

Abortive pollen germination: A mechanism of allergen release in birch, alder, and hazel revealed by immunogold electron microscopy

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Background: Pollen from early-flowering trees (eg, birch, alder, hazel) represent major seasonal allergen sources. The effects of rain on the release of allergens from tree pollen has thus far not been studied at the ultrastructural level. **Objective:** This study was designed to investigate the effects of rain on the morphology of pollens from early-flowering trees and of potential rain-induced mechanisms of allergen release. **Methods:** Freshly collected pollen grains (birch, alder, and hazel) were exposed under controlled conditions to rainwater. Changes of pollen morphology and the release of allergens were analyzed by scanning electron microscopy. The release of allergen-bearing submicronic particles was studied by field emission scanning electron microscopy and transmission electron microscopy in conjunction with immunogold staining by using antibodies with specificity for the major allergens. **Results:** Scanning electron microscopy showed that freshly isolated pollen grains from birch, alder, and hazel have abortive germination in rainwater. Abortive pollen germination is characterized by the formation of short pollen tubes, which rupture at their tips and release micronic and submicronic particles containing major allergens. Immunogold transmission electron microscopy provided evidence that the allergens are transported through the pollen tubes during germination. **Conclusions:** Rainwater-induced release of allergen-bearing submicronic particles from abortively germinated tree pollens may represent a mechanism of allergen release, with important implications on the induction of asthma as well as on current methods for measuring environmental allergen exposure. (J Allergy Clin Immunol 2003;111:1017-23.)

Key words: Pollen allergens, allergen release, immunogold electron microscopy, submicronic particles, Bet v 1

Pollen from trees of the order *Fagales* (eg, birch, alder, hazel) constitute the most important allergen sources in middle and northern Europe as well as in northern America and even in certain parts of Australia.¹ The major

Abbreviations used

FESEM: Field emission scanning electron microscopy
SEM: Scanning electron microscopy
TEM: Transmission electron microscopy

allergens of birch (Bet v 1), alder (Aln g 1), and hazel (Cor a 1) represent cross-reactive protein allergens with a molecular weight of approximately 17 kDa.^{2,3} Bet v 1, Aln g 1, and Cor a 1 have been classified as major allergens because >90% of sensitized patients react with these molecules, and more than 65% of these patients are sensitized exclusively against these allergens.^{4,5}

Methods for the measurement of allergen exposure have been developed that are either based on counting of pollen grains in air samples or that use antibody-based allergen detection systems.⁶⁻⁸ They represent a basis for controlled allergen avoidance and allow correlation of symptoms with exposure and thus allow evaluation of the effects of therapeutic measures, for example, allergen-specific immunotherapy, on allergen-induced clinical symptoms.

As far as dry pollen is concerned, it has been demonstrated that the major *Fagales* allergens represent cytoplasmic proteins, which cannot be detected on the surface of dry pollen.⁹ However, during hydration in isotonic media, such as occurs during pollen contact with the mucosa of the upper respiratory tract, these allergens elute within minutes and can induce local allergic reactions.¹⁰ The latter mechanism is responsible for symptoms of allergic rhinoconjunctivitis but cannot explain the occurrence of allergic asthma during or after the pollination period because intact pollen grains are unlikely to intrude into the deeper airways because of their size.^{11,12}

In this study, we investigated the effects of rainwater on the morphology and release of allergens from pollen of birch, alder, and hazel by using scanning electron microscopy and transmission electron microscopy in conjunction with immunogold labeling to identify and characterize possible mechanisms of rainwater-induced allergen release. Our finding that submicronic particles released after abortive pollen germination represent respirable, allergen-containing particles helps explain the induction of asthma during the tree pollen season. It also suggests that it may be important to conduct measure-

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ments of allergen exposure based on the true (eg, antibody-based allergen determination) detection of allergen molecules in the environment rather than on exclusively counting intact pollen grains.

METHODS

Pollen materials

Pollens from hazel, alder, and birch were collected from flowering catkins in Münster in springtime 2002. The pollen samples were used fresh without any storage. Rainwater was taken from different collecting points, being as much as 200 km apart from one another. The pH values of the water samples were measured with the use of indicator paper and showed small variations around a value of pH 6.

Pollen preparation

For SEM, pollen grains were dusted on aluminum stubs that had been coated with a thermosensitive glue. After short warming, pollen grains firmly stuck to the support. For comparison, pollen grains were dusted on glass coverslips without any glue. The pollen samples were covered with approximately 200 μ L of rainwater. Evaporation of the water took between 6 and 8 hours. For conventional SEM, pollen preparations were sputter-coated with gold. For FESEM immunogold labeling of Bet v 1 allergens, specimens were fixed in *p*-formaldehyde vapor and labeled according to the protocol given below.

For TEM, pollen samples were put into an Eppendorf vial, hydrated in rainwater overnight, and centrifuged. The pellet containing the pollen grains was fixed in 4% aqueous *p*-formaldehyde in 0.2 mol/L PIPES buffer, pH 7.4, for 24 hours. Then the pollen was dehydrated in an ascending series of ethanols and embedded in Lowicryl K4M at -35°C . Ultrathin sections were cut and immunogold-labeled for Bet v 1 or Bet v 1–cross-reactive allergens, as indicated below.

Antibodies

A rabbit antiserum was raised against purified recombinant major birch pollen allergen, Bet v 1, by immunization of New Zealand White rabbits by using complete and incomplete Freund's adjuvans (Charles River, Kisslegg, Germany). The rabbit antibodies were tested for cross-reactivity with the major allergens of alder (Aln g 1), hazel (Cor a 1), and hornbeam (Car b 1) by immunoblotting as described.¹³

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

Immunogold-labeling protocol

Pollen grains on aluminum stubs or ultrathin sections of embedded pollen grains were labeled for Bet v 1 and Bet v 1–cross-reactive allergens by using the indirect labeling technique (moist chamber, ambient temperature, PBS buffer at pH 7.4): (1) 5% BSA in PBS, 15 minutes; (2) Rabbit anti-Bet v 1 IgG antibodies, diluted 1:30 in PBS + 1% BSA, 2 hours; (3) PBS + 1% BSA, 1 $\times$ 5 minutes, Tris buffer + 1% BSA, pH 8.2, 2 $\times$ 5 minutes; (4) Goat anti-rabbit IgG antibodies coupled to 10 nm colloidal gold particles, diluted 1:20 in Tris buffer + 1% BSA, pH 8.2, 1 hour; (5) Tris buffer + 1% BSA, pH 8.2, 1 $\times$ 5 minutes; distilled water, 2 $\times$ 5 minutes. Controls were run replacing the specific rabbit antibody against Bet v 1 with the preimmune antibodies and by canceling steps 2 + 3 from the protocol.

Electron microscopy

Sputter-coated specimens were examined in a conventional Hitachi S 450 scanning electron microscope. After immunogold labeling, FESEM specimens were air-dried and coated with 1.5 nm Pt/C for obtaining conductivity and a good contrast in secondary elec-

tron micrographs. They were evaluated in a Hitachi S 5000 field emission scanning electron microscope, using the backscattered electron mode. Grids with ultrathin sections were air-dried and stained with heavy metals to increase contrast. They were analyzed in a Hitachi H 500 transmission electron microscope working at 75 kV.

RESULTS

Contact with rainwater induces abortive germination in pollen of hazel, alder, and birch with release of submicronic particles

To investigate the behavior of pollen grains from hazel, alder, and birch on exposure to rainwater, pollen grains were analyzed by SEM immediately after hydration and drying. Fig 1 shows preparations of hazel (Fig 1, A and B), alder (Fig 1, C and D), and birch (Fig 1, E and F) pollen grains after hydration in rainwater. At low magnification in the conventional scanning electron microscope (Fig 1, A, C, and E), germinating pollen grains can be detected that are surrounded by variable amounts of micronic and submicronic granular materials. Detailed analyses performed for single pollen grains at higher magnification are shown in Fig 1, B, D, and F. Each of these pollen tubes is ruptured at its tip and surrounded by a large number of granular and amorphous particles, among which starch grains can be identified by their characteristic shape.

Released submicronic particles contain major allergens

Next, we studied whether the submicronic particles released after rainwater-induced pollen germination may contain allergens. For this purpose, we performed immunogold electron microscopy using antibodies specific for the major allergens of birch (Bet v 1), alder (Aln g 1), and hazel (Cor a 1). Fig 2 exemplifies the localization of the major allergen of alder (Aln g 1) in cytoplasmic materials released from alder pollen by field emission scanning electron microscopy. When an area such as enclosed in the rectangle in Fig 2, A, is examined at high magnification, Aln g 1 allergens as indicated by gold particles (bright dots, arrows) are found distributed over the released submicronic materials (Fig 2, B). The corresponding control experiment with preimmune immunoglobulin (Fig 2, C and D) shows almost no labeling. Similar results were obtained when pollen grains from birch and hazel were analyzed by immunogold electron microscopy. Submicronic particles from birch and hazel pollen contained the major allergens of birch (Bet v 1) and hazel (Cor a 1), respectively (data not shown).

Expulsion of allergen-containing particles in the course of pollen germination

In dry pollen, the major allergens of birch, alder, and hazel are found predominantly in the cytoplasm but not in the pollen wall. We therefore investigated by immunogold transmission electron microscopy whether in the course of pollen germination allergen-containing cytoplasmic material is transported through the pollen tube and released after rupture of the tube. Fig 3 shows the localization of the

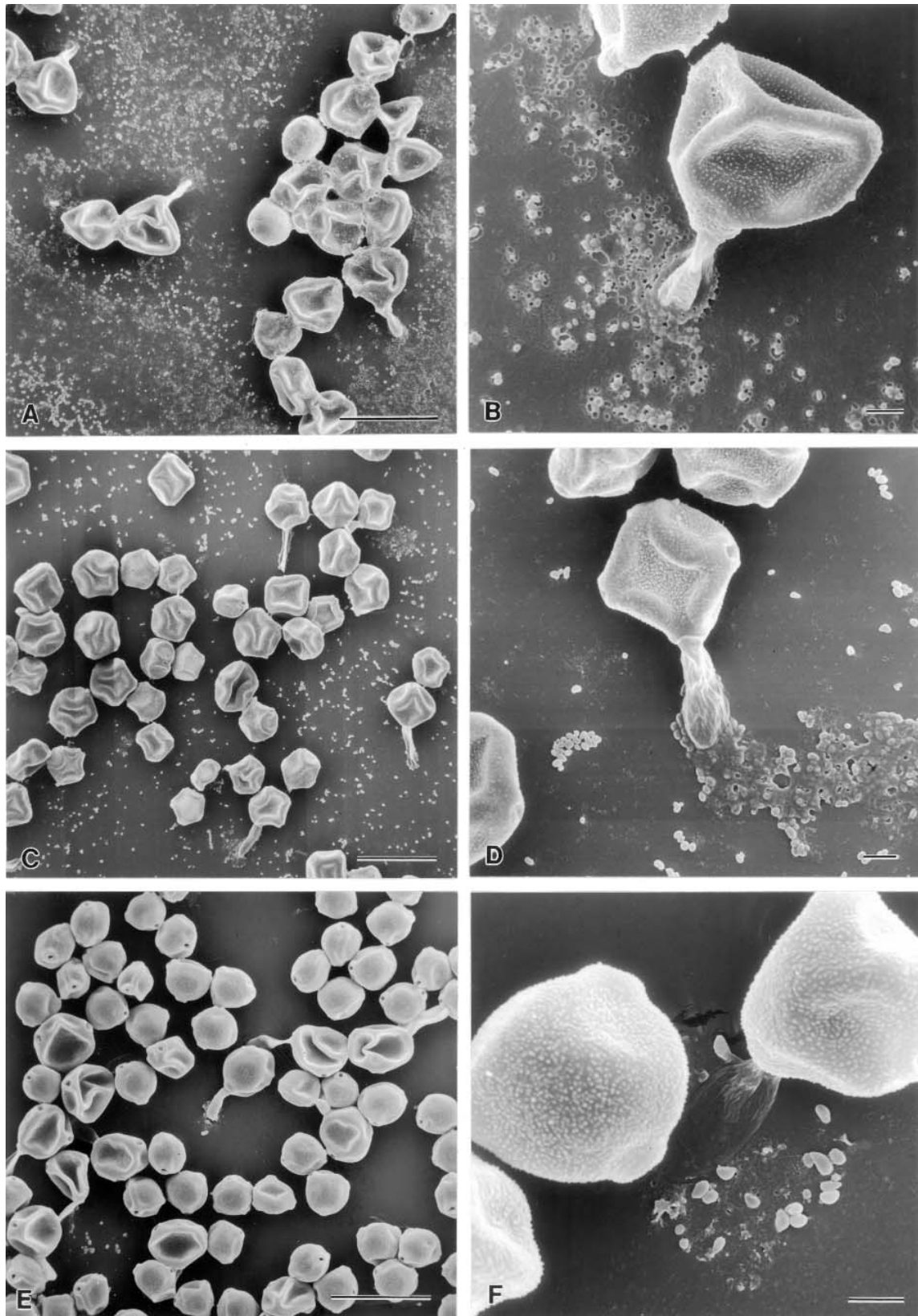


FIG 1. Conventional scanning electron micrographs of hazel (A and B), alder (C and D), and birch (E and F) pollen grains after hydration in rainwater. A, C, and E show surveys of hydrated pollen preparations at low magnification. Bar = 50 μ m. B, D, and F show details of germination tubes. Bar = 5 μ m.

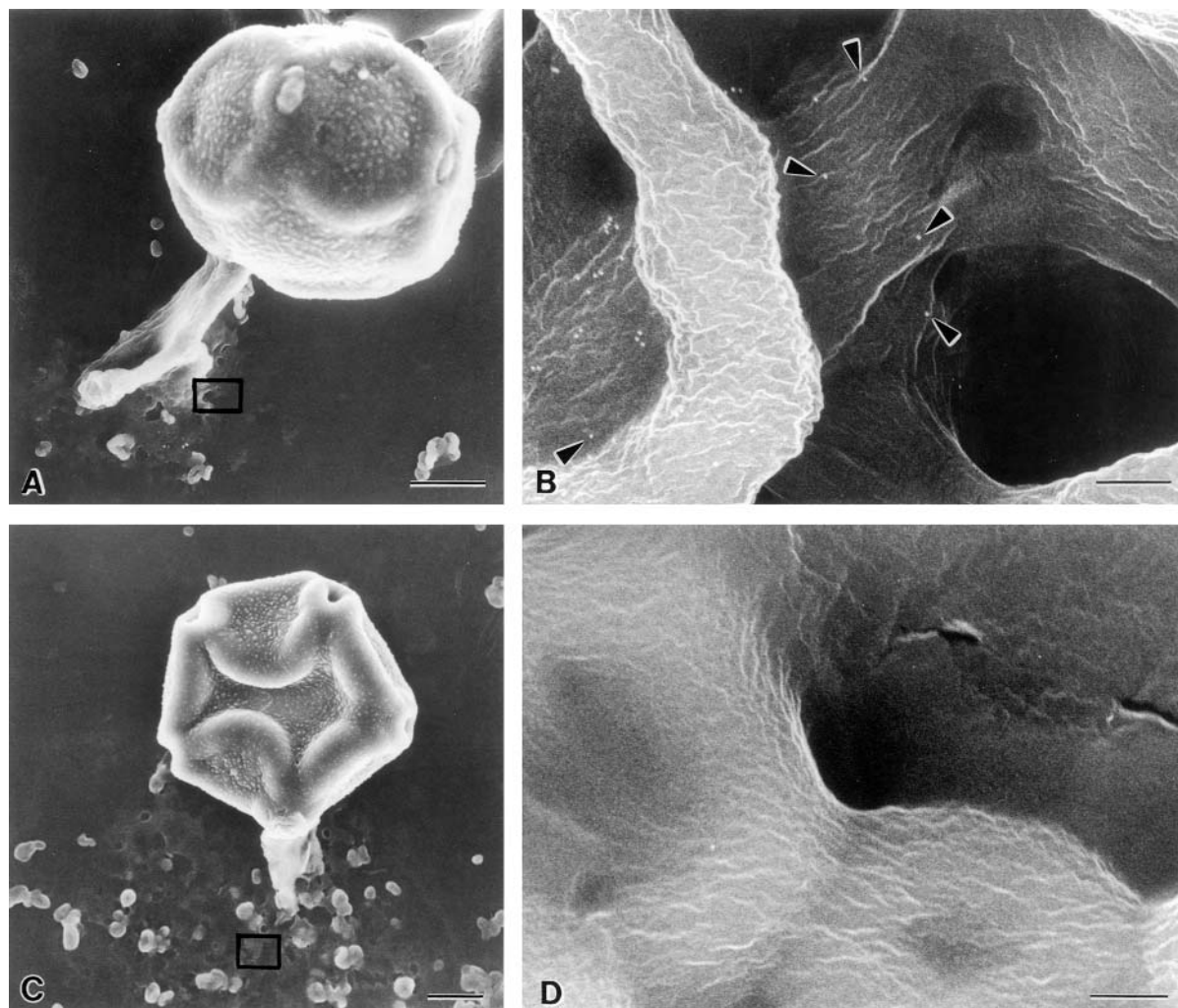


FIG 2. Field emission scanning electron micrographs of alder pollen after immunogold labeling for Aln g 1 allergens. **A** and **C**, Surveys of germinated pollen grains, with rectangle indicating an area such as chosen for analysis of gold particle labeling of Aln g 1 allergens, which is shown in **B** and **D**. **B**, Localization experiment; **D**, control. **A** and **C**: bar = 5 μ m. **B** and **D**: bar = 0.25 μ m.

major allergens of hazel (Cor a 1) and birch (Bet v 1) in ultrathin sections of germinating hazel (Fig 3, A-C) and birch (Fig 3, D-F) pollen. In the case of hazel, an early germination stage of the pollen grain was cut perpendicularly as shown in Fig 3, A. High magnification of the area enclosed in Fig 3, A, localizes the allergen (black dots represent gold particles) predominantly in the cytoplasmic matrix (Fig 3, B). The micrograph of the control experiment performed with preimmune immunoglobulin (Fig 3, C) taken from a different pollen tube but showing a similar area of the cytoplasm reveals a low degree of background staining (Fig 3, C). In the case of birch pollen, the pollen tube was cut obliquely. Released particles were recovered for analysis (Fig 3, D). The major birch pollen allergen, Bet v 1 (arrows point to gold particles), was found in the released amorphous constituents (Fig 3, E). Control experiments performed with preimmune immunoglobulin in other pollen tubes showing similar release of particles showed no staining (Fig 3, F).

DISCUSSION

This investigation shows that after contact with rainwater, pollen grains from birch, hazel, and alder can release allergens into the atmosphere by a mechanism for which we coin the term "abortive germination." This term was chosen because fresh pollen grains of birch, alder, and hazel had pollen tube growth in rainwater in a way similar to pollen tube growth on the stigma of the female reproductive organ, which is required for fertilization. In contrast to normal fertilization, however, tube growth in rainwater stops after some hours and the tube proves abortive. A similar observation was previously published in the literature. The authors found germinated pollen grains deposited on birch leaves and hypothesized that starch particles liberated from degrading pollen tubes might represent a potential allergen source in birch.¹⁴⁻¹⁶

In this study, we demonstrate by a combination of scanning and transmission electron microscopy in con-

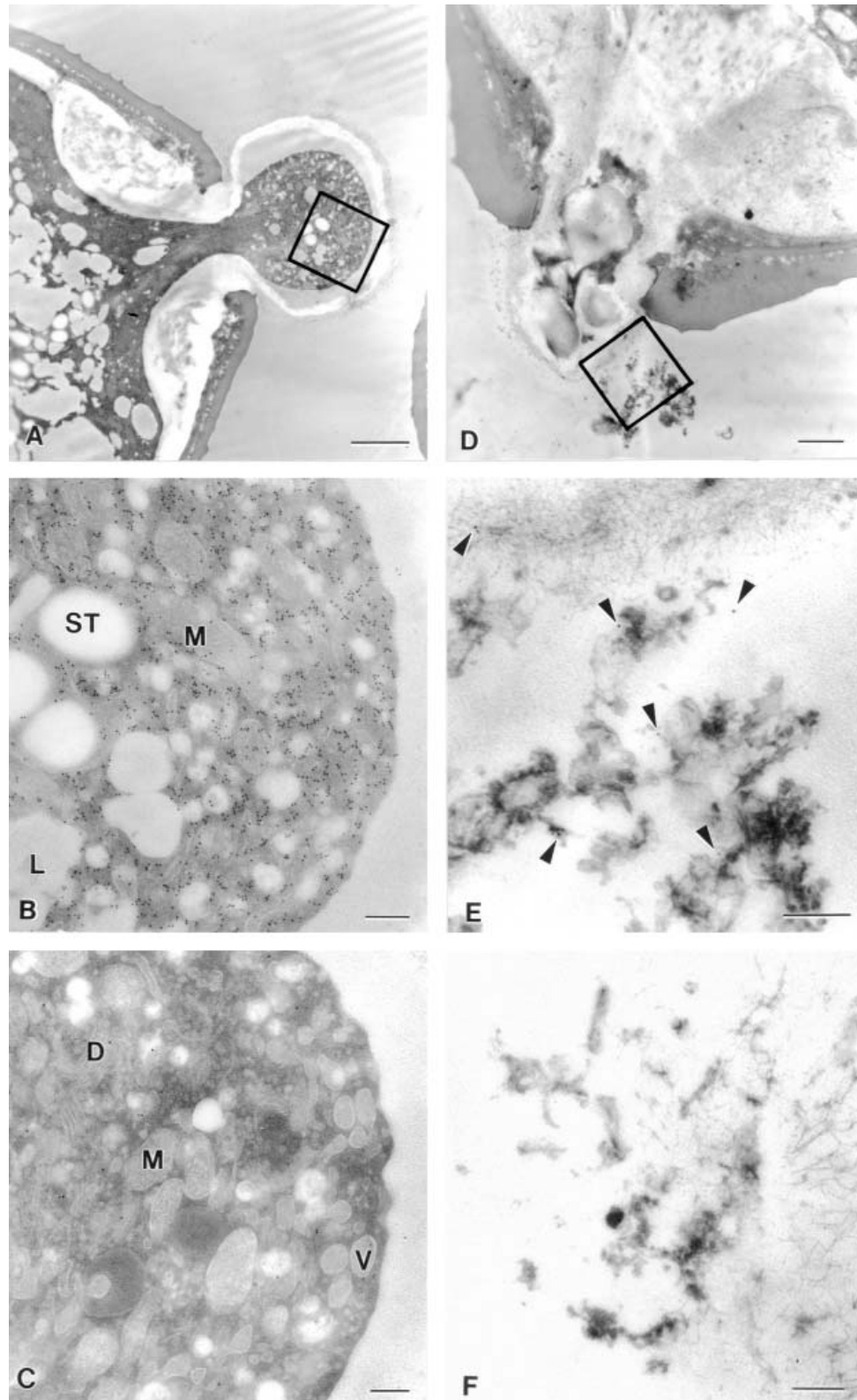


FIG 3. Transmission electron micrographs of germinated pollen grains after immunogold labeling for Cor a 1 allergens and Bet v 1 allergens, respectively. **A-C,** Hazel pollen. **D-F,** Birch pollen. **A** and **D,** surveys at low magnification. **B** and **E,** Immunogold labeling for Cor a 1 and Bet v 1, respectively. Allergens are indicated by gold particles (black dots, arrows). **C** and **F,** Control experiments with rabbit preimmune antibodies. Bar: **A** and **B,** 2 μ m; **B-F,** 0.25 μ m. **D,** Dictyosome; **L,** lipid; **M,** mitochondrion; **ST,** starch granule; **V,** vacuole.

junction with immunogold labeling of the major allergens that indeed pollen grains from birch, alder, and hazel have rainwater-induced abortive pollen germination, during which allergens are transported into the pollen tube. Furthermore, we showed that these tubes rupture and release micronic and submicronic particles of respirable size that contain the major allergens. This process occurs when fresh pollen comes into contact with hypotonic rainwater and represents a mechanism of allergen release that is different from the previously described diffusion of allergen molecules through the pollen wall¹⁰ taking place when pollen grains come into contact with an isotonic medium (eg, mucosa of the upper respiratory tract). We consider the release of small allergen-bearing particles as important because it would explain the occurrence of asthma after periods of rainfall, whereas intact, dry pollen grains, because of their size, may preferentially induce allergic symptoms in the upper respiratory tract. The release of paucimicronic, micronic, and submicronic allergen-bearing particles into the atmosphere has also been described for grass pollen^{11,12,17-19} and ragweed pollen.²⁰⁻²² However, grass pollen simply ruptures after contact with rainwater, whereas pollen from trees of the *Fagales* order (birch, alder, hazel) showed characteristic abortive germination as the mechanisms of allergen release.

In this context, it would be interesting to investigate the role of another natural fluid, for example, dew, on the morphology and physiology of pollen grains during the pollination period of plants. Dew constitutes a hypotonic medium just as rain but is a nightly event, in contrast to rain, which is occasionally available. However, an experimental setup completely different from the one chosen for the current investigation would have to be designed for obtaining information about any possible effects of dew on the production of inhalable pollen-derived particles.

The liberation of allergen-bearing small particles may be important for the induction of allergic symptoms in the deeper respiratory tract (ie, asthma) and thus would explain the occurrence of asthma attacks after periods of rainfall. The liberation of allergens from pollen grains before contact with the respiratory mucosa would, however, also allow association with other submicronic particles (eg, diesel exhaust, dust, fuel combustion)^{23,24} and hence may not only play a role in regarding the induction of symptoms but also lead to the induction of strong sensitizations caused by a possible adjuvant character of the attached particles.²⁵⁻²⁷ In this context, it should be mentioned that diesel exhaust particles have been reported to act as T_H2-driving adjuvants in murine models of allergic sensitization.²⁸

The demonstration that pollen allergens from trees (this study), grasses,^{18,19,29} and weeds²⁰⁻²² can be released in the form of submicronic particles and hence may be loaded to other particles should also lead us to consider methods for the measurement of true allergen exposure, which is based on the detection of allergen molecules rather than on pollen counts and morphology. This goal may be achieved by the analysis of air samples by using immunologic detection methods (eg, sandwich

ELISA systems) that are already used for the measurement of indoor allergen exposure.⁷

In conclusion, we demonstrate abortive pollen germination as a characteristic mechanism for the release of major tree pollen allergens with potential implications for the induction of specific clinical symptoms (eg, asthma), measurements of allergen exposure, sensitization, and perhaps prevention.

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