

Immuno-electronmicroscopic identification and localization of the antigenic proteins of tree pollen grains

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The localization of antigenic proteins on ultrathin sections of pollen grains represents an interesting approach to understanding the release mechanisms of these antigens when the pollen grains come in contact with various physiological fluids. Using different rabbit antibodies we have demonstrated the locations of these antigens in the various structures of pollen grains. We further demonstrated the cross-reactivities between alder (*Alnus incana*), birch (*Betula verrucosa*) and hazel (*Corylus avellana*) pollen allergens. Ultrathin sections of the pollen grains were prepared and allowed to react with two individually raised rabbit antibodies, (Ab-BV and Ab-ALK), against birch pollen. The sites of the Ag/Ab complex on the sections were labelled by protein A/gold, and identified in a transmission electron microscope. The two birch antibodies showed either quantitative or qualitative differences regarding their binding to various structures on the pollen sections. Using Ab-BV, the antigen-binding sites were located in the apertural region of the pollen grain and in the cytoplasm, while almost no gold labelling could be seen on the pollen surface. With the other antibodies (Ab-ALK), we could visualize the antigen-binding locations on the surface material of the pollen grains, particularly in the exine part of the wall and in the cytoplasm. A few gold particles could also be seen in the apertural region of the pollen. In hazel and alder pollen the exine part of the wall was the most densely labelled, whereas the cytoplasm and the aperture bound smaller numbers of gold particles. Cross-incubations: birch pollen incubated with antibodies against hazel (Ab-CA), or alder (Ab-AI), showed various intensities of gold labelling for each of the three species. Statistically, the differences in the number of gold particles bound per μm^2 grain section between birch, hazel and alder, were highly significant. The cross-reactivities between these antigens from the three pollen species were further tested using house-produced rabbit antisera against antigens of the three species by means of electrophoretic and autoradiographic techniques (CIE and CRIE). The three antibodies could precipitate the major IgE-binding antigen from all three pollen species.

Key words: alder; birch; cross-reactivity; hazel; immunocytochemical; immuno-electron microscopy; tree-pollen allergens.

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Tree pollen allergy is common in north European countries and represent a considerable clinical problem during early spring-time. During the last two decades several laboratories have been studying the antigenic and allergenic material of tree pollen, particularly birch (1-3, 7-9, 14, 20, 21). Results from our laboratory as well as from others have shown that the anti-

genic composition of the birch pollen extract consisted of a minimum of 33 proteins, of which one, BV45, represented the major allergenic material of birch (WHO-nomenclature Bet v I) (18).

Birch (*Betula verrucosa*) and alder (*Alnus incana*) trees belong to a common botanical family, the Betulaceae, while hazel (*Corylus avellana*)

belongs to the Corylaceae family. The cross-reactivities between the antigens from these species have also been investigated (15). An antigenic and allergenic mapping of the three species were compared by CIE and CRIE techniques (24, 25).

An understanding of the histological location of the antigens within the different layers of the pollen grain might enable the development of methods for intervention by the rapid release of these antigens upon interaction with the upper respiratory tract of allergic individuals. A preliminary localization of the antigens on ultrathin sections of their pollen grains by immunocytochemical techniques has been reported. This was done by using rabbit antisera and the protein-A/gold technique or sera from atopic patients in combination with colloidal gold-labelled secondary antibodies (9, 10).

In this communication we will use three models of antigenic proteins extracted from the pollen grains of birch, alder and hazel pollen, and two sets of individually produced antibodies. The main purpose is: 1) to localize the different antigens on the various pollen-grain structures, and 2) to further substantiate the cross reactivities between the three pollen species.

MATERIAL AND METHODS

Mature pollen from *Betula verrucosa*, *Corylus avellana* and *Alnus incana* were collected by separately gathering branches of the species at times of flowering, and placing them in vases of water at +4°C. Sheets of thin, smooth aluminium foil were placed beneath the branches, and the accumulated pollen was collected into small glass tubes by gentle shaking or brushing. These conditions secured optimal purity of the three pollen preparations. The pollen were stored at -23°C until used. Samples of each pollen species were fixed in 4% p-formaldehyde and 0.5% cetylpyridinium chloride overnight, and embedded in Lowicryl K4M resin at -35°C (9, 10). Ultrathin sections of the pollen grains were cut on a Reichert-Om-U3 ultramicrotome and placed on pioloform-coated nickel grids. Protein A from *Staphylococcus aureus* was supplied by

Sigma, Chem. Co. USA. The protein A/gold complex was prepared as described by Roth (19) using trisodium citrate as the reducing agent for the chloroauric acid. The mean diameter of the gold particles was 18 nm.

The sections were successively incubated in the following solutions:

1. 5% bovine serum albumin (BSA) in phosphate-saline buffer (PBS), pH 7.4, 75 mmol for 10 min, for the blocking of unspecific binding sites.
2. Specific rabbit antiserum, diluted 1:30 in PBS for 2 h.
3. Washing with PBS for 3 × 5 min.
4. Protein A/gold complex stock solution diluted 1:8 in PBS for 1 h.
5. The final washing was done by PBS for 3 × 10 min; distilled water for 5 min.
6. Staining was performed by a saturated solution of uranyl acetate in 50% ethanol for 3 min.

The sections were then examined and photographed in a Hitachi H 500 transmission electron microscope using Agfa-Gevaert-23 D 56 films. Controls were performed using normal rabbit IgG (Dynatech, Denkendorf, FRG).

Pollen from alder (AI), birch (BV) and hazel (CA), each containing less than 1% foreign pollen, were obtained from Allergon AB, Engelholm, Sweden. The various extracts were produced as described in previous communications (1, 21).

Three different rabbit antisera were produced, against alder (Ab-AI), birch (Ab-BV) and hazel (Ab-CA) allergen extracts respectively, as described previously (13, 21). Pools were constituted by mixing equal portions of sera from four identically treated, responding rabbits, and pooled sera were stored at -20°C until used. Rabbit antibodies against birch were also purchased from Allergologisk Laboratorium, Copenhagen, Denmark (Ab-ALK).

Sera from 10 tree pollen-allergic patients with positive history of clinical allergy to tree pollen and with serum containing specific IgE corresponding to RAST classes 2-4, to alder, birch and hazel pollen, were collected and pooled in equal amounts. The patients were not subjected

to any desensitization therapy, and donated the blood samples after informed consent. The serum pool was stored at -20°C until used.

For each experiment the number of gold particles were counted in the cytoplasm, the exine, the intine and the aperture regions within 10 pollen grain sections. The values were expressed as particle number/ μm^2 of the sections. Median and range were estimated. The U-test according to Wilcoxon-Mann-Whitney, was used to determine the differences between the median values of two experiments.

Crossed-immunoelectrophoresis (CIE) and crossed radio-immunoelectrophoresis (CRIE) were performed as presented in earlier papers (6, 17, 21, 23-25). Aliquots of 100 μg of the respective pollen extracts were used for anodic and cathodic runs, using 10 V/cm for 40 min in the first direction electrophoresis. In the second direction 500 μl of the respective rabbit antisera were incorporated in the antibody gels and run for 20 h at 2 V/cm (10, 14).

In CRIE and CIE plates were incubated overnight in 0.5 ml of the diluted serum pool. The day after, ^{125}I -anti IgE, estimated to 25×10^4 cpm was applied to each plate. On the third day the plates were placed on Cronex 4 safety films (Du Pont), using calcium intensifying tungstate screens at -90°C at different time intervals (6).

RESULTS

Immunocytochemistry

The immunocytochemical localization patterns of the antigens in the pollen grain of birch are shown in Fig. 1. Ab-ALK antibodies induced labelling by gold particles in the surface coat, the pollen wall (exine part) and the cytoplasm. Only few particles were found in the aperture region, and no gold markers were located in the intine part of the wall (Fig. 1 a,b). Ab-BV induced poor labelling of the pollen surface material, but a dense covering with gold particles in the aperture region. In addition, as for the Ab-ALK antibodies, Ab-BV also bound to the cytoplasm and the exine part of the pollen grain wall, whereas the intine was almost unlabelled (Fig. 1 c,d).

Incubation of pollen grains from the three species with Ab-BV, Ab-CA and Ab-AI and protein A/gold complexes, produced distinct gold labelling. This has, in all cases, showed significant differences compared with the small amount of background labelling observed in the controls (using normal rabbit IgG antibodies). Figs 2-4 show the electron micrographs of these immunocytochemical preparations, while Table 1 gives the median values and range of the number of the gold particles/ μm^2 .

Further incubations of the three pollen species with homologous antibodies are shown in Fig. 2. Incubation of BV pollen with Ab-BV resulted in intensive concentrations of gold particles in the cytoplasm and a moderate labelling of the exine and aperture (Fig. 2 a,b), whereas incubation of CA pollen with Ab-CA, and AI pollen with Ab-AI showed heavy labelling of the tectum part of the exine and moderate labelling of the cytoplasm. The aperture was weakly labelled (Fig. 3 a,b and 4 a,b). The differences in the concentrations of gold particles between the median values of birch and hazel, and birch and alder pollen were significant. There were no significant differences between the median values of hazel and alder pollen, except in the aperture region.

The pollen species were further incubated with heterologous antibodies. When BV pollen was incubated with Ab-CA, there was some labelling of the cytoplasm. Few gold particles were found in the exine and almost no labelling was observed in the aperture and intine (Fig. 2 c,d). The differences between the median values of the BV/Ab-BV and the BV/Ab-CA experiment were significant, as were the differences between the BV/Ab-BV and the BV/Ab-AI experiments. The latter showed an even greater decrease in cytoplasm labelling but a higher number of gold particles in the tectum part of the exine (Fig. 2 e,f). When hazel pollen was incubated with Ab-BV, there was strong labelling of the aperture and moderate labelling of the cytoplasm, exine and intine (Fig. 3 c,d). In comparison with the CA/Ab-CA experiment, the differences were significant except for the cytoplasm. The picture after incubation of hazel pollen with Ab-AI and Ab-CA was practically

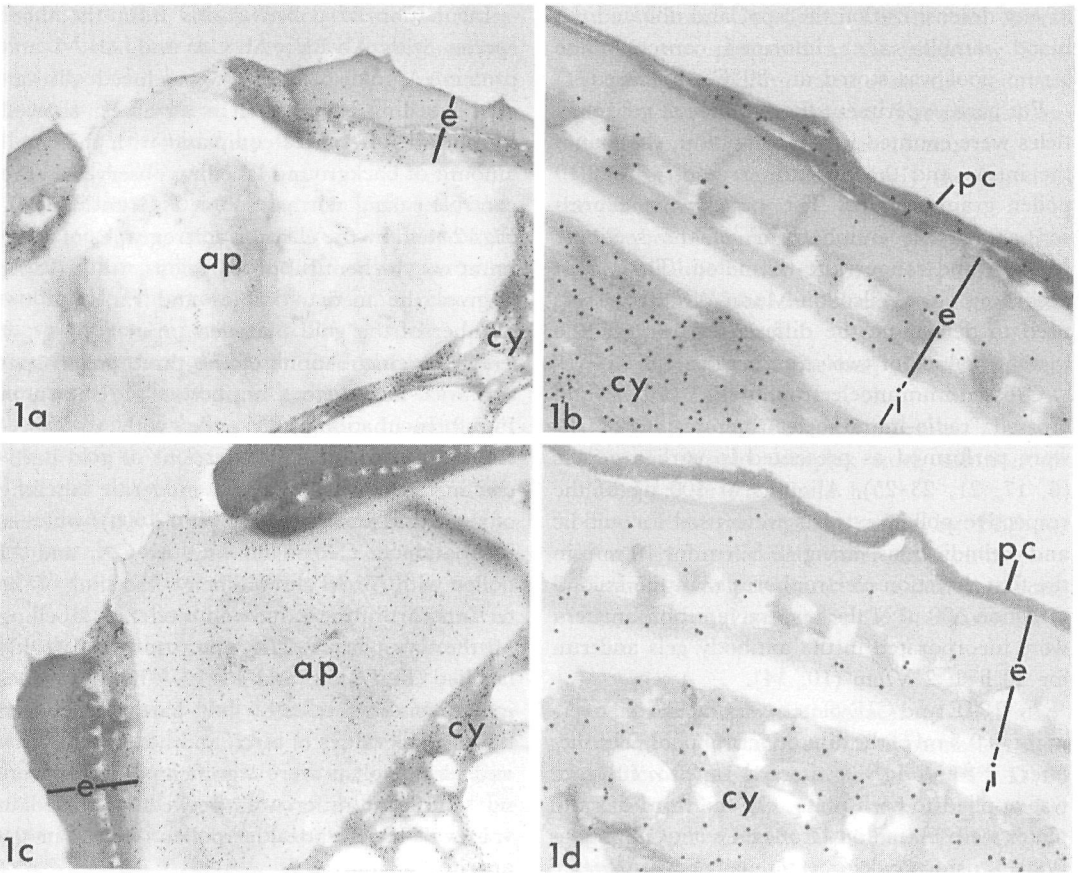


Fig. 1. Ultrathin sections of birch pollen grains (*Betula verrucosa*) after labelling with rabbit antisera and protein A/gold. ap = apertural region of the pollen grain, cy = cytoplasm, e = exine part of the pollen wall, i = intine part of the pollen wall, pc = pollen coat. a) ap incubated by Ab-ALK. There are few gold markers. The apertural region is very weakly labelled. X 42,000; b) peripheral part of birch pollen grain incubated by Ab-ALK. Gold particles are located within the pc, e, and cy. X 15,000; c) ap incubated by Ab-BV. The region is heavily labelled by gold particles. X 36,000; d) peripheral part of a birch pollen grain incubated by Ab-BV. Gold particles are located in the e and cy, while the pollen coat is unlabelled. X 15,000.

similar (Fig. 3 e,f), and showed no significant differences with regard to particular pollen compartments. When alder pollen was incubated with Ab-BV, a uniform moderate labelling was observed throughout the whole pollen grain (Fig. 4 c,d). Compared with the alder/Ab-AI experiment, the observed differences in the median values were significant, except for the cytoplasm. After the incubation of alder pollen with Ab-CA, there was moderate labelling of the cytoplasm and exine, but almost no gold particles were found in the intine and

aperture (Fig. 4 e,f). In comparison with the alder/Ab-AI experiment, only the differences in the median values of aperture and exine were significant.

The three utilized antibodies gave a different degree of gold labelling in the three pollen species investigated. Compared with birch pollen Ab-BV in hazel gave a significantly different binding to the cytoplasm, intine and aperture, but not to the exine. In alder, Ab-BV gave a significantly different binding to all pollen compartments compared with birch. Ab-CA in

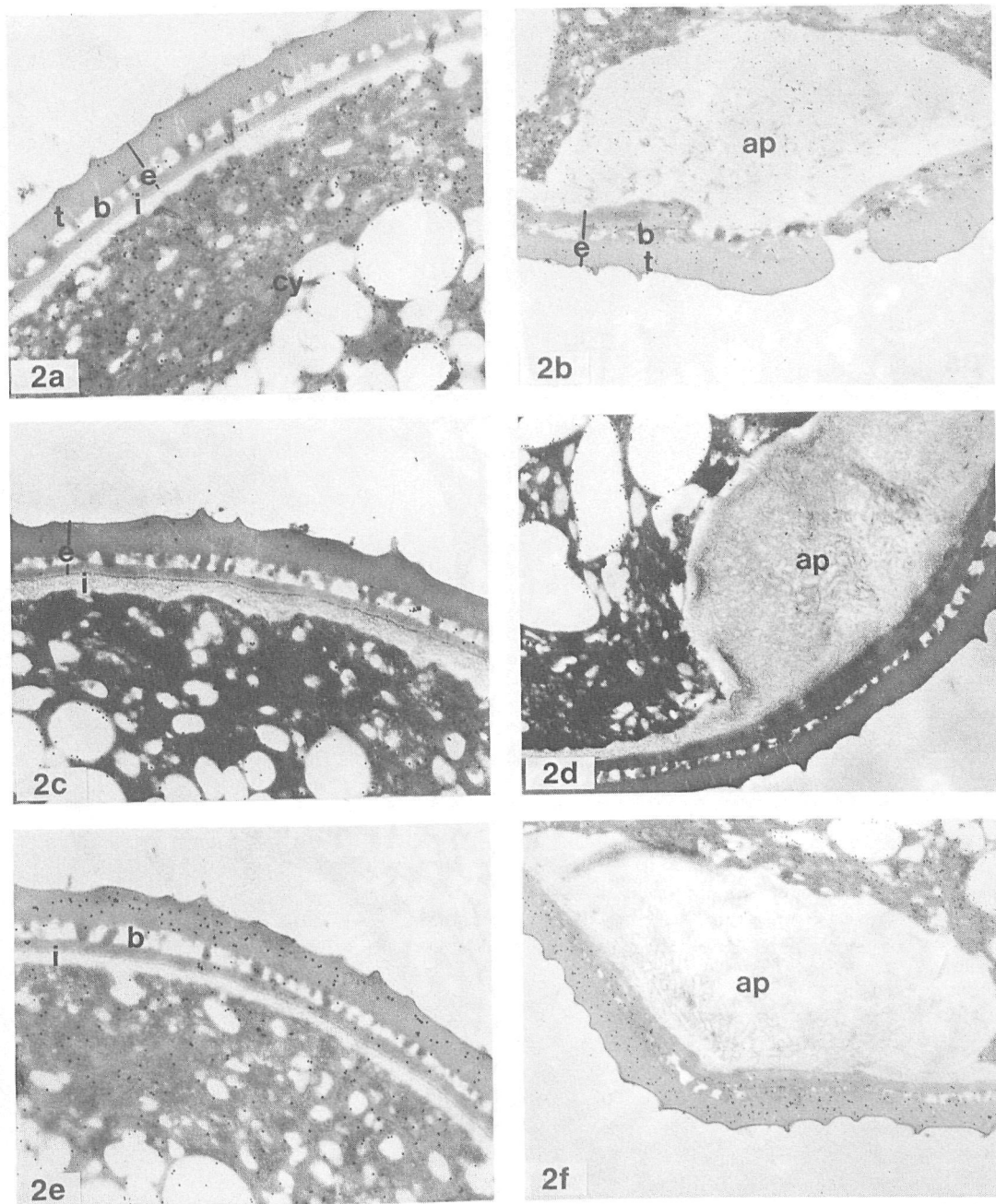


Fig. 2. Immunocytochemical analysis on ultrathin birch (BV) pollen grain sections. Gold labelling is seen as black spots located on the sections. Abbreviations as for Fig. 1. b = bacula (middle part of the pollen wall), t = tectum (outer part of exine). a) Birch against Ab-BV (X 18,000); b) Birch against Ab-BV (ap), (X 12,000); c) Birch against Ab-CA (X 18,000); d) Birch against Ab-CA (ap), (X 15,000); e) Birch against Ab-AI (X 18,000); f) Birch against Ab-AI (X 12,000).

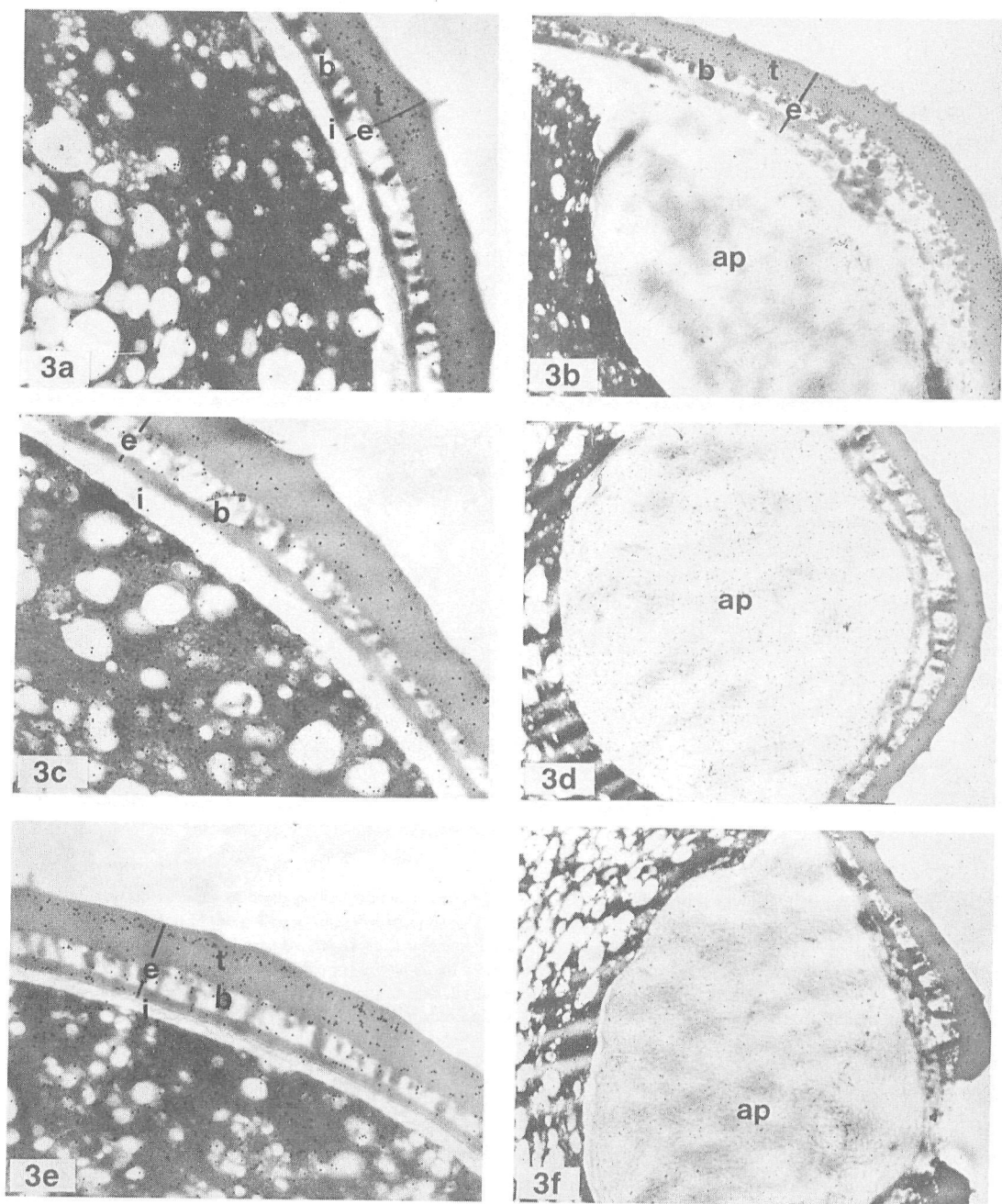


Fig. 3. Immunocytochemical illustrations of ultrathin hazel (CA) pollen grain sections. Abbreviations as for Figs. 1 and 2. a) Hazel against Ab-CA (X 18,000); b) Hazel against Ab-CA (ap), (X 10,000); c) Hazel against Ab-BV (X 18,000); d) Hazel against Ab-BV (ap), (X 9,000); e) Hazel against Ab-AI (X 18,000); f) Hazel against Ab-AI (ap), (X 10,000).

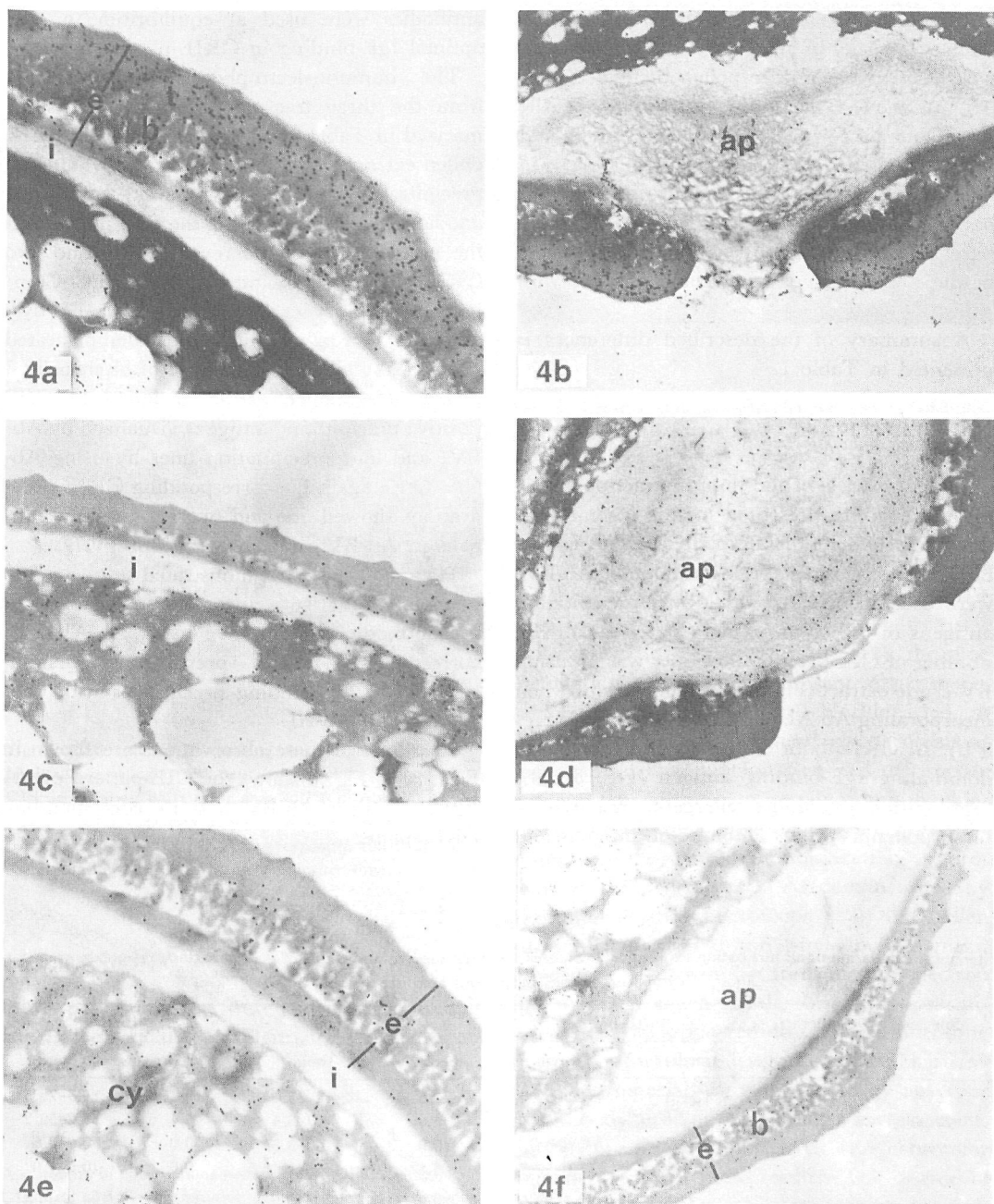


Fig. 4. Immunocytochemical photograph of ultrathin alder (AI) pollen grain sections. a) Alder against Ab-AI (X 18,000); b) Alder against Ab-AI (ap), (X 15,000); c) Alder against Ab-BV (X 18,000); d) Alder against Ab-BV (ap), (X 15,000); e) Alder against Ab-CA (X 18,000); f) Alder against Ab-CA (ap), (X 12,000).

birch pollen, gave significant differences in binding capacity to the exine and aperture compared with hazel. Alder pollen incubated with Ab-CA showed significant differences in the exine compared with hazel pollen, while Ab-AI in birch pollen lead to significant differences in binding to the aperture and cytoplasm compared with alder pollen. Further, Ab-AI in hazel pollen gave equally significant differences in the antigen bindings when compared with alder.

A summary of the described differences is presented in Table 1.

Immunoelectrophoretic results

Only few models of the immunoelectrophoretic experiments are presented in this communication. The two rabbit antibodies raised against birch pollen extracts (Ab-ALK and Ab-BV) were used to visualize analogous precipitating antigens in the birch extract (Fig. 5 a-d). The number of CIE precipitation lines was 15 using BV-crude extract (Fig. 5 a), and only four when incorporating Ab-ALK in the antibody gel (Fig. 5 b). In CRIE both antibodies used gave one dominating IgE-binding antigen (Fig. 5 c,d), representing the major allergenic material of birch pollen. Various amounts of antigens and

antibodies were used at equilibrium to give optimal IgE-binding in CRIE-plates.

The immunoelectrophoretical experiments from the three tree pollen species are summarized in Table 2. By use of Ab-AI the birch pollen extract was shown to include five anodic precipitates, while Ab-CA showed only three anodic antigens. Incubating the CIE-plates with the human serum pool revealed one and two CRIE-positive precipitates, respectively. Compared to the earlier presented BV/Ab-BV system (22), cross-reactivity was demonstrated between the allergenic material of birch pollen.

The alder pollen extract included 11 CIE-positive precipitating antigens visualized by Ab-BV, and four precipitation lines by using Ab-CA. Once again the corresponding CRIE preparations showed one and two IgE-binding antigens in Ab-BV and Ab-CA, respectively.

Hazel pollen was demonstrated to give seven precipitated antigens incorporating Ab-BV in the antibody gel, and five using Ab-AI. The corresponding CRIE preparations demonstrated two IgE-binding precipitates with both Ab-BV and Ab-AI.

Examples of these observations are shown in Figs. 5, 6. Fig. 6 shows the CIE-pattern of CA extract against Ab-BV and its corresponding CRIE-plate.

Table 1

Statistical evaluation of all incubation experiments. Figures show the median and range of gold particles/ μm^2 pollen grain for cytoplasm, exine, intine and aperture sections

Species	Organ	Ab-BV		Ab-CA		Ab-AI	
		median	range	median	range	median	range
<i>Betula verrucosa</i>	cy	44.5	34.3-57.8	26.5	24.2-35.0	18.3	14.4-25.5
	e	17.1	15.5-18.4	5.0	4.7-6.4	45.1	37.2-52.2
	i	4.9	3.6-6.3	0.0	0.0-3.8	0.0	0.0-0.6
	ap	17.1	11.0-22.3	0.6	0.0-1.4	0.0	0.0-1.0
<i>Corylus avellana</i>	cy	32.8	30.5-39.2	29.8	18.0-45.2	34.1	24.0-45.8
	e	18.4	13.9-20.5	51.7	37.4-63.8	47.4	36.7-55.0
	i	10.9	8.7-12.3	0.0	0.0-2.0	1.5	0.0-8.0
	ap	31.8	27.1-29.4	3.3	2.8-4.0	3.6	2.4-5.0
<i>Alnus incana</i>	cy	28.0	25.1-29.5	31.5	19.4-42.4	28.0	22.6-31.4
	e	23.5	18.9-28.8	19.6	17.1-21.0	51.3	42.1-70.9
	i	22.5	14.5-31.4	0.0	0.0-0.7	1.3	0.0-2.3
	ap	30.9	27.1-33.3	2.7	1.9-4.0	6.0	3.3-8.8

cy: cytoplasm, e: exine part of pollen wall; i: intine part of pollen wall; ap: apertural region of pollen grain.

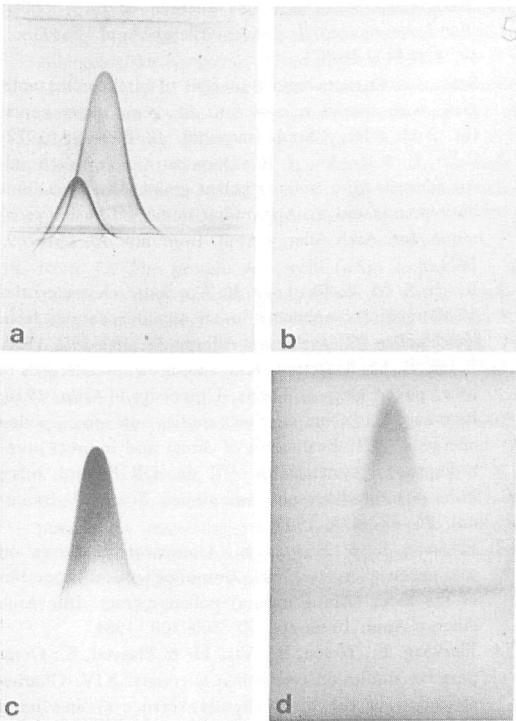


Fig. 5. CIE and CRIE showing the antigenic patterns of birch allergens using different birch pollen antibodies. a) CIE using 50 µg BV extract against 250 µl Ab-BV; b) CIE using 10 µg BV extract against 150 µl Ab-ALK; c) CRIE corresponding to a) with 2 days exposure time; d) CRIE corresponding to b) with 2 days exposure time.

DISCUSSION

Birch pollen antigens were shown to be ubiquitously located in the pollen surface coat, aper-

Table 2

A summary of observations with CIE and CRIE. Number of CIE and CRIE precipitation lines are given separately. Numbers in parentheses represent CRIE-positive lines. An example of these experiments is shown in Fig. 6.

Pollen species	Antibody		
	Ab-BV	Ab-CA	Ab-AI
<i>Betula verrucosa</i> (BV)	33 (1)	3 (2)	5 (1)
<i>Corylus avellana</i> (CA)	7 (2)	38 (1)	5 (2)
<i>Alnus incana</i> (AI)	11 (1)	4 (2)	27 (3)

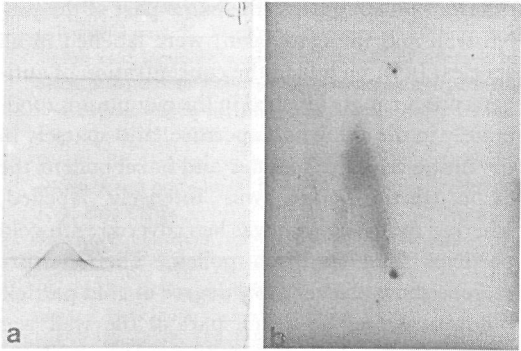


Fig. 6. Crossed immunoelectrophoresis and radiolabelled pattern of a) hazel extract against Ab-BV. b) CRIE corresponding to a) with 7 days exposure time.

tural region, exine and cytoplasm. The intine was the only layer devoid of allergenic proteins. This was more clearly demonstrated by Ab-BV than by Ab-ALK.

As shown in a previous study (11), the type of fixation used in the present experiments (aldehyde + cetylpyridinium chloride), led to precipitation of surface bound glycoproteins of the grains. This precipitation did not occur without the addition of cetylpyridinium chloride. This finding suggested that cetylpyridinium chloride caused immobilization of proteins in the pollen grain. As demonstrated by scanning electron microscope (12), the pollen surface coat covered the whole pollen grain. However, as shown by transmission electron micrographs, much of the originally precipitated material appeared lost after embedding the grains for ultramicrotomy. Since this surface coat material was not labelled by any type of antibody used in the present investigation, only stripped pollen grain areas were chosen for documentation of these results. The degree of antigen mobilization while moistening the pollen grains in the fixation fluid, was studied by measuring the protein content in the fixation solution. It became evident that the addition of cetylpyridinium chloride to aldehyde reduced the protein release into the fixation fluid.

As demonstrated immuno-electronmicroscopically, two main compartments of the three

different pollen grains (the exine part of the pollen wall and the cytoplasm) were labelled in all species. In birch pollen the concentration of antigens was strongly labelled in the cytoplasm, moderately in the exine and aperture, and sparsely in the intine regions. In alder and hazel pollens the exine (tectum part) was intensely labelled, whereas the cytoplasm was less covered with gold particles than in birch pollen. The aperture regions showed a very low degree of gold particle binding, while the intine part of the wall was practically unlabelled. Judged from these observations birch pollen could be considered to contain the broadest spectrum of antigenic compositions in tree pollen grains.

The immunocytochemical observations confirmed earlier described cross-reactions between birch, hazel and alder pollen. The various antibodies were shown to have cross-binding affinities to different pollen species.

These observations clearly demonstrate the presence of homologous epitopes, localized in the aperture regions of hazel and alder pollen, and in the tectum of birch and hazel pollen.

The very weak labelling of the wall in birch pollen after incubation with Ab-CA, indicated only partial structural similarity between wall antigens of hazel and birch pollen. This was also confirmed by studying the N-terminal amino acid sequence of the three pollen allergens (4, 5, 16). An important conclusion of this study is that the dynamic location of antigens in most of the pollen grain layers explained the rapid release of these allergens on contact with mucous fluid.

The immunoelectrophoretic investigations were in accordance with earlier observations (8, 15). However, by using Ab-CA with birch and alder extracts, two IgE binding antigens were revealed. The location of alder antigen differed from that of the major allergens of birch and alder (7, 18, 22). This observation suggested the presence of several homologous isoallergens with slightly different charges and almost identical amino acid sequences (22).

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