

Release of allergen-bearing cytoplasm from hydrated pollen: A mechanism common to a variety of grass (Poaceae) species revealed by electron microscopy

Monika Grote, PhD,^a Susanne Vrtala, PhD,^b Verena Niederberger, MD,^c
Rolf Wiermann, PhD,^d Rudolf Valenta, MD,^b and Rudolf Reichelt, PhD^a

Münster, Germany, and Vienna, Austria

Background: The release of submicronic particles from grass pollen after rainfall was suggested to be responsible for outbreaks of grass pollen asthma. Recently, we provided evidence for the release of respirable allergen-bearing particles from hydrated ryegrass (*Lolium perenne*) pollen as a possible explanation for this phenomenon.

Objective: We investigated whether water-induced release of respirable allergen-bearing particles could be a mechanism common to several members of the sweet grass family Poaceae (Gramineae).

Methods: Pollens from 6 different Poaceae species were hydrated in water and examined by means of scanning electron microscopy for release of cytoplasmic materials. Rabbit antisera raised against purified recombinant group 1 and 5 allergens were used for immunogold labeling of expelled materials by means of field emission scanning electron microscopy. In addition, group 1 and 5 allergens were immunogold-localized on ultrathin sections.

Results: Fresh Poaceae pollens expelled cytoplasmic materials containing group 1 and 5 allergens on hydration in water.

Expulsion of submicronic particles strongly decreased after 1 month of storage.

Conclusions: Our results suggest expulsion of cytoplasm after hydration as a mechanism common to pollens of important allergenic grasses. The water-induced release of respirable allergen-bearing particles from grass pollens might explain asthma attacks observed after rainfall during the grass pollen season. (*J Allergy Clin Immunol* 2001;108:109-15.)

Key words: Grass pollen allergy, asthma, allergen release, immunogold labeling, electron microscopy

Grasses (Poaceae [Gramineae]¹) belong to the most important allergen sources, both because of their worldwide distribution and because of their heavy pollen pro-

Abbreviations used

FESEM: Field emission scanning electron microscopy

TEM: Transmission electron microscopy

duction.²⁻⁴ The importance of grass pollen as potent allergen sources was established almost 130 years ago when Blackley⁵ performed provocation tests and documented the relationship between pollen exposure and allergic symptoms. The biochemical nature of group 1 grass pollen allergens was analyzed approximately 90 years later.⁶ Meanwhile, most of the important grass pollen allergens have been produced as recombinant allergens for diagnostic and therapeutic purposes.^{4,7} Although allergic reactions in easily accessible organs (skin, nose, eyes) can be explained by direct contact with intact pollen grains and subsequent elution of soluble allergens, it is still not clear how pollen allergens can intrude into the deeper airways and elicit bronchial asthma. Because of their size, the likelihood that intact grass pollen grains will reach the deeper airways is low. However, ryegrass pollen grains rupture in water and release large amounts of respirable particles (eg, starch granules).⁸ Therefore it has been suggested that grass pollen-induced asthma attacks could be caused by respiration of subcellular allergen-containing grass pollen particles.^{8,9} In this context, it was demonstrated that starch granules from ryegrass pollen contain group 5 allergens,¹⁰ and group 6 allergens were found to be associated with the polysaccharide particles (P-particles)¹¹ of timothy grass pollen.¹² We have recently obtained evidence that ryegrass pollen can expel such allergen-containing particles on hydration.¹³

The aim of our study was to investigate whether the expulsion of allergen-containing cytoplasm might be a common mechanism of allergen liberation for various clinically relevant grass species. We collected pollens from 6 different Poaceae species, and using scanning and transmission electron microscopy, we studied the release of allergen-containing subcellular particles. For this purpose, fresh or stored grass pollens were exposed to water and the presence of group 1 and group 5 major grass pollen allergens was checked in the released cytoplasmic materials through use of immunogold labeling techniques. We discuss our finding that the release of aller-

From ^athe Institute of Medical Physics and Biophysics, University of Münster; the Departments of ^bPathophysiology and ^cOtorhinolaryngology, University of Vienna; and ^dthe Institute of Botany, University of Münster.

Supported in part by grant Y078GEN of the Austrian Science Fund and by the ICP program of the Austrian Federal Ministry for Education, Science and Culture.

Received for publication January 29, 2001; revised April 5, 2001; accepted for publication April 11, 2001.

Reprint requests: Monika Grote, PhD, Institute of Medical Physics and Biophysics, University of Münster, Robert-Koch-Strasse 31, 48149 Münster, Germany.

Copyright © 2001 by Mosby, Inc.

0091-6749/2001 \$35.00 + 0 1/81/116431

doi:10.1067/mai.2001.116431

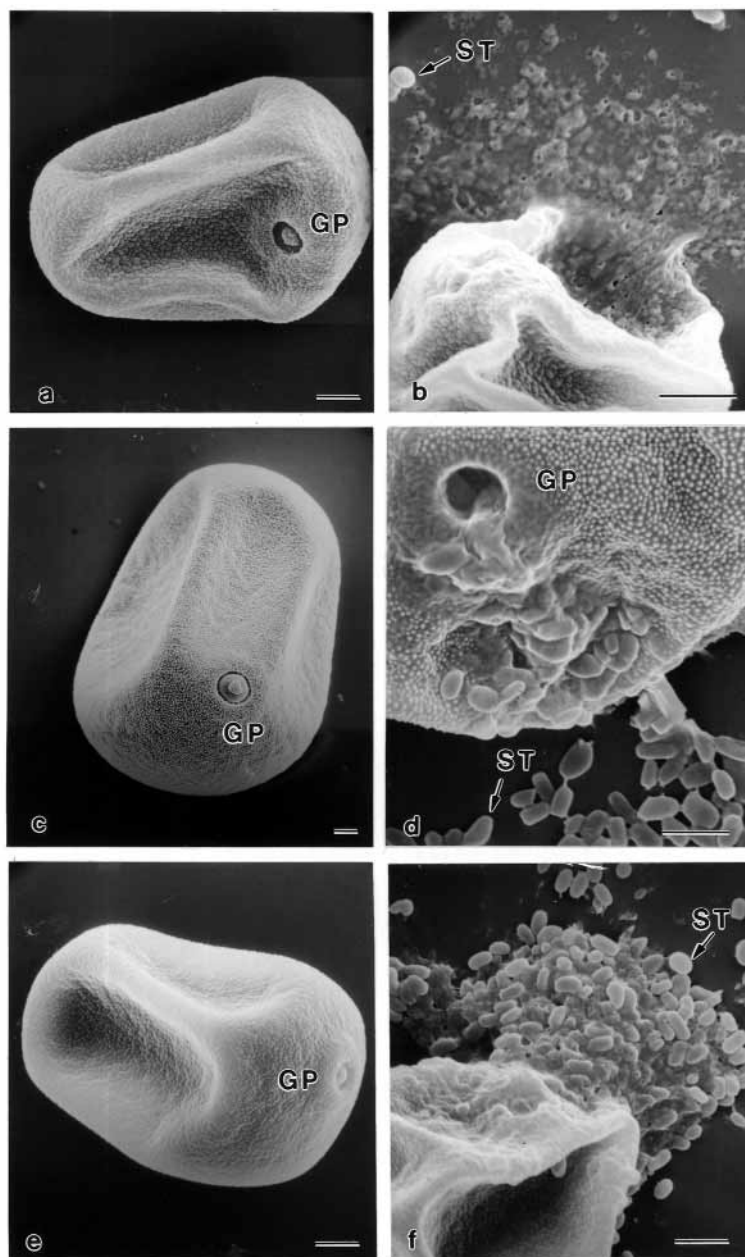


FIG 1. Gallery of conventional scanning electron micrographs of fresh Poaceae pollens in the dry (a, c, e) and hydrated (b, d, f) states. a, b, *P pratensis* (Kentucky bluegrass). c, d, *Secale cereale* (cultivated rye). e, f, *A pratensis* (foxtail grass). Bar = 2 μ m. GP, Germination pore; ST, starch granule.

gen-bearing particles of respirable size from fresh grass pollen is a common mechanism of allergen liberation of potential importance in grass pollen-induced asthma.

MATERIALS AND METHODS

Pollen materials

Pollens of *Alopecurus pratensis* (foxtail grass), *Poa pratensis* (Kentucky bluegrass), *Secale cereale* (cultivated rye), *Phleum pratense* (timothy grass), and *Lolium perenne* (ryegrass) were collected in Münster, Germany, during the spring and summer of 2000. *Sorghum bicolor* (durra, broomcorn) pollen was obtained from

Münster Botanical Gardens in late summer 2000. Pollens were used directly after shedding. From each pollen species, an aliquot was stored at ambient temperature and moisture.

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

Rabbit antisera were raised against purified recombinant group 1 (rPhl p 1) and group 5 (rPhl p 5) allergens from timothy grass pollen through immunization of New Zealand white rabbits (Charles River, Kisslegg, Germany). Cross-reactivity of the antisera with group 1 and group 5 allergens from other grass species and use for immunogold electron microscopy was demonstrated.^{13,14}

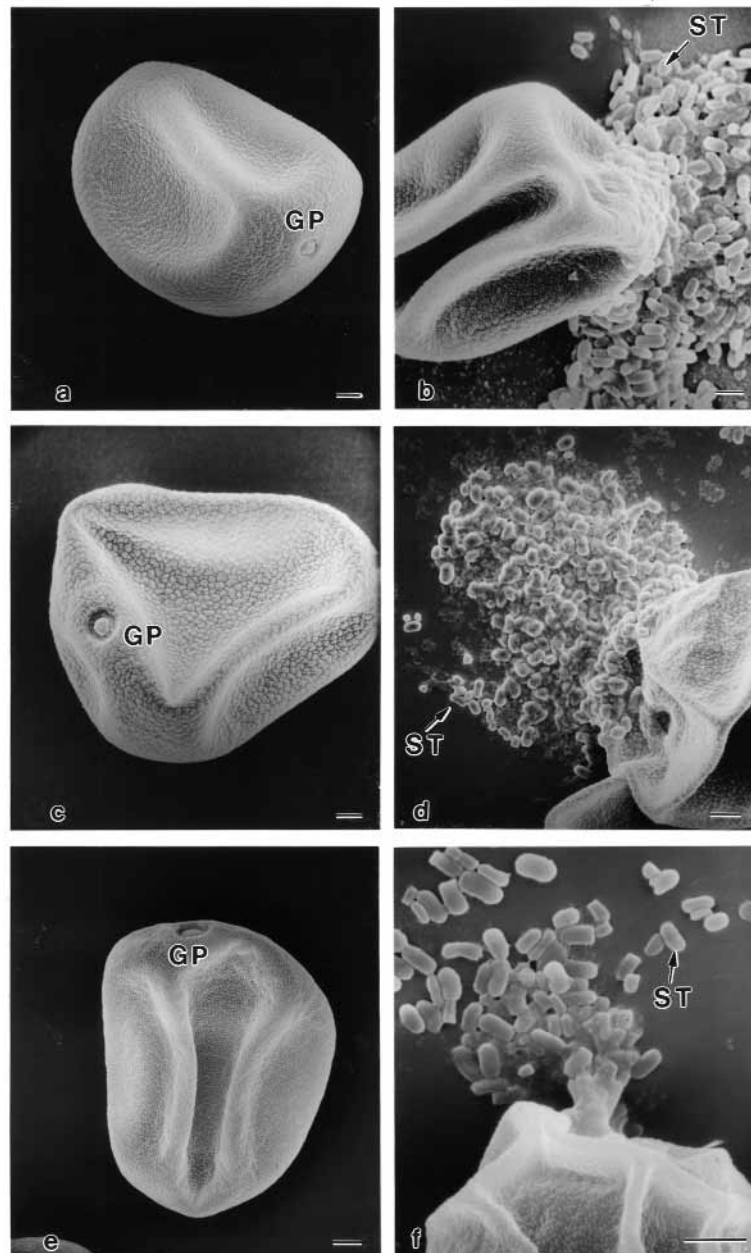


FIG 2. Gallery of conventional scanning electron micrographs of fresh Poaceae pollen in the dry (a, c, e) and hydrated (b, d, f) states. a, b, *Sorghum bicolor* (durra, broomcorn). c, d, *P pratense* (timothy grass). e, f, *L perenne* (ryegrass). Bar = 2 μ m. GP, Germination pore; ST, starch granule.

Gold-conjugated antibodies

Goat anti-rabbit IgG antibodies coupled to colloidal gold particles with a diameter of 10 nm were obtained from British BioCell (Plano, Wetzlar, Germany).

Conventional scanning electron microscopy

Fresh pollen grains from each species were dusted on 3 glass cover slips. The first coverslip was covered with double-distilled water and the second with rainwater; the third coverslip remained dry. After evaporation of the fluids at room temperature, all cover slips were sputtercoated with gold. Images were recorded with a

conventional scanning electron microscope (S-450, Hitachi Ltd, Japan). Photographs were taken with Agfapan 100 films.

After 1 month of storage, samples were again taken from each pollen species and treated as described above (for the fresh pollen).

Field emission scanning electron microscopy, transmission electron microscopy, and immunogold labeling protocols

Details regarding our field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), and immunogold labeling protocols have been provided previous-

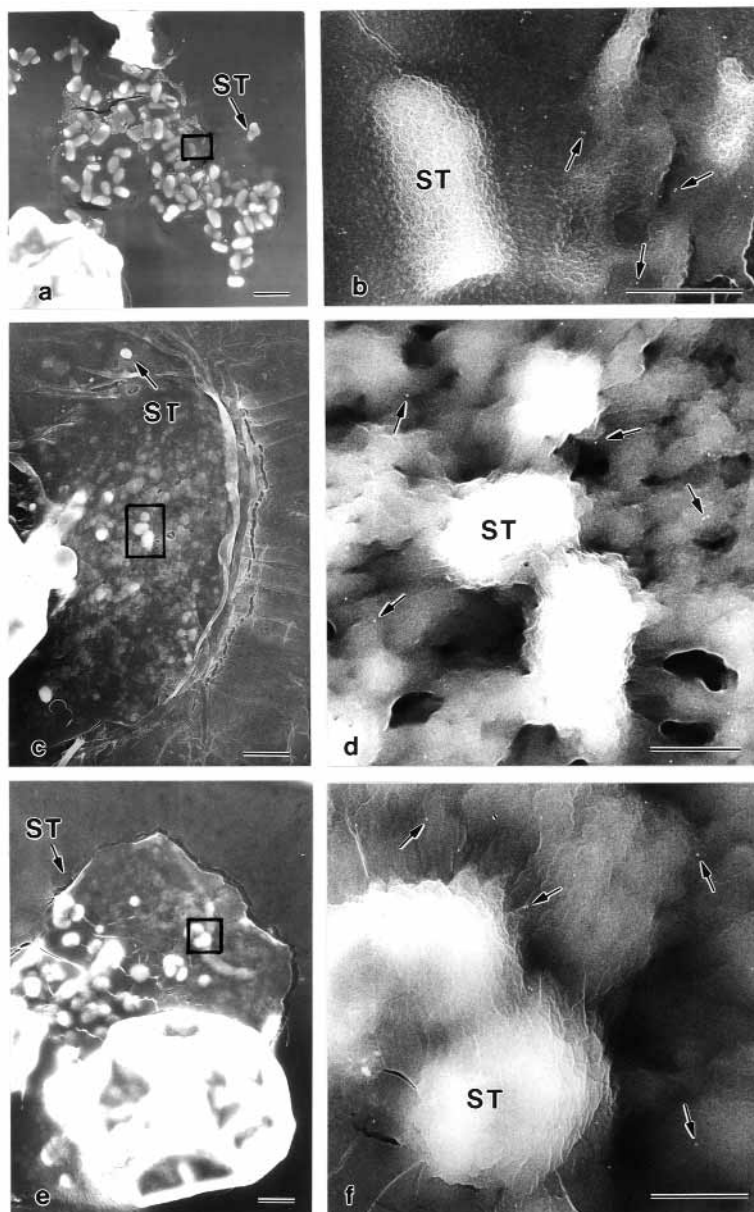


FIG 3. Field emission scanning electron micrographs show immunogold detection of allergens in 3 different hydrated Poaceae pollen species: *Secale cereale* (cultivated rye; a and b), *P pratensis* (Kentucky bluegrass; c and d), and *A pratensis* (foxtail grass; e and f). a, c, e, Surveys of the expelled pollen materials. The rectangle indicates the area chosen for allergen detection by immunogold labeling at higher magnification (b, d, f). b, Group 1 allergens. d, Group 5 allergens. f, Group 1 allergens. Gold particles (arrows) are randomly distributed over the expelled materials. Bar = 2 μ m (a, c, e), 1 μ m (b, d, f). ST, Starch granule.

ly.¹³ In brief: For FESEM, fresh pollen grains were glued to an aluminum support, hydrated in distilled water, air-dried, and fixed in acrolein vapor. Specimens were incubated with rabbit anti-group 1 and anti-group 5 antibodies or preimmune antibodies and then by gold-conjugated secondary antibodies. Next, specimens were again air-dried, coated with platinum/carbon and examined by means of FESEM. For TEM, pollen grains were hydrated in distilled water, fixed in glutaraldehyde, dehydrated in ethanol, and embedded in resin. Sections were immunogold-labeled, as described above, and examined by means of TEM. As a negative control, sections of

unhydrated birch pollen grains were immunogold-labeled and examined by means of TEM.

RESULTS

Water exposure induces expulsion of cytoplasm from 6 different Poaceae pollens

Fig 1, a, c, and e and Fig 2, a, c, and e show a gallery of dry grass pollen grains representing 6 different

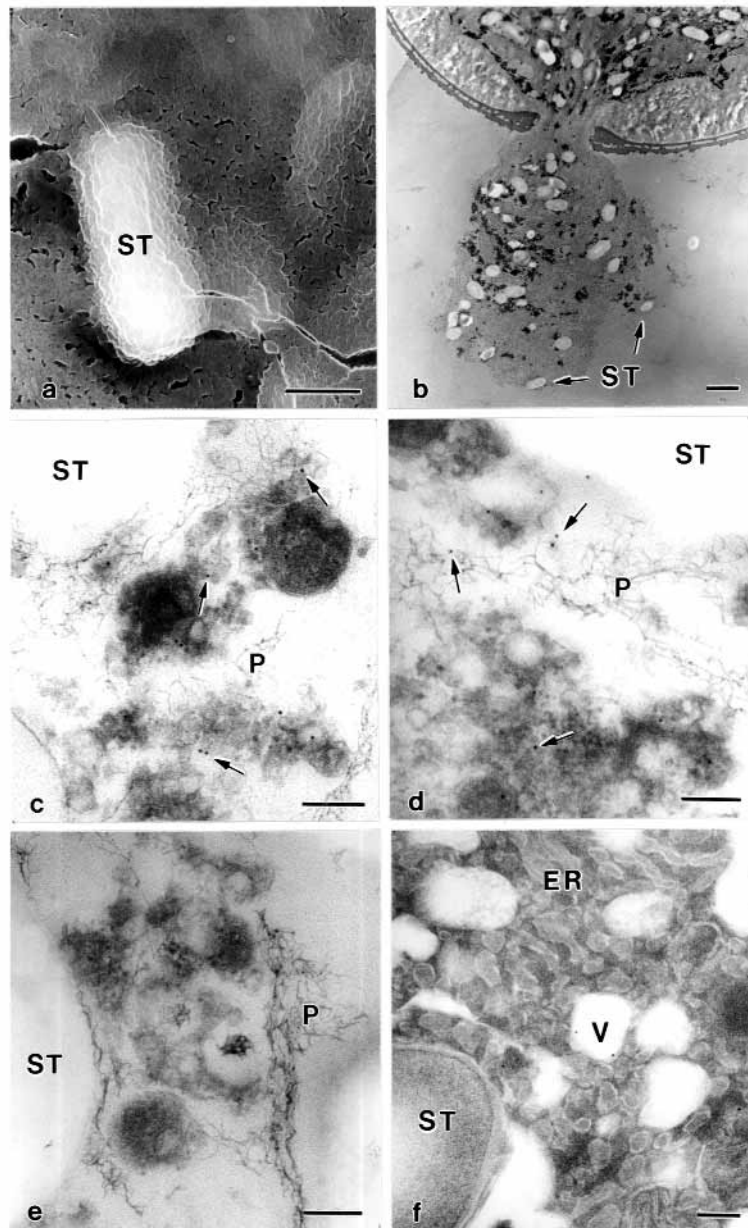


FIG 4. **a**, Field emission scanning electron micrograph of cytoplasm expelled from *Secale cereale* pollen after incubation with group 1 preimmune antibodies. No gold particles are visible. **b** through **e**, TEMs of hydrated *P pratense* (timothy grass) pollen after immunogold labeling for allergens: **b**, Survey of a pollen grain shows the cytoplasm bulging out of the grain. **c**, Group 1 allergens. **d**, Group 5 allergens. Gold particles (arrows) indicate that allergens are associated with dark cytoplasmic materials. **e**, Control experiment with group 5 preimmune antibodies shows low background staining. **f**, Negative control. Unhydrated birch pollen cytoplasm shows very few nonspecifically bound gold particles after incubation with group 5 grass pollen allergens. Bar = 0.5 μ m (**a**), 5 μ m (**b**), 0.2 μ m (**c-f**). ER, Endoplasmic reticulum; P, P-particle debris (degraded polysaccharide material); ST, starch granule; V, vacuole.

Poaceae species. Each pollen grain is spheroidal, has 1 germination pore, and shows a smooth surface with tiny spikes protruding from the exine.

After hydration in distilled water (Fig 1, **b**, **d**, and **f** and Fig 2, **b**, **d**, and **f**), all tested pollen species expel particles through the germination pore (best visible in Fig 1, **d**). In the released materials, starch granules are most conspicuous. Smaller particles and amorphous materials are also

released. In the picture gallery, this is best recognized in *P pratensis* and *P pratense* pollen (Fig 1, **b** and Fig 2, **d**, respectively). There was no difference in the number of burst pollen grains between hydration in distilled water and hydration in rainwater.

After 1 month of storage, pollen grains of all 6 species had almost completely lost their ability to expel cytoplasm on hydration (micrographs not shown).

FESEM and TEM demonstrate the existence of group 1 and group 5 allergens in materials expelled from 4 species of Poaceae pollen (other than *L perenne*)

Fig 3 shows that group 1 and group 5 allergens can be localized in the materials released from pollen of *Secale cereale* (group 1 allergens; Fig 3, *a* and *b*), *P pratensis* (group 5 allergens; Fig 3, *c* and *d*) and *A pratensis* (group 1 allergens; Figs 3, *e* and *f*). Gold labels indicating the sites of allergens seem randomly distributed. The controls (shown in Fig 4, *a* for *Secale cereale* group 1 preimmune antibodies) exhibit little or no background staining.

TEM examination of the expelled materials (shown in Fig 4, *b* through *e* for *P pratense*) demonstrates that group 1 (Fig 4, *c*) and group 5 (Fig 4, *d*) allergens are found in the amorphous, electron-dense (dark) structures representing the remnants of the cytoplasmic pollen architecture. Starch granules are unlabeled by gold particles. The control preparations (shown in Fig 4, *e* for group 5 preimmune antibodies) show little nonspecific staining. Unhydrated birch pollen, used as a negative control, did not cross-react with grass pollen anti-group 1 antibodies (Fig 4, *f*) or anti-group 5 antibodies.

DISCUSSION

The results of the present investigation strongly suggest that expulsion of allergen-containing respirable materials is a general mechanism of allergen release in the sweet grasses (Poaceae). Thus in grass pollens, at least 2 separate mechanisms of allergen release are operative:

1. Rapid diffusion of allergens in isotonic media (eg, nasal secretions), which has been demonstrated for many different pollens and many different allergens.¹⁵ Allergen diffusion from pollen grains might thus be responsible for the induction of immediate allergic symptoms on accessible mucosal surfaces (eg, allergic rhinitis, conjunctivitis) and in the skin.
2. Rapid expulsion of respirable allergen-containing submicronic materials after hydration in hypotonic media (eg, during rainfall). Because of the small size of the subcellular pollen particles, they might preferentially reach the deeper airways and induce symptoms of bronchial asthma.¹⁶

In contrast to previous investigators,¹⁰ we could not localize group 5 allergens inside the starch granules. We hypothesize that the allergens might have diffused out of the starch during hydration. In any case, attachment of allergen-containing cytoplasm to the surface of starch granules might explain the observation that starch granules can elicit bronchial asthma in sensitized patients.

In contrast to the sweet grasses, sour grasses (rushes and sedges) did not expel cytoplasm after exposure to water (unpublished observation). Likewise, birch pollen grains did not burst on hydration.

The question is why certain pollen species expel their cytoplasm whereas others do not. A possible explanation

is that grass pollen grains (reportedly) have thinner walls than birch pollen, which might favor "bursting."¹⁷ However, grass pollen grains do not rupture along the wall; rather, they expel particles via the germination pore. We therefore assume that differences in the chemical composition between grass and birch pollen cytoplasm might play a decisive role. Amyloplasts (found in birch and grass pollen) are osmotically largely inactive. However, the large amounts of polysaccharide particles (P-particles)¹¹ typically found in grass pollen cytoplasm could form a pool of osmotically active molecules, possibly developing osmotic pressures sufficient for the expulsion of cytoplasm.

Interestingly, we found that grass pollens stored for 4 weeks at ambient temperature and moisture lose their ability to expel cytoplasm. In this context, it is remarkable that episodes of grass pollen-induced asthma were reported to occur *during* the flowering season of grasses, when high loads of pollen grains could be detected in the environment.^{8,18} Because morphologic examination of air samples does not allow unambiguous identification of pollen from different grass species and because pollen allergens can be found in micronic atmospheric aerosol fractions¹⁹⁻²¹ and attached to other environmental particles (eg, diesel exhaust),²² antibody-based measurements of grass pollen allergen exposure can be considered.²³ In this context, it is worthy of note that antibody-based detection assays for the identification of species-specific grass pollen allergens are becoming available.²⁴

In conclusion, our study suggests the liberation of allergen-bearing subcellular particles from hydrated grass pollens as a general mechanism that might be responsible for grass pollen-induced asthma attacks after rainfall.

We thank Ms U. Malkus for excellent assistance in the preparation of specimens and Ms G. Kiefermann for expert photographic work.

REFERENCES

1. Judd WS, Campbell CS, Kellogg EA, Stevens PF. Plant systematics: a phylogenetic approach. Sunderland (MA): Sinauer Associates; 1999.
2. Freidhoff LR, Ehrlich-Kautzky E, Grant JH, Meyers DA, Marsh DG. A study of the human immune response to *Lolium perenne* (rye) pollen and its components, Lol p 1 and Lol p 2 (rye I and rye II). J Allergy Clin Immunol 1986;78:190-201.
3. Esch RE. Grass pollen allergens. In: Lockey RF, Bukantz SC, editors. Allergens and allergen immunotherapy. New York: Marcel Dekker; 1999. p. 103-20.
4. Suphioglu C. What are the important allergens in grass pollen that are linked to human allergic disease [editorial]? Clin Exp Allergy 2000;30:1335-41.
5. Blackley CH. Experimental researches on the causes and nature of Catarrhus aestivus (hayfever or hay-asthma). London: Dawson's of Pall Mall; 1959 (originally publication: London: Balliere, Tindall & Cox; 1873).
6. Johnson P, Marsh DG. "Isoallergens" from ryegrass pollen. Nature 1965;206:935-7.
7. Valenta R, Steinberger P, Duchene M, Kraft D. Immunological and structural similarities among allergens: prerequisite for a specific and component-based therapy of allergy. Immunol Cell Biol 1996;74:187-94.
8. Suphioglu C, Singh MB, Taylor P, Bellome R, Holmes P, Puy R, et al. Mechanism of grass-pollen-induced asthma. Lancet 1992;339:569-72.
9. Bellomo R, Gigliotti P, Treloar A, Holmes P, Singh MB, Knox B. Two consecutive thunderstorm associated epidemics of asthma in the city of Melbourne. Med J Aust 1992;156:834-7.

10. Singh MB, Hough T, Theerakulpisut P, Avjioglu A, Davies S, Smith PM, et al. Isolation of a cDNA encoding a newly identified major allergenic protein of rye grass pollen: intracellular targeting to the amyloplast. *Proc Natl Acad Sci U S A* 1991;88:1384-8.
11. Heslop-Harrison J. Aspects of the structure, cytochemistry and germination of rye (*Secale cereale* L.) *Ann Bot* 1979;44(Suppl 1):1-47.
12. Vrtala S, Fischer S, Grote M, Vangelista L, Pastore A, Sperr WR, et al. Molecular, immunologic, and structural characterization of Phl p 6, a major allergen and P-particle-associated protein from timothy grass (*P pratense*) pollen. *J Immunol* 1999;163:5489-96.
13. Grote M, Vrtala S, Niederberger V, Valenta R, Reichelt R. Expulsion of allergen-containing materials from hydrated rye grass (*Lolium perenne*) pollen revealed by using immunogold field emission scanning and transmission electron microscopy. *J Allergy Clin Immunol* 2000;105:1140-5.
14. Niederberger V, Laffer S, Fröschl R, Kraft D, Rumpold H, Kapiotis S, et al. IgE antibodies to recombinant pollen allergens (Phl p 1, Phl p 2, Phl p 5, and Bet v 2) account for a high percentage of grass pollen specific IgE. *J Allergy Clin Immunol* 1998;101:258-64.
15. Vrtala S, Grote M, Duchene M, van Ree R, Kraft D, Scheiner O, et al. Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity. *Int Arch Allergy Immunol* 1993;102:160-9.
16. Suphioglu C. Thunderstorm asthma due to grass pollen. *Int Arch Allergy Immunol* 1998;116:253-60.
17. Schäppi GF, Taylor PE, Staff IA, Suphioglu C, Knox RB. Source of Bet v 1 loaded inhalable particles from birch revealed. *Sex Plant Reprod* 1997;10:315-23.
18. Schäppi GF, Taylor PE, Pain MC, Cameron PA, Dent AW, Staff IA, et al. Concentrations of major grass group 5 allergens in pollen grains and atmospheric particles: implications for hay fever and allergic asthma sufferers sensitized to grass pollen allergens. *Clin Exp Allergy* 1999;29:633-41.
19. Agarwal KM, Swanson MC, Reed CE, Yunginger JW. Airborne ragweed allergens: association with various particle sizes and short ragweed plant parts. *J Allergy Clin Immunol* 1984;74:687-93.
20. Schumacher MJ, Griffith RD, O'Rourke MK. Recognition of pollen and other particulate aeroantigens by immunoblot microscopy. *J Allergy Clin Immunol* 1988;82:608-16.
21. Spieksma FTM, Kramps JA, van der Linden AC, Nikkels BH, Plomb A, Koerten HK, et al. Evidence of grass-pollen allergenic activity in the smaller micronic atmospheric aerosol fraction. *Clin Exp Allergy* 1990;20:273-80.
22. Knox RB, Suphioglu C, Taylor P, Desai R, Watson HC, Peng JL, et al. Major grass pollen allergen Lol p 1 binds to diesel exhaust particles: implications for asthma and air pollution. *Clin Exp Allergy* 1997;27:246-51.
23. Tovey E, Marks G. Methods and effectiveness of environmental control. *J Allergy Clin Immunol* 1999;103:179-91.
24. Lovburg U, Baker P, Tovey E. A species-specific monoclonal antibody to *Cynodon dactylon*. *Int Arch Allergy Immunol* 1998;117:220-3.

Availability of Journal back issues

As a service to our subscribers, copies of back issues of *The Journal of Allergy and Clinical Immunology* for the preceding 5 years are maintained and are available for purchase until inventory is depleted. The following quantity discounts are available: 25% off on quantities of 12 to 23, and one third off quantities of 24 or more. Please write to Mosby Subscription Customer Service, 6277 Sea Harbor Dr, Orlando, FL 32887, or call (800) 654-2452 or (407) 345-4000 for information on availability of particular issues and prices. If unavailable from the publisher, photocopies of complete issues may be purchased from Bell & Howell Information and Learning, 300 N Zeeb Rd, Ann Arbor, MI 48106, (313) 761-4700.