

Expulsion of allergen-containing materials from hydrated rye grass (*Lolium perenne*) pollen revealed by using immunogold field emission scanning and transmission electron microscopy

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Background: Several studies demonstrated episodes of grass pollen-induced allergic asthma after heavy rainfalls. It has been hypothesized that these asthma attacks might be due to the release of respirable allergen-bearing particles from pollen cytoplasm.

Objective: In this study we investigated the release mechanism of the most potent and frequently recognized grass pollen allergens, group 1 and group 5, from freshly harvested and subsequently hydrated rye grass pollen at the ultrastructural level.

Methods: Rabbit antisera against purified recombinant group 1 and group 5 allergens were used to investigate, by using field emission scanning and transmission immunogold electron microscopy, the allergen release from rye grass pollen grains into isotonic aqueous solutions or water.

Results: Pollen grains exposed to isotonic aqueous solutions remained intact and released allergens by means of diffusion. However, pollen grains hydrated in distilled water or rainwater expelled starch grains and cytoplasmic debris of respirable size. Group 1 and group 5 allergens were observed on and within these materials.

Conclusions: Exposure of rye grass pollen to water leads to an expulsion of subcellular allergen-containing pollen components of respirable size. Our ultrastructural data thus support the idea that this release of allergen-containing respirable pollen materials may be a cause of asthma attacks after heavy rainfalls. (*J Allergy Clin Immunol* 2000;105:1140-5.)

Key words: Thunderstorm asthma, rye grass pollen, allergens, immunogold labeling, electron microscopy

Grass pollens belong to the most potent allergen sources in cool temperate climates, where almost 40% of allergic patients have grass pollen allergy.¹ Most grass

Abbreviations used

FESEM: Field emission scanning electron microscopy

TEM: Transmission electron microscopy

pollen allergens are intracellular proteins and glycoproteins ranging between 5 and 60 kd in molecular weight that are rapidly eluted when pollen grains come into contact with aqueous solutions, as happens on the surface of mucous membranes.^{2,3} They are thus able to trigger immediate, as well as late, reactions in patients. Ultrastructural studies performed with birch and grass pollen have shown that during hydration in isotonic media, pollen grains remain intact, but allergens are eluted in a diffuse manner through the pollen wall layers (intine and exine) by as yet undefined mechanisms.^{3,4} The rapid diffusion of allergens out of pollen grains on accessible surfaces of the human body (eg, conjunctiva, nose, and skin) can thus explain symptoms of type I allergy (allergic rhinoconjunctivitis and dermatitis) in places that can be reached by intact pollen grains. However, because of their size, grass pollen grains have a rather low probability to reach the deeper airways and trigger asthma. Therefore it is not clear how grass pollen allergens can elicit symptoms of allergic asthma. The finding that grass pollen grains can rupture in water and liberate large amounts of respirable particles suggests, however, that episodes of grass pollen-induced asthma, frequently reported after heavy rainfalls, may be caused by these particles.^{5,6} Support for this hypothesis came from the observation that starch granules released from rye grass pollen induced IgE-mediated bronchial constriction in sensitized patients⁷ and that the environmental concentrations of allergen-containing starch granules were elevated after heavy rainfall.⁸

In this study we investigated the release of two of the most important grass pollen allergens, group 1 and group 5 allergens, from rye grass pollen that had been exposed to water or isotonic solutions by using high-resolution field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM). We studied the morphology of rye grass pollen grains after hydration in water or isotonic solutions and used rabbit antisera

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raised against purified recombinant group 1 and group 5 allergens to monitor the release of allergens by the immunogold labeling technique. Furthermore, we discuss the clinical relevance of our findings that rye grass pollen exposed to water opens and expels small, respirable, allergen-containing subcellular particles for the elicitation of asthma attacks in patients allergic to grass pollen.

METHODS

Pollen material

Pollen from rye grass (*Lolium perenne*) was collected from flowering plants on the University campus in Münster in June 1999. Pollen samples were used immediately after shedding or up to 1 month after shedding. During storage, they were kept at room temperature. Commercial *L perenne* pollen was obtained from Sigma (St Louis, Mo).

Preparation and characterization of rabbit anti-group 1 and anti-group 5 antisera

Recombinant timothy grass pollen allergens, rPhl p 1 and rPhl p 5, were expressed in *Escherichia coli* BL21(DE3) and purified as previously described.⁹ Specific antisera against purified rPhl p 1 and rPhl p 5 were raised in rabbits by using complete and incomplete Freund's adjuvants (Charles River, Kisslegg, Germany). Rabbit antisera and the corresponding preimmune sera were tested for reactivity to nitrocellulose-blotted natural grass pollen extracts from timothy grass (*Phleum pratense*), sweet vernal grass (*Anthoxanthum odoratum*), cultivated oat (*Avena sativa*), Bermuda grass (*Cynodon dactylon*), rye grass (*L perenne*), Australian reed (*Phragmites australis*), Kentucky blue grass (*Poa pratensis*), cultivated rye (*Secale cereale*), cultivated wheat (*Triticum sativum*), and maize (*Zea mays*) as previously described.¹⁰ The rabbit anti-rPhl p 1 antiserum cross-reacted with group 1 allergens from all species, and the rabbit anti-rPhl p 5 antiserum identified group 5 allergens in all species except maize and Bermuda grass. Antisera used for immunogold electron microscopy were affinity purified by using protein G sepharose (Pierce, Rockford, Ill). Immunoglobulin contents of affinity-purified rabbit antisera and preimmune sera were adjusted to comparable levels according to spectrophotometry at 280 nm.

Gold-conjugated antibodies

Goat anti-rabbit IgG antibodies coupled to colloidal gold particles with a diameter of 15 nm were obtained from British BioCell (Plano, Wetzlar, Germany)

Preparation for FESEM

Aluminum supports (5 × 10 mm) were warmed up to 40°C, thinly coated with a melting glue (Tempfix; Plano, Wetzlar, Germany), and cooled down to room temperature to harden the glue. Then pollen grains were dusted onto the coated supports to create a monolayer. The supports were briefly warmed up again to 40°C to make the glue sticky and to "point-fix" the pollen grains to the surface of the glue.

For hydration, 50 µL of double-distilled water or rainwater was pipetted on the pollen preparations. Stereo light microscopic observation found most pollen grains in place after hydration. Only a small number of grains did not stick to the surface of the coated support.

Water evaporated within 2 hours from the pollen preparations. The supports were then briefly warmed again to 40°C to fix any cytoplasmic contents released from the pollen grains during hydration to the surface of the coated support. After that, dry and hydrated pollen preparations were exposed to acrolein vapor (Riedel-de-Haen, Seelze, Germany) for a chemical fixation period of at least 7

days to prevent diffusion of water-soluble allergens during the subsequent labeling steps.⁴

For ruling out the possibility that glueing of the pollen to the aluminum supports might influence its behavior during hydration, parallel preparations were made by using uncoated glass cover slips as support for dry pollen grains. When a drop of water was added, pollen grains swam up but soon settled down. After drying, the expulsion of pollen material could be imaged in a conventional scanning electron microscope. However, it was not possible to perform any labeling on these pollen preparations because they became detached from the cover slip during the subsequent incubation steps.

Preparation of pollen for TEM

For immunocytochemistry, small aliquots of pollen were placed in Eppendorf vessels and hydrated for 2 hours in distilled water or rainwater. The suspensions were gently centrifuged, the supernatants were discarded, and the pellets were resuspended in buffered glutaraldehyde for 2 hours. After fixation, the pollen was dehydrated in ethanol and embedded in Lowicryl K4M resin at -30°C according to standard techniques.¹¹

For morphologic observations, hydrated pollen samples were fixed in glutaraldehyde, postfixed in osmium tetroxide, and embedded in Spurr's resin according to standard techniques.¹¹ Ultrathin sections of both preparations were cut on a Reichert Ultracut S ultramicrotome (Leica, Bensheim, Germany).

Immunogold labeling procedure and electron microscopy of labeled pollen

Aluminum supports with pollen preparations or nickel grids with ultrathin pollen sections were successively incubated with the following solutions at room temperature in a moist chamber with PBS buffer at pH 7.4 and TRIS buffer at pH 8.2: (1) 5% (wt/vol) BSA in PBS buffer for 15 minutes; (2) rabbit anti-group 1 Ig diluted 1:5 in PBS and BSA and rabbit anti-group 5 Ig diluted 1:30 in PBS and BSA for 2 hours; (3) PBS buffer for 5 minutes and TRIS and BSA buffer for 2 × 5 minutes; (4) goat anti-rabbit IgG antibodies coupled to gold particles with a diameter of 15 nm diluted 1:20 in TRIS and BSA buffer for 1 hour; and (5) TRIS and BSA buffer for 5 minutes and distilled water for 2 × 5 minutes.

The following controls were run: (1) replacement of the primary antibody by the preimmune Ig; (2) replacement of the primary antibody by PBS buffer; and (3) incubation of the specimens with the gold-conjugated secondary antibody alone.

For analyzing immunogold-labeled pollen preparations in FESEM, preparations were dehydrated in a graded series of ethanols and air-dried from absolute ethanol. They were coated with a 1.5-nm thick layer of platinum-carbon in a BAF 300 apparatus (Balzers, Liechtenstein). Images were recorded at 10 kV by using an in-lens field emission scanning electron microscope (S-5000, Hitachi Ltd) with secondary or backscattered electrons. Photographs were taken with Agfapan 100 films.

For analyzing ultrathin pollen sections after immunogold labeling in TEM, sections were stained with uranyl acetate and lead citrate and analyzed in a transmission electron microscope (H500, Hitachi Ltd, Japan, operated at 75 kV or EM 410; Philips, Eindhoven, the Netherlands, operated at 60 kV). Photographs were taken with Agfa 23D56 films.

Conventional scanning electron microscopy for unlabeled pollen preparations

After hydration and air-drying, pollen preparations were sputter-coated with gold (about 50 nm). Images were recorded with a conventional scanning electron microscope (S-450, Hitachi Ltd, Japan) operated at 20 kV. Photographs were taken with Agfapan 100 films.

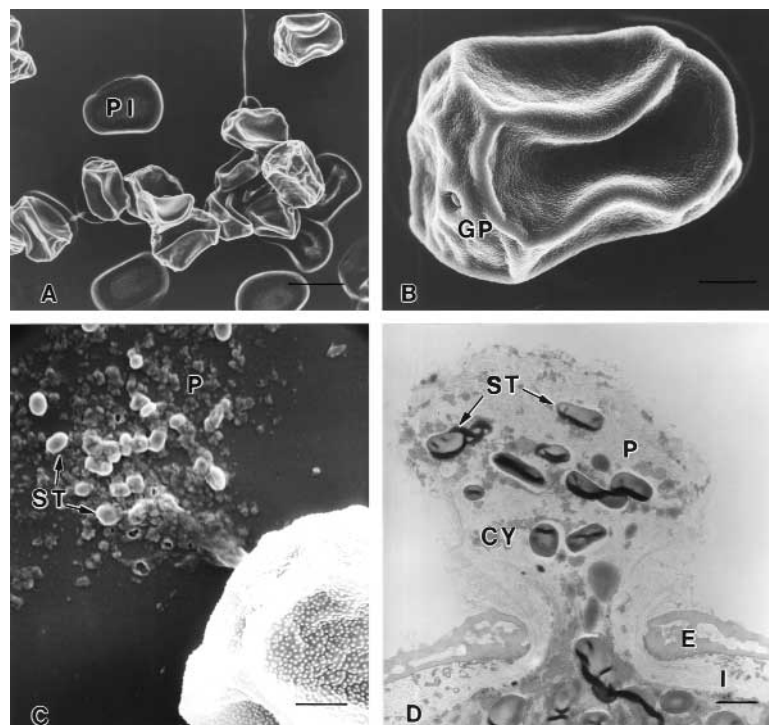


FIG 1. Survey of dry and hydrated pollen grains of *L. perenne*. **A**, Low-magnification FESEM image of dry pollen grains dusted on the surface of an aluminum support and fixed by means of melting glue. Some grains have been detached from the surface (imprints). **B**, FESEM micrograph of the pollen grain in the right upper corner of **A** at higher magnification. **C**, Scanning electron micrograph and **D**, transmission electron micrograph of a hydrated pollen grain and the cytoplasmic materials released from the germination pore. CY, Cytoplasm; E, exine; GP, germination pore; I, intine; P, polysaccharide particles; PI, pollen imprint; ST, starch. (Magnifications: **A**, 410 $\times$, bar = 25 μ m; **B**, 1870 $\times$, bar = 5 μ m; **C**, 4500 $\times$, bar = 2 μ m; **D**, 7100 $\times$, bar = 1 μ m.)

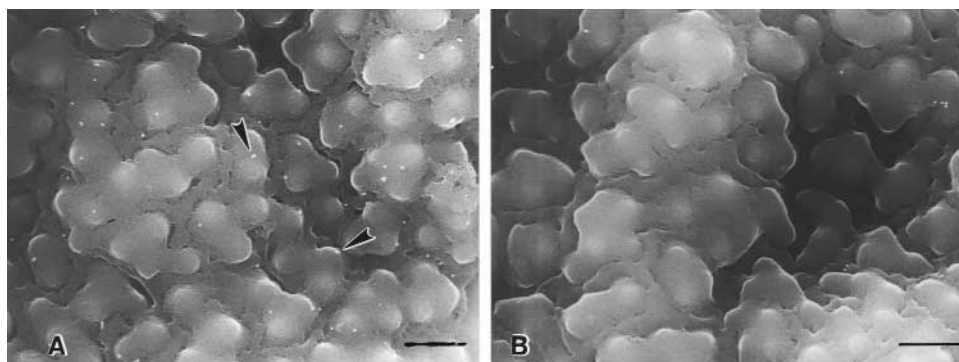


FIG 2. High-magnification FESEM micrographs of the surface of dry *L. perenne* pollen grains. **A**, After incubation with antibodies against group 1 allergens and 15-nm colloidal gold-conjugated secondary antibodies, the surface of the exine bears many gold particles (bright dots, arrowheads). **B**, Control preparations with preimmune Ig are nearly devoid of gold markers. (Magnifications: **A** and **B**, 20,400 $\times$, bar = 0.5 μ m.)

RESULTS

Freshly shed pollen grains open in water but not in isotonic solutions

Fig 1, A, shows a survey of dry *L. perenne* pollen grains dusted onto the surface of an aluminum support and fixed by means of a melting glue. Most of the pollen grains stick to the glue-coated surface, but some have been detached, leaving only their imprints. A single dry

pollen grain is shown at higher magnification on Fig 1, B. It looks shrunk with deep indentations. The surface is decorated with numerous small spikes. There is one single germination pore (Figs 1, B and C). After hydration in distilled water or rainwater, the pollen grains expel their cytoplasmic content through the single germination pore (Figs 1, C and D). Among the released constituents, numerous starch granules and plenty of small roundish bodies are conspicuous. The latter presumably represent

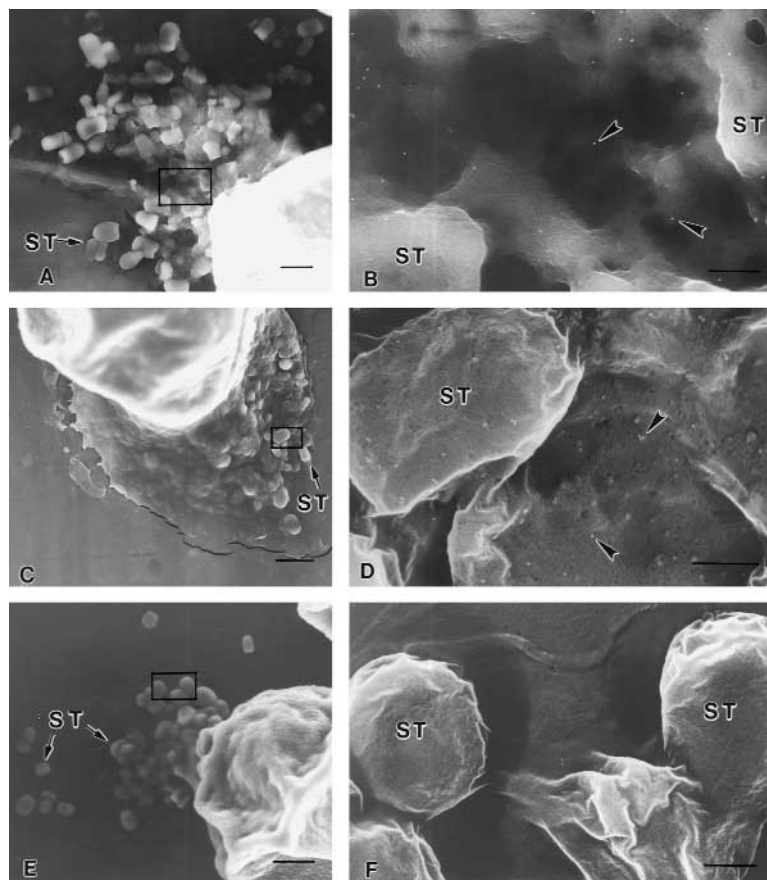


FIG 3. FESEM micrographs of hydrated *L perenne* pollen grains after probing the expelled cytoplasmic materials with antibodies against group 1 and group 5 allergens and 15-nm gold-conjugated secondary antibodies. **A**, **C**, and **E** show low-magnification micrographs of hydrated pollen grains, indicating the area used for high-magnification recording of gold particle labeling (rectangle). **B** shows group 1 allergens, **D** shows group 5 allergens, and **F** shows control labeling with group 5 preimmune Ig. On **B** and **D**, no preference of the gold marker (bright spots, arrowheads) for starch granules is observed. ST, Starch. (Magnifications: **A**, 2720 $\times$, bar = 2 μ m; **B**, 19,740 $\times$, bar = 0.5 μ m; **C**, 1720 $\times$, bar = 4 μ m; **D**, 26,400 $\times$, bar = 0.5 μ m; **E**, 1880 $\times$, bar = 4 μ m; **F**, 20,450 $\times$, bar = 0.5 μ m.)

polysaccharide particles,¹² probably intermingled with cytoplasmic debris. Expulsion of cytoplasmic materials was not observed in the commercial pollen, and it decreased rapidly in pollen stored for more than 1 month after shedding. It was also not observed when pollen grains were hydrated in isotonic solutions, such as physiologic sodium chloride solution (data not shown).

Only group 1 allergen is found on the surface of dry *L perenne* pollen grains

After incubating dry pollen grains with antibodies against group 1 allergens and 15-nm colloidal gold-conjugated secondary antibodies, the pollen surface shows many randomly distributed gold particles (Fig 2, A). Control preparations with preimmune Ig exhibit only a few nonspecifically adsorbed gold particles (Fig 2, B). Group 5 allergens could not be detected on the surface of dry *L perenne* pollen grains (micrographs not shown).

Group 1 and group 5 allergens are found among the cytoplasmic materials expelled from *L perenne* pollen

Fig 3, A, shows a hydrated pollen grain after immuno-gold labeling for group 1 allergens at low magnification. Within the expelled materials, many starch granules are discernible. High magnification of the area indicated in Fig 3, A, shows gold particles distributed on the surface of the released cytoplasmic materials without preferential labeling of starch granules (Fig 3, B). After incubating hydrated pollen with antibodies against group 5 allergens, results are similar to those obtained with group 1 allergens: gold labeling occurs on the surface of the expelled cytoplasmic materials without any preference for starch granules (Figs 3, C and D).

When hydrated pollen was incubated with preimmune Ig, almost no gold labeling was found on the surface of

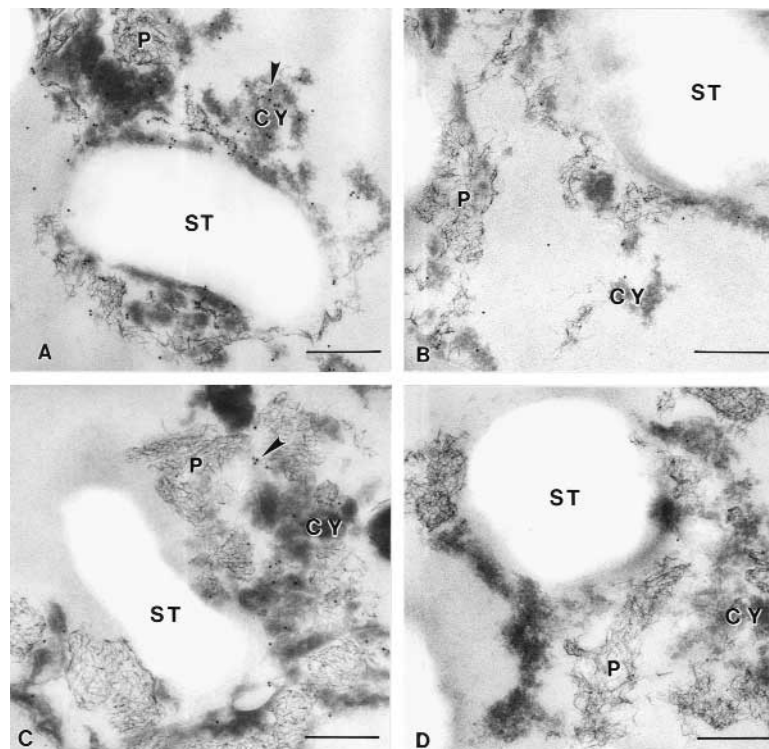


FIG 4. TEM micrographs of ultrathin sections through the cytoplasmic materials released from *L. perenne* pollen. After labeling for group 1 allergens (**A**) and group 5 allergens (**C**), the gold marker (dark spots, arrowheads) is found in the electron-dense cytoplasmic debris. **B** and **D** show the controls after incubation with anti-group 1 preimmune antibodies and anti-group 5 preimmune antibodies, respectively. CY, Cytoplasm; P, polysaccharide particles; ST, starch. (Magnifications: **A**, 31,000 $\times$, bar = 0.5 μ m; **B**, 32,700 $\times$, bar = 0.5 μ m; **C**, 31,000 $\times$, bar = 0.5 μ m; **D**, 29,530 $\times$, bar = 0.5 μ m.)

the released materials. This is demonstrated in Figs 3, *E* and *F*, for group 5 allergens. All other kinds of control gave similar results. The surface of hydrated pollen grains also labeled both for group 1 and group 5 allergens (micrographs not shown).

Group 1 and group 5 allergens are localized in the cytoplasm and not in the reserve materials of the hydrated pollen grain

Ultrathin sections of hydrated *L. perenne* pollen reveal immunogold labeling for group 1 and group 5 allergens confined to the electron-dense areas of the expelled materials. These areas are the remnants of the cytoplasmic matrix intermingled with destructed cell organelles. Polysaccharide particles and starch granules (amyloplasts) are free from gold particles (Figs 4, *A* and *C*). The control with preimmune Ig shows low background labeling (Figs 4, *B* and *D*).

DISCUSSION

In the present investigation we used high-resolution scanning electron microscopy in combination with an immunogold labeling technique to visualize how freshly shed rye grass pollen grains expel allergen-containing

cytoplasmic material through their germination pore when they are brought into contact with water, a process which naturally occurs during periods of heavy rainfall.

Among the cytoplasmic materials released from the pollen, starch grains (amyloplasts) and polysaccharide¹² particles are most prominent. Unfortunately, the latter could no longer be detected after labeling, presumably because of an inadequate preservation of these polysaccharides by aldehyde fixation.¹³ Other cell organelles could not be identified in the released cytoplasmic materials. This is not too surprising because the expulsion of cytoplasmic materials seems to be a rather violent event, rupturing membranes and muddling up the 3-dimensional architecture of the pollen grain's interior.

Using rabbit antisera raised against purified recombinant group 1 and group 5 allergens, we could demonstrate that the cytoplasmic debris contained major allergens. We suggest that these debris particles can act as small respirable allergen carriers, either attached to the simultaneously released starch granules or bound to other respirable particles of the environment (eg, diesel exhaust particles). The massive release of allergen-containing respirable subcellular particles from hydrated grass pollen grains may explain the frequent occurrence of asthma attacks after heavy rainfalls or thunderstorms. It may further explain why allergenic material can be

detected in air samples containing particles smaller than intact pollen grains. In this connection we could not detect allergens inside the starch granules, which were previously reported to contain group 5 allergens.^{4,14,15} This may be due to the fact that group 5 allergens were dissolved from the starch granules during hydration. However, we demonstrated that allergen-containing (group 1 and group 5) subcellular debris was attached to the surface of starch granules. This might explain previous findings that starch granules elicit bronchial hyperactivity in grass pollen-sensitized asthmatic individuals.

There are also reports that pollen from other plant species (birch and ragweed) can release allergen-containing particles of respirable size that can be detected in air samples comprising particles smaller than pollen grains.¹⁶⁻¹⁸ The latter finding emphasizes that it may be important to analyze air samples not only regarding pollen counts but also to determine allergen contents in air samples containing small-sized respirable particles with specific antibody probes.

In conclusion, our study reveals, at the ultrastructural level, the release of two of the most important grass pollen allergens from hydrated pollen grains by expulsion of respirable subcellular particles. In line with previous investigations, this release mechanism may explain the frequent observation that asthma attacks frequently occur after periods of heavy rainfall.

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