

Release of allergens as respirable aerosols: A link between grass pollen and asthma

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Background: Asthma incidence has long been linked to pollen, even though pollen grains are too large to penetrate into the airways where asthmatic responses originate. Pollen allergens found in small, respirable particles have been implicated in a number of asthma epidemics, particularly ones following rain-fall or thunderstorms.

Objective: The aim of this study was to determine how pollen allergens form the respirable aerosols necessary for triggering asthma.

Methods: Flowering grasses were humidified and then dried in a controlled-environment chamber connected to a cascade impactor and an aerosol particle counter. Particles shed from the flowers were analyzed with high-resolution microscopy and immunolabeled with rabbit anti-Phl p 1 antibody, which is specific for group 1 pollen allergens.

Results: Contrary to what has been reported in other published accounts, most of the pollen in this investigation remained on the open anthers of wind pollinated plants unless disturbed—eg, by wind. Increasing humidity caused anthers to close. After a cycle of wetting and drying followed by wind disturbance, grasses flowering within a chamber produced an aerosol of particles that were collected in a cascade impactor. These particles consisted of fragmented pollen cytoplasm in the size range 0.12 to 4.67 μm ; they were loaded with group 1 allergens.

Conclusion: Here we provide the first direct observations of the release of grass pollen allergens as respirable aerosols. They can emanate directly from the flower after a moisture-drying cycle. This could explain asthmatic responses associated with grass pollination, particularly after moist weather conditions. (*J Allergy Clin Immunol* 2002;109:51-6.)

Key words: Grass, pollen, allergen, aerosol, asthma, air pollution

It is only particles smaller than approximately 5 μm that can reach the lower respiratory tract where asthmatic responses originate.^{1,2} Nevertheless, pollen allergens appear to play a key role in the increasing incidence of

Abbreviations used

DEP: Diesel exhaust particle
RH: Relative humidity
SEM: Scanning electron microscopy
TEM: Transmission electron microscopy

asthma, even though pollen grains are so large (diameter, >20 μm) that they deposit in the upper airways when inhaled. Grass pollen allergens are predominantly located within the cytoplasm of mature pollen grains,³ but atmospheric samples of small respirable particles often contain these allergens, even when pollen counts are low.⁴⁻⁶ Epidemics of so-called thunderstorm asthma, during which hospital admissions rise within hours of storm passage, coincide with the appearance of grass pollen allergens in the respirable aerosol.⁷⁻¹³

Efforts to explain the presence of pollen allergens in respirable aerosol particles have focused on pollen rupture. When a fresh ryegrass pollen grain is immersed in water, the *zwischenkörper* and the intine layers of the apertural pore rupture, the result being release of the pollen contents, including up to 700 small (diameter, 0.6 to 2.5 μm) starch granules, through the mouth of the pollen pore.^{13,14} Suphioglu et al¹⁴ speculated that these starch granules form the respirable allergen aerosols after rainfall.

Pollen allergens have recently been localized in the fragmented cytoplasm released from pollen grains that had been ruptured in water on a glass slide.¹⁵ Ruptured grass pollen was also effective in producing positive cutaneous reactions in a cohort of patients with thunderstorm asthma.¹⁶ Nebulized pollen cytoplasm containing starch granules elicits bronchospastic responses when inhaled by asthmatic patients.¹⁴ Particles resembling starch granules have been filtered from the air after rainfall,^{13,14} but how pollen that ruptured in water could produce a free aerosol remained to be explained.

Wind-pollinated plants, such as grasses, are believed to dehisce (open) their anthers under dry conditions and immediately shed their pollen into the air and onto surrounding surfaces.¹⁷⁻²¹ Researchers have speculated that pollen rupture occurs in the airways,²² during rainfall on surfaces such as leaves,²³ or in the air.¹⁴ Germinated olive (*Olea europaea* L) pollen has been found inside anthers after rainfall, but this was thought to be premature germination occurring before anther opening.²⁴

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Supported in part by the Sunair Children's Foundation of Altadena, Calif.

Received for publication July 9, 2001; revised September 27, 2001; accepted for publication October 3, 2001.

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0091-6749/2002 \$35.00 + 0 1/81/120759

doi:10.1067/mai.2002.120759

This article reports the first direct observations of the formation of allergen-loaded aerosols from pollen and provides the link to rainfall and other meteorologic conditions. We describe laboratory approaches to replicate a natural mechanism for the release of pollen allergen-loaded aerosols so they can be characterized for their ability to trigger asthma. To determine where and under what conditions the respirable particle release occurs, we examined flowering in 2 major allergenic grasses.

METHODS

Plant collection

Flowering stems of ryegrass (*Lolium perenne* L) and Bermuda grass (*Cynodon dactylon* L) were harvested from the grounds of the California Institute of Technology in Pasadena, Calif, and placed indoors in a vase of water. Collections were replenished early in the morning every second day. A 40-W lamp provided supplementary lighting. Some flowers were wetted with water by means of a misting spray or a nebulizer.

Controlled environment experiment

Flowering stems were also placed in a Plexiglas chamber connected to a Sierra cascade impactor (flow rate, 3.0 L/min), a model 226 Laser Aerosol Particle Counter (Royco Inst Inc; flow rate, 0.3 L/min; lower detection limit, 0.12 μ m), and temperature and relative humidity (RH) probes. Old anthers were removed, and fresh anthers were induced to open within the chamber for analysis. This was designed to minimize the potential effect of previous environmental exposures on the mature pollen and anthers. The controlled-environment chamber was monitored to optimize flowering of grasses through control of RH, temperature, airflow, and lighting. Air entered the chamber through a filter to eliminate any background aerosol.

We used approximately 15 flowering stems of ryegrass, each 14 mm long, and after 2 to 3 hours of exposure to 60% RH at 27°C, these produced as many as 300 anthers. We then humidified the air in the chamber by passing the incoming air through an evaporator and reducing the flow rate to allow transpiration to raise the humidity. The plants were left in high humidity for 1 to 6 hours. The samplers then pulled dry air into the chamber (2 to 3 L/min). In later experiments, the humidified Plexiglas container was opened and the plants were wetted with a fine mist produced by a water sprayer. All experiments were replicated at least 3 times.

Pollen rupture and anther analysis

Pollen was collected from open anthers by contact with a glass microscope slide or coverslip. To assess pollen rupture, either a drop of water (or 10% sucrose) was applied or water droplets were condensed onto the pollen. Preparations were observed by means of a Zeiss light microscope through use of bright-field optics and recorded by means of a Panasonic CCD video camera and recorder. Still frames from every 10 seconds of recording were digitized and then printed. Some coverslips were air-dried for scanning electron microscopy (SEM). Moistened anthers and cover slips were mounted on SEM stubs with carbon conductive paint and then air-dried and sputter-coated (Technics Hummer Sputter Coater) with platinum/palladium or gold. Specimens were viewed with an Etec Autoscan scanning electron microscope at 20 kV.

Ultrastructural analysis and immunolocalization

For transmission electron microscopy (TEM), coverslips removed from the cascade impactor were anhydrously fixed in the

vapor from 1.7% paraformaldehyde in 2,2-dimethoxy propane for 10 minutes. They were then infiltrated in a drop of LR gold resin (Ted Pella, Inc, Redding, Calif) for 24 hours at 4°C. Each sample was inverted and placed on top of a gelatine capsule filled with LR gold resin containing 1% benzil. Samples mounted on capsules were inverted and polymerized at 4°C in a refrigerator with a 40-W lamp. Sections 100 nm thick were collected on polyvinyl butyral-coated gold grids and immunolabeled for group 1 allergens with rabbit anti-Phl p 1 antibody. This antiserum was raised against purified recombinant Phl p 1 (group 1 allergen) as previously described.¹⁵ The primary antibody was detected with 15-nm gold-goat antirabbit (Sigma Chemical Co, St Louis, Mo). For controls, primary antibody was omitted, the antibody was preincubated with recombinant Phl p 1, the antibody was serially diluted, or a preimmune serum or irrelevant antibody (rabbit anti-Bet v 1) was used, according to published protocols.³ For each antibody, immunolabeling was performed on at least 3 separate grids with sections attached. Preparations were viewed with a Philips CM 120 transmission electron microscope.

RESULTS

Grass pollen rupture

Approximately 72% of freshly collected grass pollen ruptured in water within 5 minutes (Fig 1, *a-d*). Slight wetting, as would occur in dew formation or by sedimenting fog droplets, also triggered rupture of fresh pollen (Fig 1, *e*). The time required for rupture increases with the age of the pollen, and the fraction of grains that rupture decreases. After 48 hours of storage at 27°C, less than 10% of the pollen ruptured. Commercially purchased pollen stored for more than 1 year at 4°C had less than 1% pollen rupture. The pollen contents released in solution bind tightly to the underlying surface. These particles are very difficult to remove from the surface of a glass slide or leaf because of intense adhesion forces. Fresh grass pollens exposed to isotonic aqueous solutions remained intact. Grass pollen is thus unlikely to rupture in isotonic fluids.

Grass plants flowering in a quiescent environment produced anthers that opened (dehiscenced), but most of the pollen on the anther surface was retained until disturbed. A heavy water spray on the flowers washed off the pollen without producing aerosol. However, a light misting of water or humidifying of the plants caused the anthers to close and encapsulate the pollen. On drying, the anthers reopened. This could be repeated 3 times in 2 hours.

Chamber humidification and drying of grass flowers

After flowers were placed in a Plexiglas chamber (Fig 2) and exposed to a RH of 53% to 67% for 2 to 3 hours at 27°C, the anthers extended from the flowers, split longitudinally, and unfurled. For the next 2 days, there appeared to be no appreciable stress on the flowers, inasmuch as they continued to produce anthers that contained viable pollen. Most of the pollen grains remained on the anthers. The air sampled contained no free respirable particles even after the plants were shaken or airblown. Much pollen was released on disturbance of the dried anthers, but most collected onto the bottom of the chamber. Some did collect in the top stage of the impactor, but positioning of the sam-

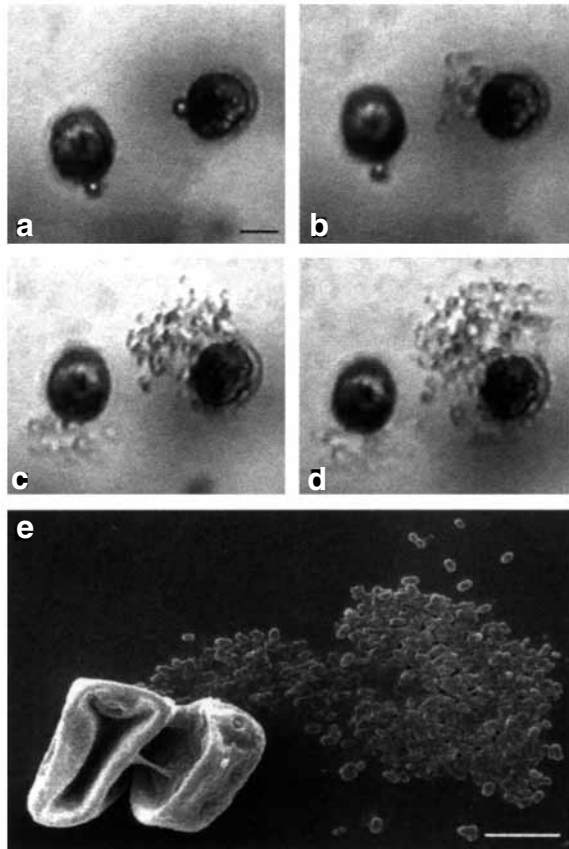


FIG 1. a-d, Time sequence of pollen rupture (1 frame every 10 seconds) from video light microscopy. Fresh ryegrass pollen was placed in a drop of water; the first photo was taken after 35 seconds. e, Ryegrass pollen collected directly from anthers by contact with a microscope slide and exposed to sedimenting mist droplets. Samples were air-dried, gold-sputter coated, and viewed with SEM. Bar = 10 μ m.

pler near the top of the chamber limited deposition of pollen grains to within the cascade impactor.

In an experiment with undisturbed grass flowers, the air in the chamber was humidified by passing the incoming air through an evaporator for 2.5 hours at a reduced flow rate of 0.3 L/min. Within 1.5 hours, RH increased to 88% at 29°C and the anthers closed again, trapping the pollen. The chamber was further humidified until the anthers were wet with condensed water vapor (92% RH, 31°C). The samplers then pulled dry air into the chamber for approximately 1 hour at 3.3 L/min before the ryegrass anthers reopened at <80% RH at 27°C. Within another hour of drying (65% RH, 25°C) pollen cytoplasmic debris had deposited on the lower stages of the impactor. The sample contained an estimated 49,192 particles in the 0.2- to 3.5- μ m size range. Three hours later, plants were disturbed by shaking or by blowing air across the flowers. Optical particle counts increased 6-fold, and a further 1342 particles were counted in the range 0.87 to 4.67 μ m. The cascade impactor was checked every hour during the experiment and 15 minutes after plants were disturbed. Bermuda grass released smaller respirable par-

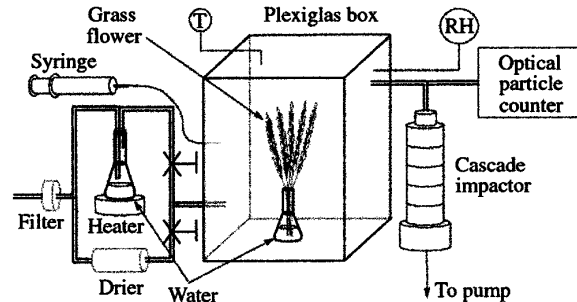


FIG 2. Apparatus used to measure respirable particle release. Filtered air is either humidified or dried before entering the Plexiglas chamber. Size-classified aerosol samples are collected with the cascade impactor. An optical particle counter was used to size particles online in the Bermuda grass experiments. The 100-mL syringe was used to generate air pulses. T, Temperature probe; RH, relative humidity probe.

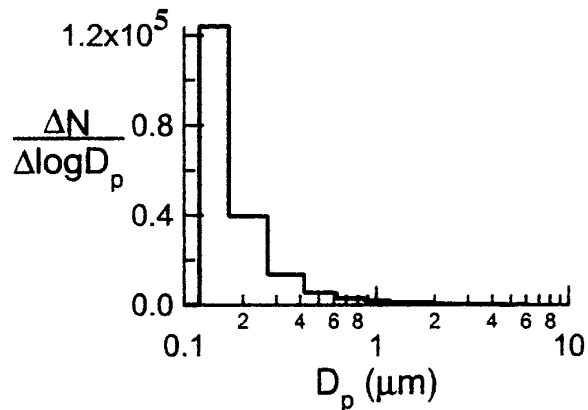


FIG 3. Size distribution of particles shed from Bermuda grass flowers as measured by means of the optical particle counter. Flowers were humidified in a Plexiglas chamber for 6 hours, air-dried for 6 hours, and then disturbed by wind.

ticles, but only when the plants were disturbed by wind or shaking (46,761 particles were optically detected in the range 0.12 to 2.37 μ m). This count increased to 163,724 after plants were humidified for 6 hours and then air-dried for 6 hours before collection (Fig 3). Yields were further increased when flowering grass was misted with water after humidification. This was achieved by opening the chamber to shorten wetting times, but increased drying times were required.

Ultrastructural analysis of anthers and respirable particles

After samples from the lower stage of the cascade impactor were fixed and embedded for TEM, viewing of sections revealed that the aerosolized particles were composed of pollen cytoplasmic debris. Particles varied in diameter from 0.12 to 4.67 μ m and contained nuclei, mitochondria, starch granules, and other organelles, often enveloped within membranes (Fig 4, a). Heterogeneous particles were observed, such as a vegetative nucleus with

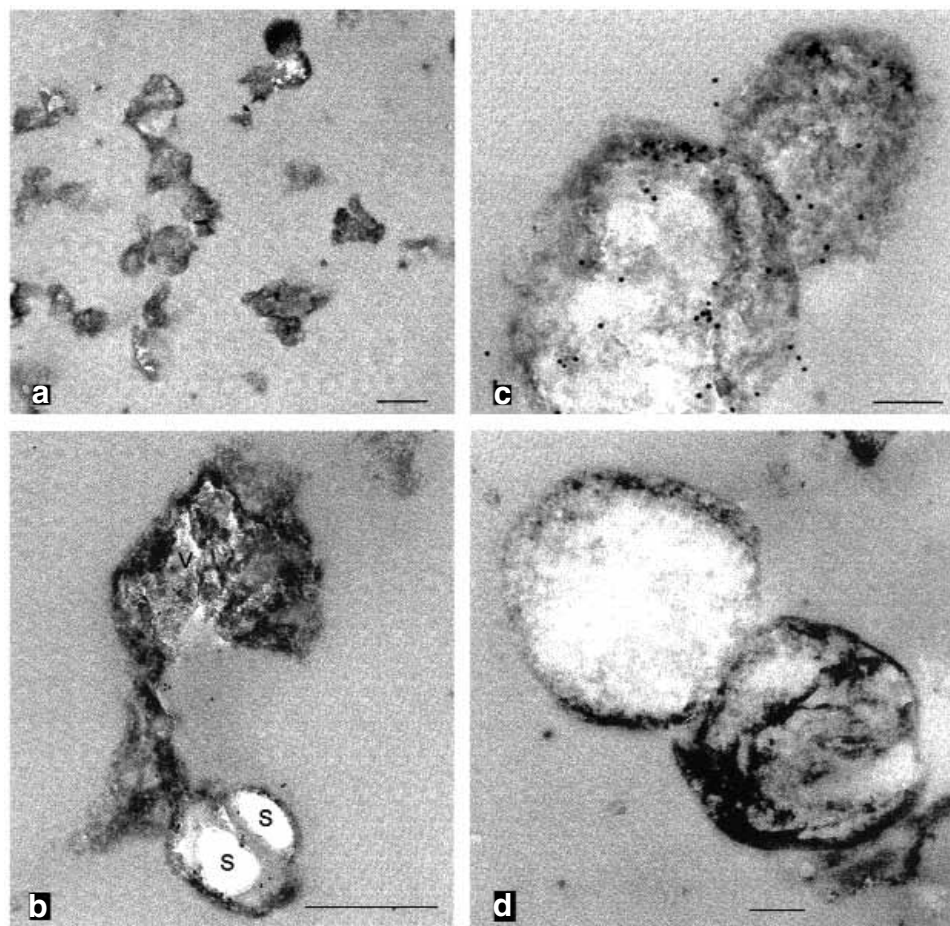


FIG 4. TEM of particles collected from the lower stage of the cascade impactor. **a, b**, Bar = 1 μ m. V, Vegetative nucleus; S, starch granule. **c**, Immunolabeled section shows localization of group 1 allergens. **d**, Omission of the primary antibody control for immunolabeling. Bar = 0.2 μ m.

2 small starch granules and cytoplasm attached (Fig 4, *b*). Grass pollen-specific group 1 allergens were localized in sections of the particles produced from pollen-laden anthers and collected in a cascade impactor (Fig 4, *c*). Starch granules were unlabeled, but attached cytoplasmic debris was often labeled. The controls, in which the primary antibody was omitted (Fig 4, *d*), antibody was preincubated with recombinant Phl p 1, or a preimmune serum or irrelevant antibody (rabbit anti-Bet v 1) was used, showed no significant labeling above background counts.

Ultrastructural analysis of ryegrass anthers that had opened and were then misted with a fine spray of water showed that (1) most of the pollen remained on the anthers and (2) numerous micronic particles were attached to the pollen surfaces (Fig 5, *a*). In another anther, several pollen grains had ruptured and remained attached to the orbicules that lined the anther surface (Fig 5, *b*). The anther surface is lined with orbicules (diameter, 0.4 to 0.5 μ m) that remain in place during flowering (Fig 5, *c*). The surface of the orbicules has many excrescences, their pattern being similar to that of the excrescences located on the pollen grain surface.

DISCUSSION

Grass pollen development occurs within the aqueous milieu of the anther sac.²⁵ On drying, the anther splits open longitudinally and exposes mature pollen to the air. Unlike the authors of previously published reports,¹⁷⁻²¹ we observed that pollen grains remained on the open anther until there was sufficient disturbance to shed the pollen. This was found on flowering plants both indoors and outdoors. This is the first time that pollen has been reported to remain on the anthers of wind-pollinated plants. In the literature, anther dehiscence (or opening) and pollen shedding (or release) are often described without recognition that a significant amount of time elapses between these 2 events. Quantification of the wind speeds or other forces required to remove pollen from anthers in a variety of flowering plants remains to be assessed.

Anther closure during rainfall has previously been observed for insect-pollinated plants²⁶; it is suggested that this protects lily pollen until the return of the butterfly pollinators.²⁷ If, however, the moisture were sufficient to rupture grass pollen grains within the closed

anther, pollen fragments might disperse and produce a respirable aerosol. After grass flowers were harvested and placed in a Plexiglas chamber connected to a cascade impactor and a particle counter, the anthers opened. Each ryegrass anther possessed approximately 5000 pollen grains. Bermuda grass produces 80% fewer anthers and 66% less pollen per anther than ryegrass.²⁸ A cycle of wetting and drying did rupture pollen on the anthers. Even with the gentle airflow through the chamber, the cytoplasmic debris from ruptured pollen can form respirable particles that are loaded with allergens and thus probably capable of triggering an asthmatic response in susceptible people.

The observed release of micron- and submicron-sized particles from the anther suggests that the decorations of the anther and pollen surfaces dramatically reduce the binding of these small particles to their surface. The cytoplasmic debris forms an aerosol with smaller size fractions and more numerous particles than the starch granules previously hypothesized as being released. Allergen deposition in the lower airways might thus be even greater than speculations have suggested.

Our observations of the release of subcellular pollen components after a cycle of humidifying, wetting, and drying of flowering grasses show how light rainfall, dew, fog, and watering of lawns can rupture pollen grains within the anthers. After drying, disturbances such as wind, recreational activities, and lawn-mowing can cause the release of allergen-loaded particles of respirable size as a free aerosol, creating a potential trigger for asthma. The incidence of asthma epidemics associated with thunderstorms during the grass pollen season might now be explained. Climate changes occurring during a thunderstorm could lead to pollen rupture on the flowers and subsequent dispersal of respirable particles. Previously observed wind flow patterns concentrating allergens at tree level might maximize the exposure of susceptible people to these allergen-loaded particles.²⁹ Our observations might also account, in part, for the asthmatic symptoms observed in susceptible people throughout the grass flowering season after any moist weather conditions, not just during thunderstorms.

This article has explored the production of respirable pollen allergens from only 2 grasses. Continuing work in our laboratories on wind-pollinated trees, weeds, and molds suggests the occurrence of related respirable aerosol release mechanisms, though the mechanisms differ substantially in detail.

Asthma prevalence has increased alarmingly in recent decades, though the cause remains unclear.³⁰⁻³³ Pollutant particles from combustion systems such as motor vehicles have been linked to the incidence of allergic asthma. Diesel exhaust particles (DEPs) are a major contributor to the urban respirable aerosol mass (18% in Pasadena, Calif³⁴) and have been implicated as a cause of allergic rhinitis and asthma in mice and human beings.³⁵ Mice were exposed to diesel exhaust aerosol and then sensitized with ovalbumin.³⁶ Subsequent airway challenges with ovalbumin increased airways hyperreactivity and

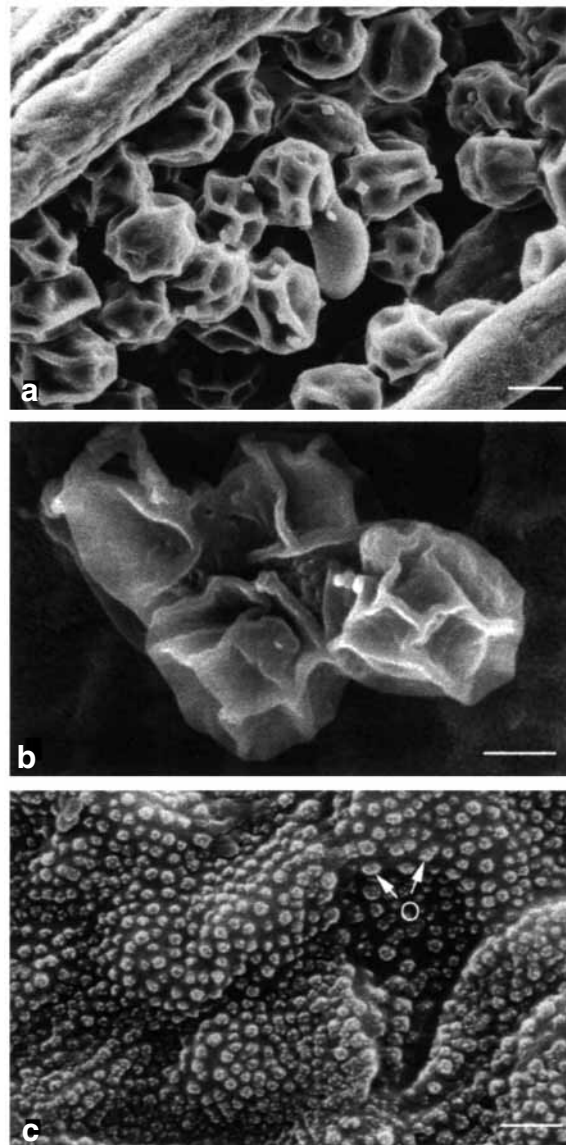


FIG 5. SEM of freshly collected anthers. **a, b,** Open anthers have been wetted and then dried in still air. **Bar** = 10 μ m. **c,** Open anther collected from a ryegrass flower after air-drying and pollen removal. **Bar** = 2 μ m. **O,** Orbicules.

inflammation in mice exposed to DEPs in comparison with mice exposed to ovalbumin and saline aerosol, in which little airway inflammation was found. When human bronchial epithelial cells from asthmatic subjects were cultured with DEPs, they released greater quantities of proinflammatory cytokines than epithelial cells from nonasthmatic subjects.³⁷ Soluble organic chemicals extracted from DEPs increased degranulation of murine mast cells³⁸ and might amplify inflammation caused by respirable allergens.

Because of their small size, aerosols of pollen allergens and fine combustion particles might deposit in similar regions of the respiratory tract. Synergistic combinations of allergen-loaded pollen debris and polycyclic

aromatic hydrocarbons, found in fine combustion aerosols, might explain the increased prevalence of pollen-induced asthma in the last half century.

We thank Dr I. Staff, Dr T. Spurr, and P. Koen for assistance with microscopy and J. Wang for assistance with air sampling. We thank Professor J. Richards at Caltech, and we are grateful to the School of Botany, University of Melbourne, and the Departments of Botany and Agriculture, La Trobe University, for provision of facilities.

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