein stain Coomassie Blue was used to detect proteins in the sections (0.25% in acetic acid : ethanol : water, 1:3:6 by vol.). Cytochemical detection of the enzyme acid phosphatase was carried out as described by Knox & Heslop-Harrison (1970) using α -naphthyl acid phosphate as substrate in a simultaneous coupling reaction with hexazotized Pararosaniline.

Results

SPECIFICITY OF ANTISERA

Antiserum to the purified Group 1 allergen preparation was tested by immunodiffusion against both the original antigen and whole pollen extract and two precipitin bands were obtained (Fig. 1A). Antigen A produced a single precipitin band against its homologous antiserum by immuno-diffusion (Fig. 1B). However, when tested against whole pollen diffusate, an additional faint precipitin band was detected. No cross-reactivity was detectable between Antigen A and anti-Group 1 allergen serum, nor between Group 1 allergen and anti-Antigen A serum by immunodiffusion techniques.

EFFECT OF FIXATION ON ANTIGENIC DETERMINANTS

The effects of methanol, paraformaldehyde, glutaraldehyde and freeze drying on the antibody combining sites of the antigens were tested by a modification of the

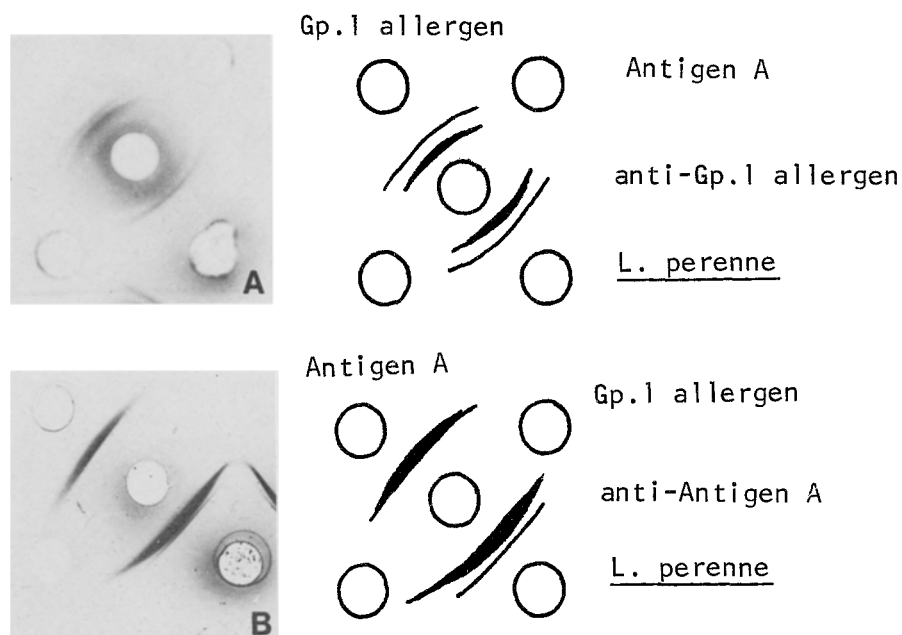


Fig. 1. Immunodiffusion analysis of (A) anti-Group 1 allergen serum and (B) anti-Antigen A serum used for immunofluorescence studies.

radioimmunoassay. As far as possible, the conditions of the anther fixation were reproduced in experiments in the plastic microtitre trays. The only fixative to reduce markedly the binding of specific antisera to antigen was 2.5% glutaraldehyde (Fig. 2) which reduced binding by 15%. Methanol, paraformaldehyde or freeze-drying did not significantly affect antibody binding.

For Group 1 allergen-anti Group 1 allergen IgG and Antigen-A-anti Antigen A IgG the glutaraldehyde treatment was significantly different from the control ($P = 0.01$) using a one-way analysis of variance.

EFFECT OF FITC ON ANTIGEN BINDING CAPACITY OF SPECIFIC IgG

Both FITC-IgG and unconjugated IgG were tested by immunodiffusion and the radio immunoassay for their capacity to bind their eliciting antigen. The FITC did not alter the antigen binding capacity of either anti-Group 1 IgG or anti-Antigen A IgG for their respective antigens (Fig. 3).

CYTOCHEMISTRY OF THE POLLEN WALL OF MATURE RYEGRASS POLLEN

Light microscope cytochemical methods have been used to define the structure of the grass pollen wall. The exine was delineated by its yellow-orange autofluorescence and by its brilliant yellow fluorescence after staining with Auramine O (Fig. 4B). It appeared as a double line around the periphery of the grain. It was not stained by

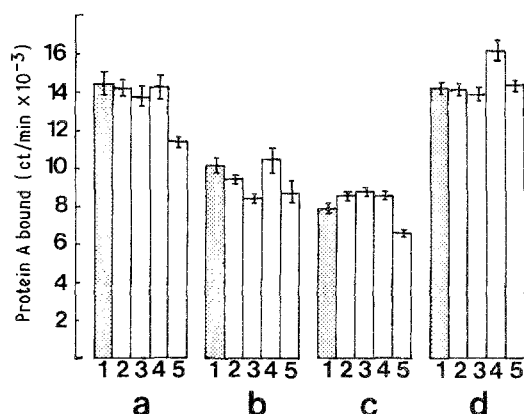


Fig. 2. Effects of various fixation treatments on antibody binding of Group 1 allergen and antigen A of ryegrass pollen as detected by radioimmunoassay. Microtitre tray wells were coated with *L. perenne* pollen extract, Group 1 allergen or Antigen A and then fixation treatments performed in the antigen coated wells. Treatments: (1) untreated antigen; (2) antigens freeze-dried for 16 h; (3) anhydrous methanol for 1 h at 4°C; (4) 4% paraformaldehyde for 1 h at 4°C; (5) 2.5% glutaraldehyde for 1 h at 4°C. After each treatment, homologous IgG was added and binding was detected by [¹²⁵I]protein A (20×10^3 ct/min/well). (a)–(d) refer to the antigen–antibody system (a) Group 1 allergen – anti-Group 1 IgG; (b) *L. perenne* pollen extract – anti-Group 1 IgG; (c) Antigen A – anti-Antigen A IgG; (d) *L. perenne* pollen extract – anti-Antigen A IgG. The histogram shows counts of [¹²⁵I]protein A bound and markers indicate standard error of mean of at least four replicates. For (a) and (c), treatment 5 was significantly different from the other treatments ($P = 0.01$) using a one-way analysis of variance.

the PAS reaction (Fig. 4A), or by Toluidine Blue except that the innermost nexine layer, apparently that termed the Z-layer by Heslop-Harrison (1979), was stained dark green. The intine was stained red by the PAS reaction, especially at the aperture where it forms a plug-like structure (Zwischenkorper) (Fig. 4A). The meso-apertural intine formed a thin PAS-positive line around the inner face of the nexine. The intine was unstained by Calcofluor or Toluidine Blue, except at the apertural plug which was stained red by Toluidine Blue. After staining with Coomassie Blue, the exine stained pale blue, the intine purple, and the cytoplasm deep purple (Fig. 4C).

In order to check that the resin-embedding technique employed had not abolished the activity of enzymic proteins in the intine of the pollen grains, acid phosphatase activity was demonstrated after the various treatments. Acid phosphatase activity was present in the intine at the aperture, and in the peripheral cytoplasm of the grains in all treatments.

LOCALIZATION OF ANTIGENS IN POLLEN FROM MATURE ANTHERS USING THE DIRECT METHOD

In order to minimize the problem of autofluorescence (cf. Knox *et al.*, 1980), material

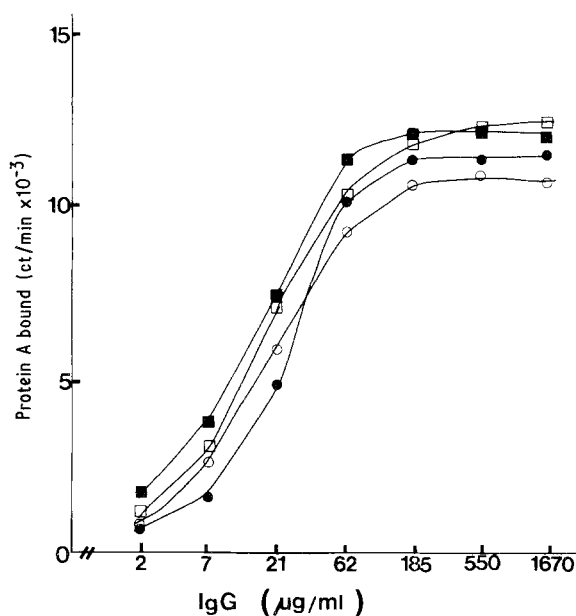


Fig. 3. Effect of FITC conjugation upon binding of IgG to specific antigen, as detected by radioimmunoassay. Wells were coated with either Antigen A or Group 1 allergen and serial dilution of homologous FITC-IgG or homologous IgG were added. Total ct/min protein A added 20×10^3 . ■—■ Group 1 allergen and homologous IgG. □—□ Group 1 allergen and FITC-homologous IgG. ●—● Antigen A and homologous IgG. ○—○ Antigen A and FITC-homologous IgG. Each point is the mean of two determinations. Background and normal IgG controls were 150 ct/min.

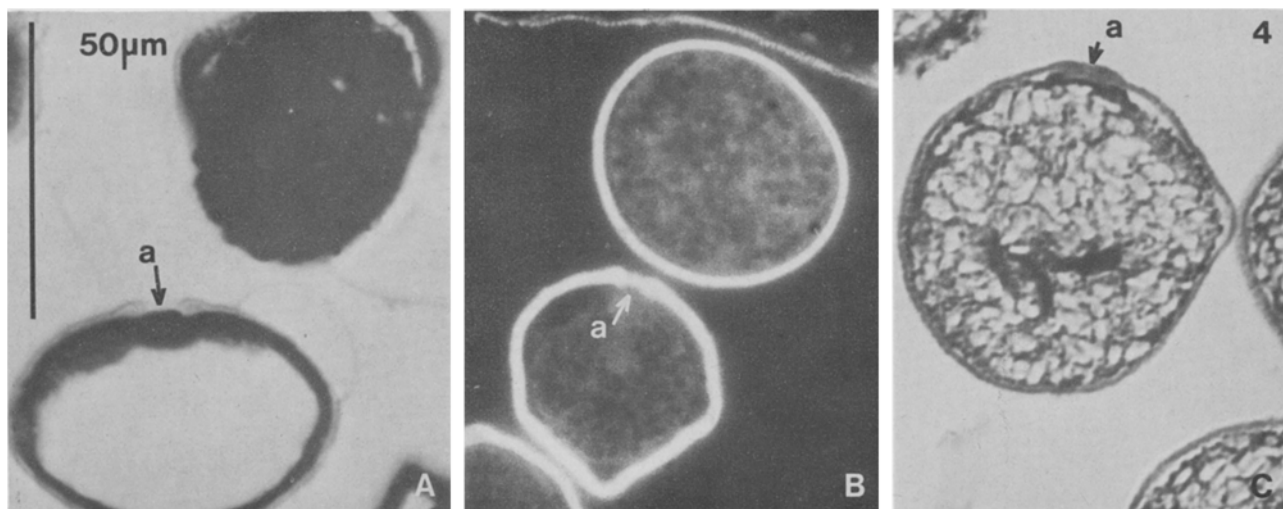


Fig. 4. Cytochemistry of the ryegrass pollen grain. Anthers fixed in methanol. (A) PAS reaction for carbohydrate showing positive staining of intine. (B) Auramine O, showing intense fluorescence of exine. (C) Coomassie Blue staining for proteins. a, aperture.

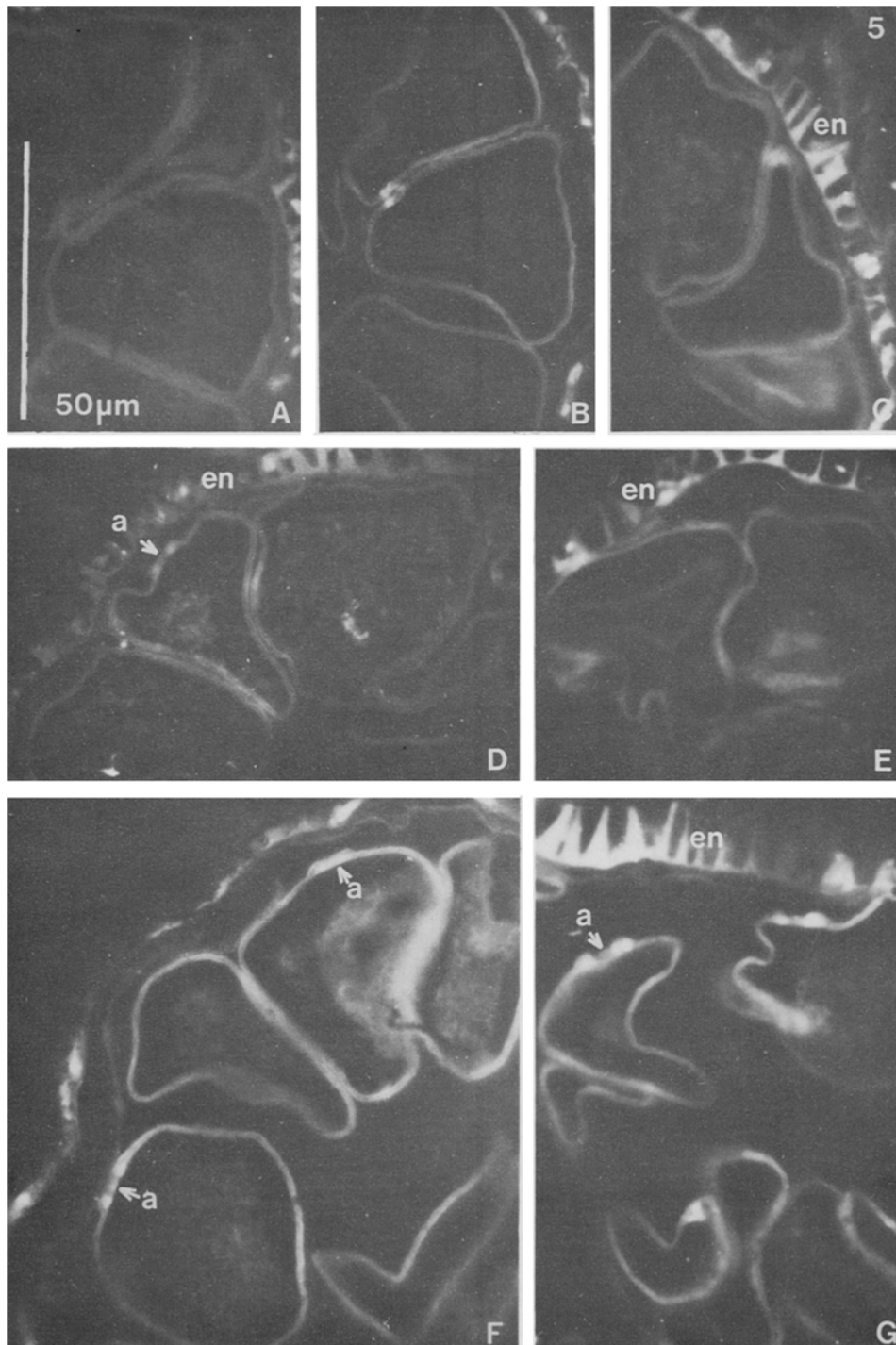


Fig. 5. Immunofluorescent localization of antigens in freeze-dried anthers of ryegrass using sections mounted in water (A–C) or in methanol (D–G). (A) Control, treated with FITC-pre-immune IgG. (B) Test section treated with FITC-anti-Group 1 IgG. (C) Test section treated with FITC-anti-Antigen A serum. Note non-specific autofluorescence of radial wall thickenings of endothecium (en). (D) Autofluorescence of unstained section. (E) Control, treated with FITC-pre-immune IgG. (F) Test section treated with FITC-anti-Antigen A IgG. (G) Test section treated with FITC-anti-Group 1 allergen. Specific fluorescence associated with cytoplasm and wall sites. a, aperture of pollen wall. (B–G) Scale as shown in (A).

was freeze-dried, embedded, sectioned and processed for immunofluorescence studies as rapidly as possible.

Freeze-dried pollen

In these anthers, the grains were shrunken irregularly, but had remained *in situ* within the anthers as the apertures were generally disposed in a regular manner towards the orbicule-lined surface of the loculus (Fig. 5F, G). In preliminary tests, using the direct method of immunofluorescence, the plastic sections were floated on water for stretching and mounting. FITC fluorescence of the grains was not visibly greater than the autofluorescence of controls (Fig. 5A–C). However, when sections were floated on methanol or polyethylene glycol, or simply pressed dry onto adhesive-coated slides, differences were obtained (Fig. 5D–G). Specific FITC fluorescence was associated with the cytoplasm and pollen wall layers.

Aldehyde-fixed anthers

After both glutaraldehyde and paraformaldehyde fixation for 1 h at 4° C, both control and test sections showed high levels of exine and autofluorescence. Cytoplasmic autofluorescence was noticeable after glutaraldehyde fixation (Fig. 6A, B) and specific FITC-fluorescence could not be detected (Fig. 6A–D).

LOCALIZATION OF ANTIGENS IN POLLEN FROM DEVELOPING ANTHERS USING THE INDIRECT METHOD

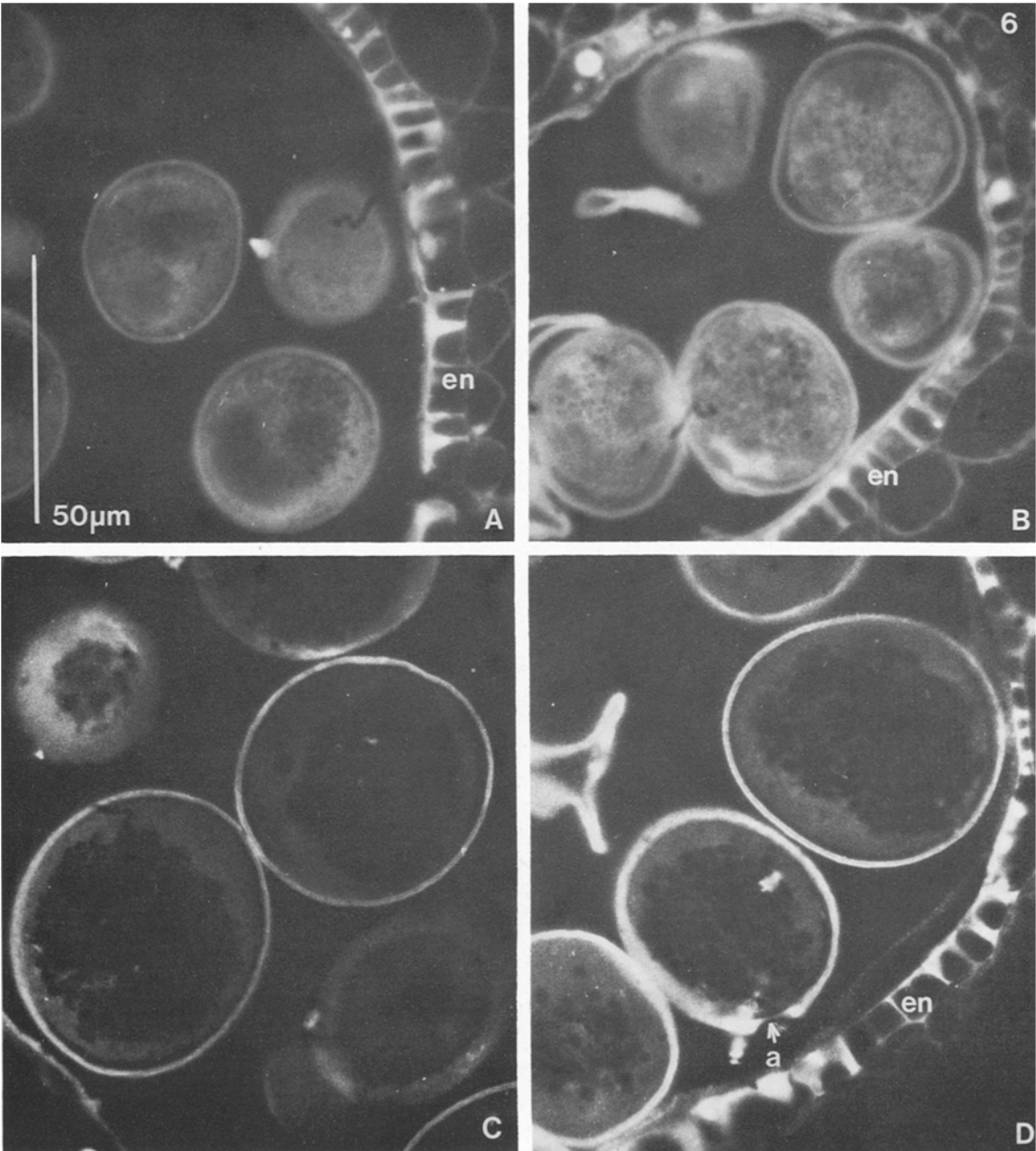
In these studies the indirect method was used, as it gave a greater intensity of specific FITC-fluorescence than the direct method (cf. Knox *et al.*, 1980).

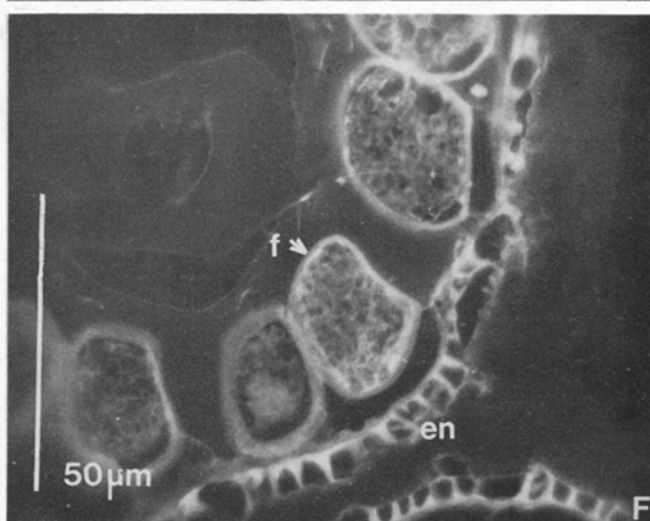
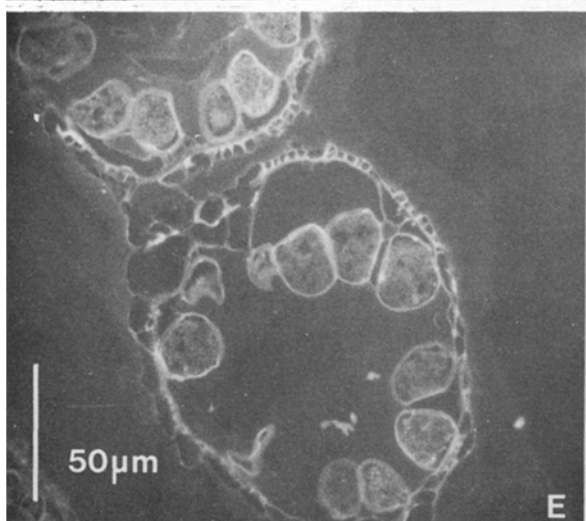
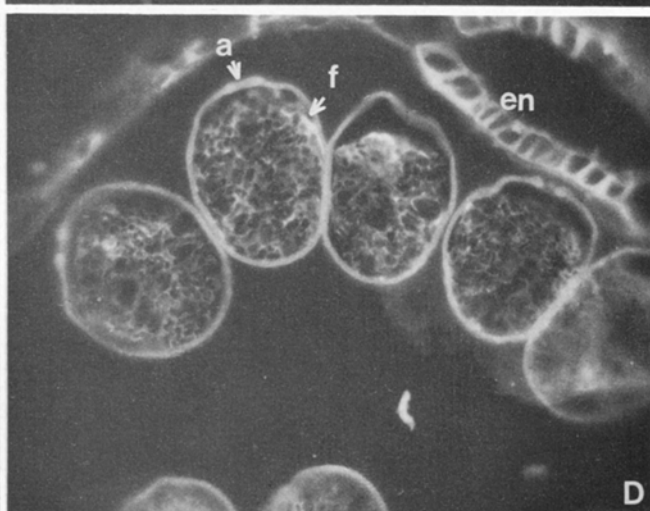
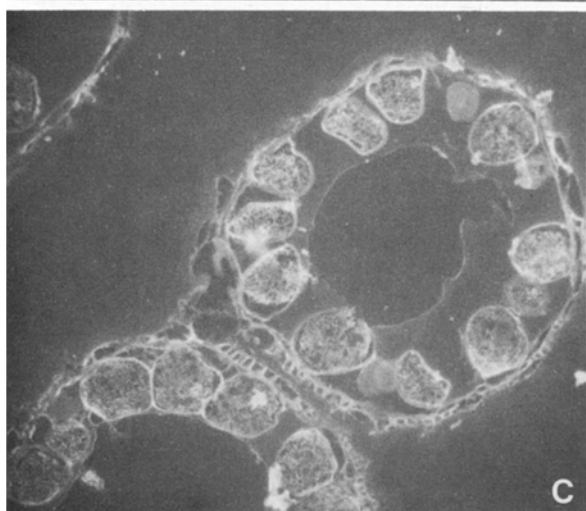
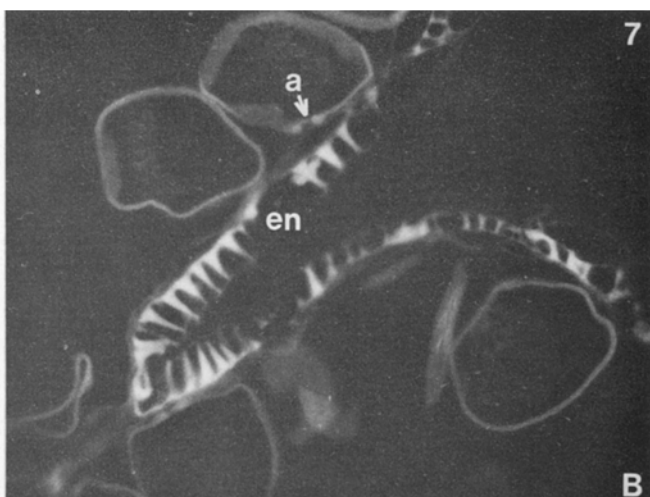
Fig. 6. Use of direct immunofluorescence technique following aldehyde fixation of anthers. (A and B) paraformaldehyde, (A) control treatment with FITC-pre-immune IgG; (B) treated with FITC-anti-Group 1 allergen IgG. (C and D) glutaraldehyde, (C) treated with FITC-pre-immune IgG; (D) treated with FITC-anti-Antigen A IgG. Note absence of any specific fluorescence in test sections. (B–D) Scale as shown in (A).

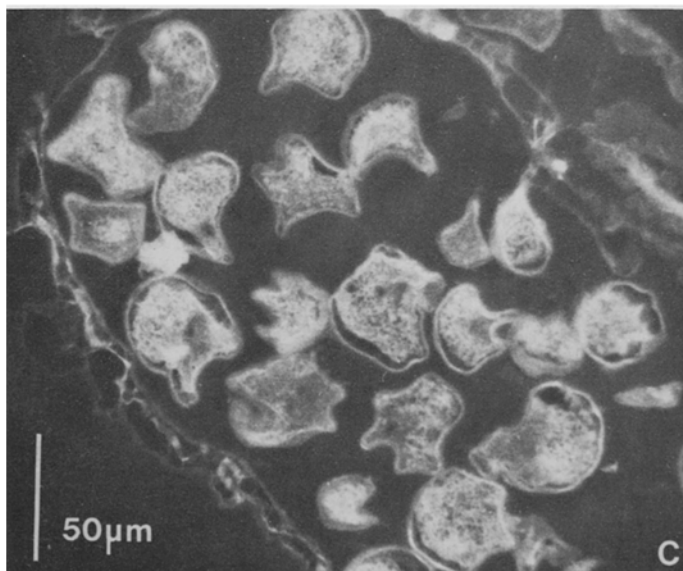
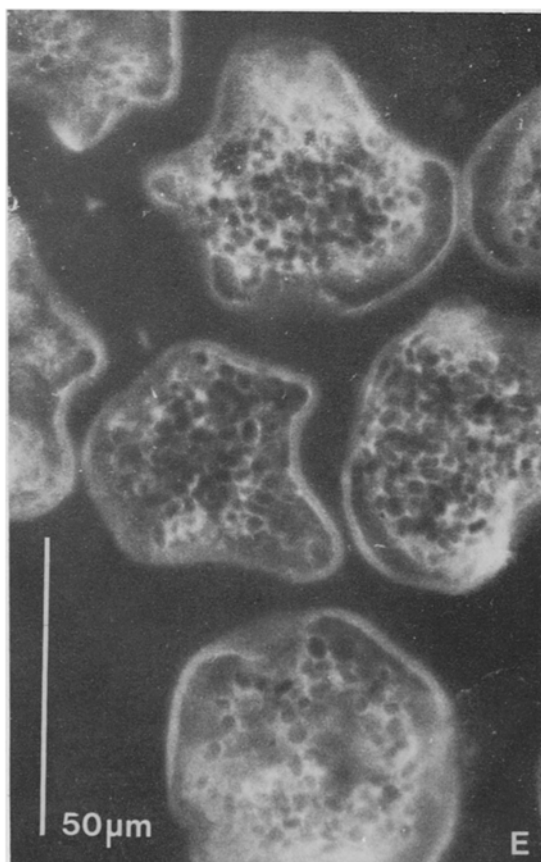
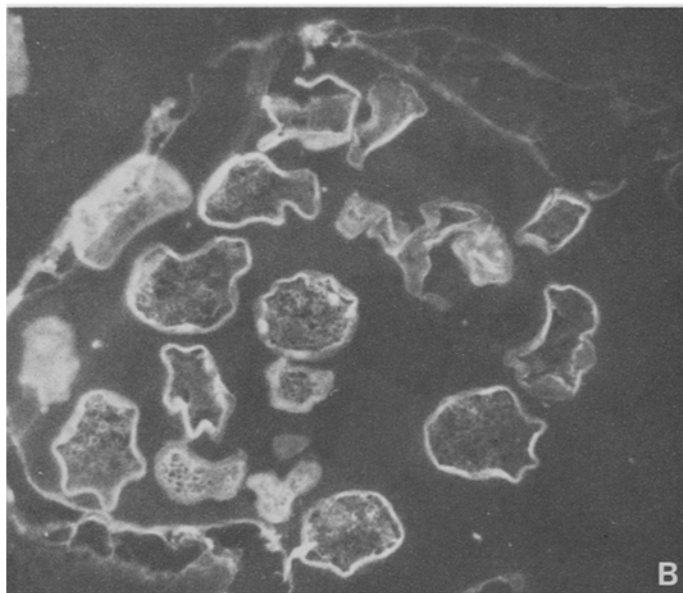
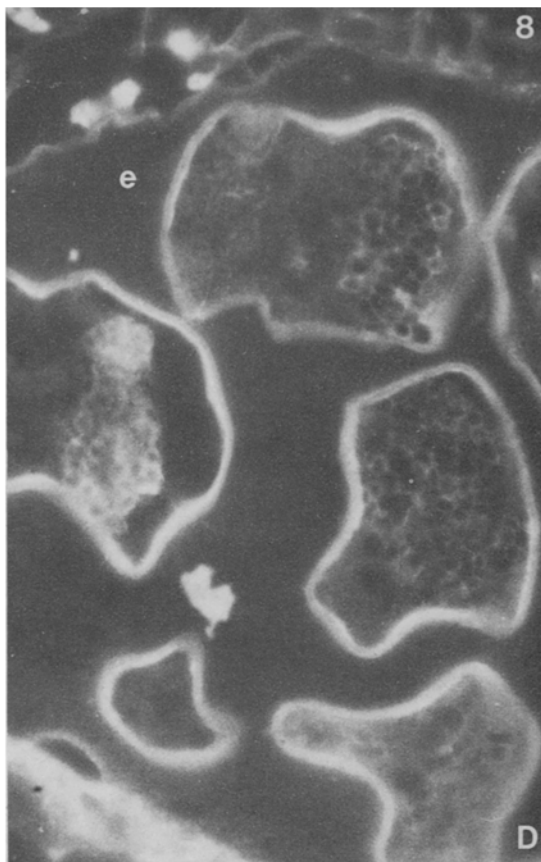
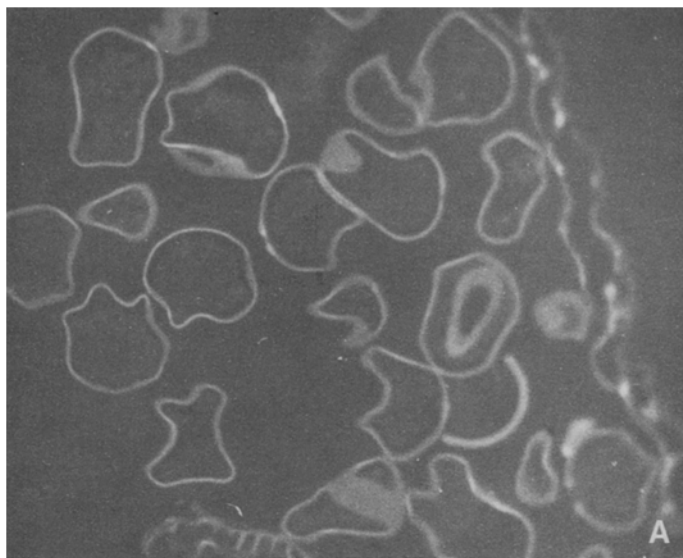
Fig. 7. Localization of ryegrass pollen antigens in developing anthers at early maturation period (anther length 3.5 mm), freeze-dried anthers, indirect immunofluorescence. (A and B) Control sections, showing autofluorescence of exine and endothecium (en) of anther wall. (C and D) Incubated with anti-Group 1 allergen IgG. (E and F) Incubated with anti-Antigen A IgG. Note specific fluorescence (f) in cytoplasm especially at periphery. Intine is not differentiated at this stage in development. (A and C) Scale as shown in (E). (B and D) Scale as shown in (F).

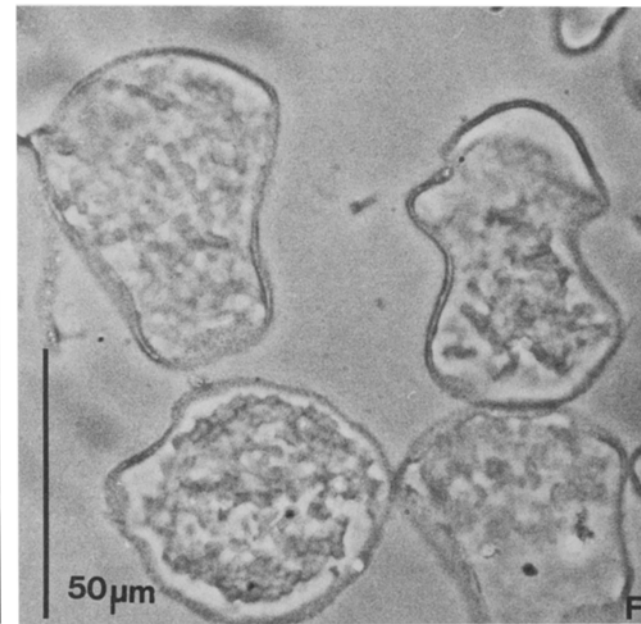
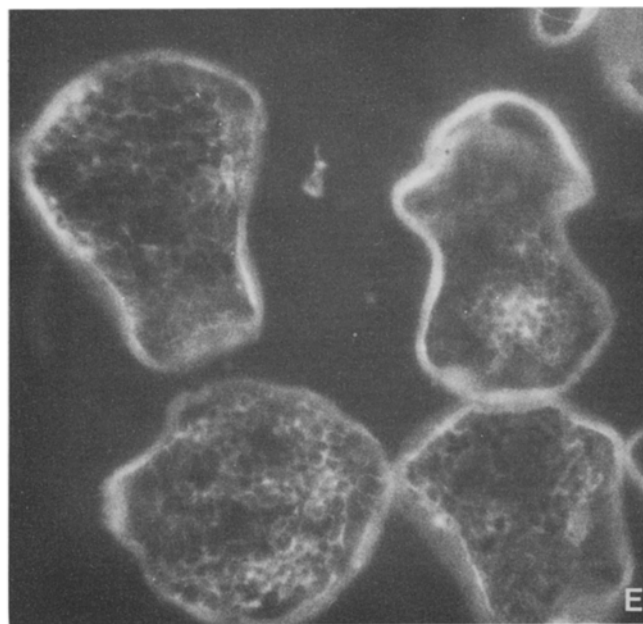
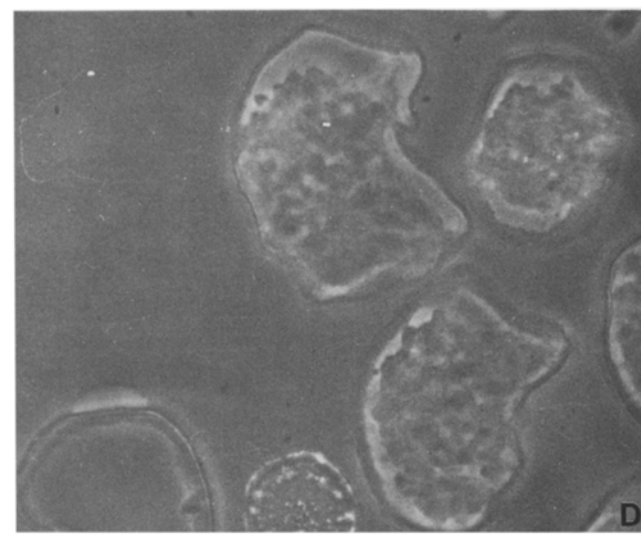
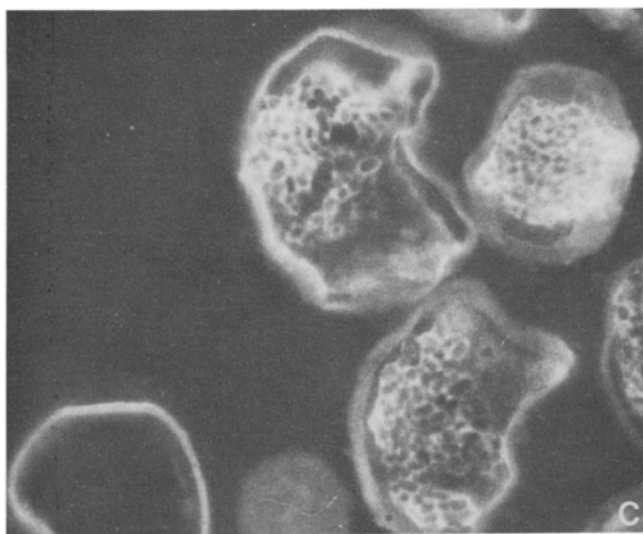
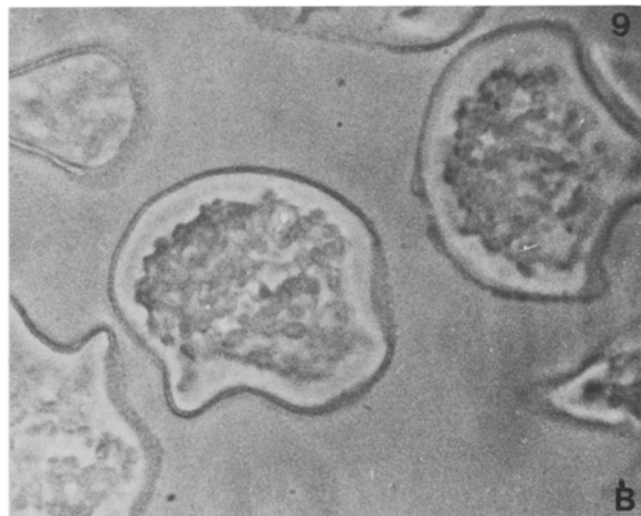
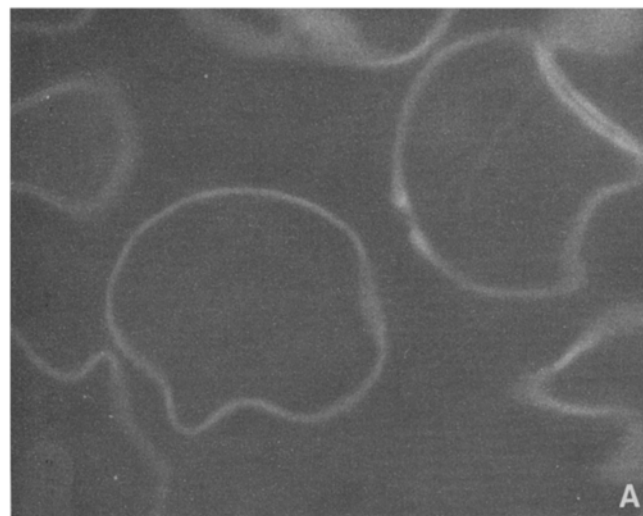
Figs. 8–9. Localization of ryegrass pollen antigens in mature anthers approximately 24 h before dehiscence (anther length 4.5 mm); freeze-dried anthers, indirect immunofluorescence.

Fig. 8. (A) Control, pre-immune IgG; (B) anti-Antigen A IgG; (c) anti-Group 1 allergen IgG; (D) detail of section treated with anti-Antigen A IgG; (E) detail of section treated with anti-Group 1 allergen IgG. Note specific fluorescence in cytoplasm and in pollen wall (e), especially the intine. (A and B) Scale as shown in (C). (D) Scale as shown in (E).









Anthers were first sampled early in the maturation period, after division of the microspore nucleus, and after starch grain synthesis. In control sections treated with pre-immune IgG, only light orange-yellow fluorescence (Fig. 7A, B) was detectable, identical with sections mounted in PBS as autofluorescence controls. After treatment with specific IgG, the greenish-yellow fluorescence of FITC was present in the cytoplasm and especially intense at the periphery (Figs. 7C–F), but virtually absent from the wall. These grains contained abundant starch grains, and the intine had hardly begun to differentiate.

A second series of anthers were sampled late in the maturation period, about 24 h before dehiscence. In controls, only exine autofluorescence was detected (Figs. 8A, 9A, B). Specific fluorescence was present in the cytoplasm with antisera to both glycoproteins (Figs. 8A–D, 9C, D) and was in some cases concentrated at the periphery (Fig. 9E, F). Intensive FITC-fluorescence was detected in the pollen wall, especially in the intine (Figs. 8D, E, 9C–F). The concentration of specific fluorescence around the starch grains in the cytoplasm is especially striking (Figs. 8E, 9C, D). Mature anthers, freeze-dried just before dehiscence, showed specific fluorescence in both the pollen cytoplasm and wall sites.

Discussion

Effects of fixation on grass pollen antigens

Use of the solid phase radioimmunoassay has permitted quantification of the effects of fixation on antigen–antibody binding. Surprisingly, the treatments employed for cytochemical studies showed little effect, although there was a 15% inhibition of antibody binding after glutaraldehyde fixation. Quantitative immunoprecipitation studies of the effects of aldehyde fixation on mammalian antigens (McLean & Nakane, 1974; Van Ewijk *et al.*, 1980) revealed inhibition of antibody binding even after fixation periods as brief as 15 min.

In a recent electron microscope study of the localization of a prostaglandin synthesizing enzyme in mouse fibroblasts, Rollens & Smith (1980) found that fixation of the cells in 0.05% glutaraldehyde in PBS for 5 min at 4° C completely abolished specific antibody binding. However, fixation (4 h, 4° C) in a mixture of 75 mM lysine, 2% paraformaldehyde and 10 mM periodic acid following the method of McLean & Nakane (1974) allowed specific antigen–antibody interactions. Craig *et al.* (1979), using qualitative methods, did not detect any inhibition with pea storage

Fig. 9. (A and B) Control section treated as Fig. 8A. (A) Fluorescence illumination; (B) phase-contrast image; (C–D) treated with anti-Group 1 allergen IgG; (C) fluorescence illumination; (D) phase-contrast image; (E–F) treated with anti-Antigen A IgG; (E) fluorescence illumination; (F) phase-contrast image. Note specific fluorescence is associated with cytoplasm and pollen wall especially in layer just beneath the exine as seen by comparison with phase contrast image. (A–E) Scale as shown in (F).

protein antigens. One explanation of our results is that the remarkable antigenic stability to the fixatives used is conferred by their adsorption to the PVC of the microtitre trays. Such stability has been recorded in systems where proteins are linked covalently to Sepharose and are resistant to degradation (Axen *et al.*, 1969).

The absence of specific staining following aldehyde fixation in ryegrass pollen may indicate that the antigens are able to diffuse rapidly from the grains, even from cytoplasmic sites under the conditions used. This has been noted before with other pollen types after aldehyde fixation (Knox, 1973; Knox *et al.*, 1975). Alternatively, the situation might be expected to be similar to animal tissue where Carnegie *et al.* (1980) have shown that glutaraldehyde induces tissue autofluorescence which obscures specific antibody fluorescence. Indeed, in the present study, glutaraldehyde induced noticeable cytoplasmic as well as wall autofluorescence of ryegrass pollen.

Freeze-drying has the advantage that the pollen grains are obviously preserved in a dehydrated state, judging by the shape of the grains. After brief cold methanol fixation, the grains are round as though partial hydration had occurred (Knox *et al.*, 1980). The freeze-drying technique used was a standard one, and more rapid methods for freezing tissues are available and might give improved resolution, with less possibility of diffusion artifacts. The possibility that such artifacts could account for some of the changes in localization revealed in the developmental study (see below) cannot be ruled out with the methods that have been used here, even though care was taken to freeze the anthers as rapidly as possible.

Immunofluorescent localization of grass pollen antigens

The present investigation is the first attempt to localize antigens in mature grass pollen. Antisera to the principal allergen, group 1 allergen of ryegrass, were employed to locate the sources of this component in mature dry grains. Antisera to a second purified antigen, antigen A, were available for comparison. Specific fluorescence was found in the cytoplasm especially of the periphery, and in the wall in both intine and exine sites. The pollen antigens of canary grass, *Phalaris tuberosa*, have been previously localized in the intine using antisera to whole pollen diffusates and the indirect immunofluorescence method (Knox & Heslop-Harrison, 1971). Freeze-sectioned anthers, containing developing pollen prior to dehydration of the anther cavity, were used. Mature anthers of grasses are very difficult to freeze-section because of the presence of air in the anther loculi and because of the dehydrated state of the grains.

Our observations suggest that there is a transfer of antigens from cytoplasmic to wall sites in the maturing ryegrass pollen grain. Such a process is possible in grass pollen because of two factors (1) the presence of exine micropores, channels penetrating radially through the inner nexine layer as well as the outer tectum; and (2) the progressive dehydration of the pollen grains within the anther cavity as dehiscence approaches, which is likely to be accompanied by physical forces tending

to remove water from the grains. Recently, Heslop-Harrison (1979) has revealed striking ultrastructural changes associated with the mature pollen of rye, *Secale cereale*, and its entry into the dry, dormant state which would support the proposition that there is a transfer of pollen antigens suggested by our immunocytochemical study.

In an ultrastructural study of pollen development in *Artemisia vulgaris*, Rowley & Dahl (1977) showed that granular fibrillar material, which accumulated in the exine arcades during the later stages of development, was present over the external surface of the grain at maturity. These observations of aldehyde-fixed material strongly suggest surface transfer of the wall proteins during pollen maturation.

The exine localization of the grass pollen antigens in mature pollen is a surprising result in view of the absence of proteinaceous material in the exine arcades of other grass pollens when viewed in radial section by transmission electron microscopy (Rowley, 1964; Skvarla & Larson, 1966; Christensen & Horner, 1974; Barlow, 1978; Heslop-Harrison, 1979; Young *et al.*, 1979). In these studies, the pollen proteins may have been lost during aldehyde or osmium tetroxide fixation, since the grass pollen antigens are remarkably mobile, even diffusing from the plastic sections floating on water. In a preliminary immunoelectron microscopic study of Antigen A and Group 1 allergen localization, we have found that the antigen is present in the arcades of the exine in mature pollen (Knox *et al.*, 1980; Vithanage *et al.*, 1980).

We are fortunate that cytochemical methods are available for differentiating the two pollen wall layers, the polysaccharide intine layer and the outer sporopollenin layer, the exine. These have recently been elegantly described by Heslop-Harrison (1979) for the large pollen grains of rye, *Secale cereale*, and the ryegrass pollen walls appear to be similar cytochemically. The intine of ryegrass did not fluoresce after staining with the fluorescent brightener dye, Calcofluor, while the intine of *Secale* gave an intense fluorescence (Heslop-Harrison, 1979) perhaps induced by the solvent treatment used to solubilize the exine prior to staining whole grains. In this study, sections of intact grains were used. Calcofluor has also been shown to stain the intine of *Populus* pollen (Ashford & Knox, 1981).

In earlier immunofluorescence studies of the allergenic pollen of ragweed *Ambrosia* spp., the major allergen, Antigen E, was shown to be concentrated within the polysaccharide matrix of the intine, and was also associated with the exine (Howlett *et al.*, 1973, 1975). The presence of antigens in the arcades of the exine was first reported for the pollen of *Malvaviscus* by Heslop-Harrison *et al.* (1973) and since then, exine antigens have been detected in several different pollen types (see review by Knox *et al.*, 1975; Howlett *et al.*, 1979). Ultrastructural studies of these pollen types have confirmed the presence of material within the exine arcades and thus has been shown to originate by transfer later in pollen development from the tapetal cells that surround the anther locus (Heslop-Harrison *et al.*, 1973). Thus, the exine proteins of these types of pollen have a parental (sporophytic) origin. Ultrastructural studies are in progress to determine the origin of the wall glycoproteins of ryegrass.

Acknowledgements

We are indebted to Dr A. E. Clarke for helpful discussions, especially about the radioimmunoassay techniques, and Dr P. Y. Ladiges for advice on statistics. We thank the Australian Research Grants Committee, the Sir A. E. Rowden-White Foundation and the Asthma Foundation of Victoria for financial support and the University of Melbourne for a postgraduate research award to B.J.H.

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