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Source of Bet v 1 loaded inhalable particles from birch revealed

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Abstract Allergenic proteins present in pollen grains, when inhaled, interact with the airways to cause an attack of asthma in susceptible humans. In one system, grass pollen grains rupture osmotically in rainfall, releasing allergen-containing inhalable particles into the atmosphere. In contrast, birch tree pollen grains do not rupture under these conditions, yet the major allergen, Bet v 1, has been detected in the atmosphere in inhalable particles of unknown origin. It is possible that Bet v 1 may diffuse from intact settled pollen grains and the allergenic material may again become airborne, interacting with settled fine particles from other sources prior to re-suspension. This study investigates the mechanism for the release of birch pollen allergen-containing inhalable particles from pollen grains. We propose the hypothesis that (1) airborne birch pollen grains settle on nearby leaf surfaces; (2) then, following light rainfall, the grains germinate and, (3) later, pollen tubes burst, releasing inhalable particles carrying Bet v 1 into the atmospheric aerosol.

We used microscopic analyses of pollen behaviour following anther opening, a Burkard volumetric trap for pollen counts and a high volume air sampler with a two-stage cascade impactor for quantitative immunochemical analyses of Bet v 1. On dry days of high birch pollen count (48 grains/m³, 1.5 ng/m³ of Bet v 1), we found that the surfaces of birch leaves became coated with pollen. This “pollen rain” is a source of secondary emission of allergens into the atmosphere. We observed that following light rainfall (<1 mm per day), about 80% of the birch pollen grains germinated, producing pollen tubes, especially in the sticky surface secretions of leaf glands.

These pollen tubes may grow up to 300 µm in length prior to rupturing, each releasing about 400 starch granules coated with allergen molecules that may, after drying, be dispersed into the aerosol. On these days following light rainfall, the highest atmospheric levels of Bet v 1 (1.18 ng/m³) are associated with inhalable particles. Following heavy rainfall, both pollen and inhalable particles are washed from the atmosphere. Immunoprinting studies show that Bet v 1 is associated with starch granules rather than the smaller orbicules. Bet v 1 is present in the atmosphere in large particles, i.e. in particular pollen grains and in inhalable particles, i.e. in particular starch granules.

Key words Bet v 1 · Birch pollen · Inhalable fine particles · Release mechanisms · Asthma

Introduction

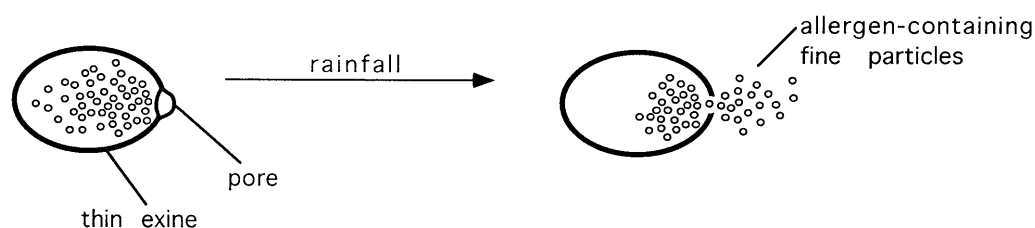
Pollen from trees of the family Betulaceae (e.g. birch, alder, hazel and hornbeam) is amongst the main causes of type I allergic reactions in the external environment in temperate zones (Wüthrich et al. 1995). Up to 80% of all pollen-allergic individuals are hypersensitive to allergens from the Betulaceae (Jarolim et al. 1989). The major birch (*Betula verrucosa* Ehrh. = *B. pendula* Roth.) pollen allergen, Bet v 1, is a polypeptide of 17 kDa (Ipsen and Lowenstein 1983). More than 96% of all birch pollen-allergic patients possess IgE antibodies to Bet v 1, and 60% show exclusive reaction to this major allergen (Jarolim et al. 1989). The cDNA of Bet v 1 shows 55% sequence identity with a pea disease resistance response gene and Bet v 1 may be involved in the pathogen resistance of pollen (Breiteneder et al. 1989). Bet v 1 can be rapidly released from hydrated pollen (Vrtala et al. 1993) and, recently, ribonuclease activity has been attributed to this allergen (Bufe et al. 1996).

Birch pollen allergens have been recorded in atmospheric fine particles smaller than pollen grains, even when no birch pollen grains were present (Schäppi et al. 1996). The concentration of these fine particles has been

Dedicated to the memory of Professor R. Bruce Knox who passed away suddenly on 30 August 1997

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(a) Grass



(b) Birch

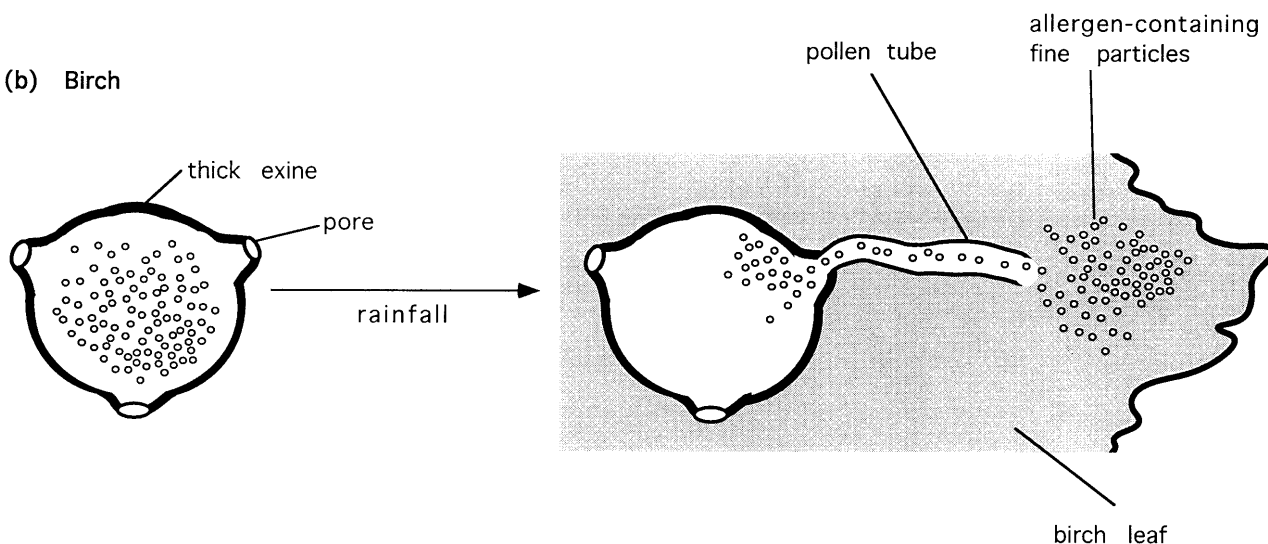


Fig. 1a, b Diagram showing the two pathways for release of natural allergen-containing inhalable particles from pollen grains. **a** A grass pollen grain ruptures in rainwater by osmotic shock, releasing about 700 allergen-loaded starch granules. **b** A birch pollen grain germinates on a leaf; the pollen tube ruptures, releasing about 400 allergen-loaded starch granules

shown to increase after rainfall (Rantio-Lehtimäki et al. 1994). Unlike birch pollen grains (22 µm in diameter), the fine particles are small enough to enter human airways (Wilson et al. 1973) and cause allergic reactions in the distal parts of the lungs. Little is known about the mechanism of release of allergen-loaded fine particles from pollen grains. Several hypotheses have been proposed, but not clearly substantiated. One hypothesis is that pollutant chemicals and particulates that have been detected on pollen grains modify the release of allergens from pollen (Behrendt et al. 1992). Other hypotheses are that sedimented pollen releases its allergens when moistened (i.e. rainfall) and the allergenic material may become airborne after drying (Rantio-Lehtimäki et al. 1994), eluted allergens might be transferred from the pollen to fine particles from other sources (Hidy 1984), fragments might be formed by the slow, physical degradation of birch pollen grains (Solomon et al. 1983) and orbicules may act as carriers for birch pollen allergens (El-Ghazaly et al. 1995).

Grass pollen grains have been shown to rupture in rainwater by osmotic shock, each grain releasing about 700 allergen-loaded starch granules (Fig. 1a) (Suphioglu

et al. 1992). Free group 1 allergen molecules dispersed in the aerosol are also able to bind to other particulate matter, including diesel exhaust carbon particles in polluted air (Knox et al. 1997). Like group 1 allergens of grass pollen, Bet v 1 occurs in the cytosol of the mature pollen grain (Grote 1991). Using immunogold labelling with a monoclonal antibody, Bet v 1 has been located predominantly in the starch granules (El-Ghazaly et al. 1996). Yet our observations show that birch pollen has a very strong exine and, unlike grass pollen, does not osmotically rupture in water. These data suggest that the origin of small particles containing birch allergens needs to be investigated further. We wished to test the hypothesis that (1) airborne birch pollen grains settle on nearby leaf surfaces, (2) then, following light rainfall, the pollen grains germinate and, (3) later, the pollen tubes burst, releasing inhalable particles carrying Bet v 1 into the atmospheric aerosol (Fig. 1b).

Accordingly, in our aerobiological study, we used microscopic and immunological techniques to reveal the mechanism of the release of inhalable particles loaded with birch pollen allergen from pollen grains into the atmosphere.

Materials and methods

Sampling of birch pollen and of particles in two different size classes

The birch pollen count was performed using a Burkard volumetric trap (Burkard Manufacturing, Rickmansworth, Hertfordshire, UK)

according to the manufacturer's instructions. Continuous sampling of particles of different aerodynamic diameters was performed during the 1996 birch pollen season (16 October–15 November) by means of a high volume cascade impactor (GMWS-2310, General Motor Works, Cleves, USA) at a height of about 15 m above ground on the campus of the University of Melbourne. An air stream (1 130 l/min) was drawn through fibre filters (Pallflex, Putnam, Conn., USA). Two fractions, each containing particles of a different size, were deposited. The first stage collected particles with an aerodynamic diameter greater than 7.2 μm ; the second stage collected particles with a diameter less than 7.2 μm . Every 24 h, the filters were replaced and the samples were removed and frozen at -20°C until required.

Quantification of Bet v 1 in particles

To estimate the Bet v 1 concentrations in the two different-particle-size fractions, a monoclonal antibody-based two-site binding assay was used (enzyme-linked immunosorbent assay, ELISA). Bet v 1 was quantified directly after extraction from particulate filters into phosphate buffered saline (PBS) containing 0.1% v/v BSA and 0.01% Thimerosal. Each well of a microtitre plate was coated with 100 μl of purified monoclonal antibody (mAb) 3B4F11D6 (Allergopharma Joachim Ganzer, Reinbek, Germany) specific to Bet v 1, at 10 $\mu\text{g}/\text{ml}$ in sodium carbonate buffer and the microtitreplate was left overnight at 4°C . All subsequent incubations were performed at room temperature. After blocking and then two subsequent washes with PBS containing 0.05% v/v Tween-20 and 0.01% Thimerosal, 100 μl of Bet v 1 standard solution (concentration range 1–800 ng/ml) and the samples were added to the wells. After 1 h of incubation, the plates were washed three times and incubated with 100 μl of biotinylated mAb 2E10G6G7 at 1:15 000 dilution, which is specific to a different epitope of Bet v 1. Again, after 1 h of incubation, the plates were washed three times. A volume of 100 μl of streptavidin-peroxidase (Sigma, Deisenhofen, Germany) was added and the plates were incubated for 30 min. Then O-phenylenediamine was used as the substrate and the resulting absorption was quantified by means of a photospectrometer at 492 nm.

Scanning electron microscopy imaging of pollen grains

To observe pollen grains using scanning electron microscopy, birch leaves were collected from trees growing on the campus of the University of Melbourne and from Pheasant Creek, Victoria, Australia, during the 1996 birch pollen season. Pieces of the leaves were mounted onto stubs with conductive carbon paint (Probing and Structure, Brisbane, Queensland, Australia) and then frozen in liquid nitrogen slush under vacuum. The mounts were then transferred into a JSM-40 scanning electron microscope (SEM) on a cold stage at -190°C and sputter coated with gold for 3 min prior to viewing.

Light microscopy investigations

The number of pollen grains on leaves was determined by gently washing birch leaves collected from trees growing on the campus of the University of Melbourne during the 1996 birch pollen season in water and filtrating the supernatant through Whatman Number 1 filter paper (Whatman, Maidstone, England). The pollen grains and pollen tubes were stained on the filters with a 1:1 mixture of Calberla's stain and 0.1% lactophenol cotton blue and observed using Nomarski optics on an Olympus BH2 light microscope. Pollen grains were considered as germinated if the length of the observed pollen tubes exceeded the diameter of one pollen grain.

The association of germinating pollen with leaf surface glands was observed by mounting pieces of leaf on a glass slide in a mixture of the fluorochromes calcofluor white and auramine O and viewing with incident fluorescence microscopy using a violet exciter filter.

Freshly dehiscent pollen (90% FCR-positive) was collected and germinated *in vitro* in either distilled water (pH 6) or 10% sucrose/water in a petri dish. *In vivo* studies were performed by applying pollen onto collected birch leaves and incubating under 100% relative humidity in an enclosed petri dish. The pollen grains were regularly observed during the following 72 hours using Nomarski optics on an Olympus BH2 light microscope.

Sampling and morphological identification of particles

For morphological identification, particles were sampled during the 1996 birch pollen season under a birch tree growing on the campus of the University of Melbourne by a modified cascade impactor (Andersen Samplers, Atlanta, Ga., USA) operated at a flow rate of 25 l/min, which permitted separation of particles into six classes according to particle size ($>7\ \mu\text{m}$, 4.7–7 μm , 3.3–4.7 μm , 2.1–3.3 μm , 1.1–2.1 μm and 0.65–1.1 μm). The particles were deposited on microscope slides covered in glue (10% Dow Corning). The slides were coated and viewed with an SEM. The slides were also examined using immunotyping to detect Bet v 1 and iodine/potassium iodide (IKI) staining to detect starch granules.

Immunotyping of Bet v 1

Twenty minutes were allowed for Bet v 1 transfer from the particles attached to microscope slides to a polyvinylidene difluoride membrane that was wetted for 1 min in methanol and rinsed in PBS before use. After blotting, the membranes were blocked (in 10% w/v skim milk powder in PBS) for 1 h at room temperature, washed once in PBS containing 0.05% v/v Tween-20 (TPBS) and twice in PBS only, 5 min each wash, with vigorous shaking. The membranes were incubated for 2 h with mAb 3B4F11D6 specific to Bet v 1 (diluted 1:500 in antibody buffer 0.5% BSA in PBS). After washing once with TPBS and twice in PBS alone for 5 min each wash, the membranes were incubated with affinity isolated anti-mouse Ig raised in sheep which is horseradish peroxidase conjugated (diluted 1:200) for 2 h, and then washed in TPBS and PBS. The membranes were developed in peroxidase substrate (0.06% 4-chloro-1-naphthol, 0.006% hydrogen peroxide and 20% methanol in PBS) at 37°C for 10 min and development was stopped by washing in distilled water. The membranes were examined under light microscopy for antigen-positive purple spots and photographed.

Detection of starch granules

The microscope slides were mounted in IKI reagent which stains starch particles blue-black. They were viewed by bright-field light microscopy and the stain patterns were compared to the corresponding immunoprints.

Results

Bet v 1 occurs in pollen and fine particles

Before considering the evidence in favour of our hypothesis, it is necessary to show that Bet v 1 occurs in fine particles in the Melbourne atmosphere. In spring 1996, birch pollen occurred in the atmosphere over a 48-day period (Fig. 2a) and the peaks are almost precisely correlated with highest temperatures. During 30 of these days, Bet v 1 was identified by quantitative immunological analysis (ELISA) of high volume sampler/cascade impactor filters to provide quantitative estimates of the allergen in large particles ($>7.2\ \mu\text{m}$) and in fine particles ($<7.2\ \mu\text{m}$) (Fig. 2b, c). Sampling periods with high numbers of birch

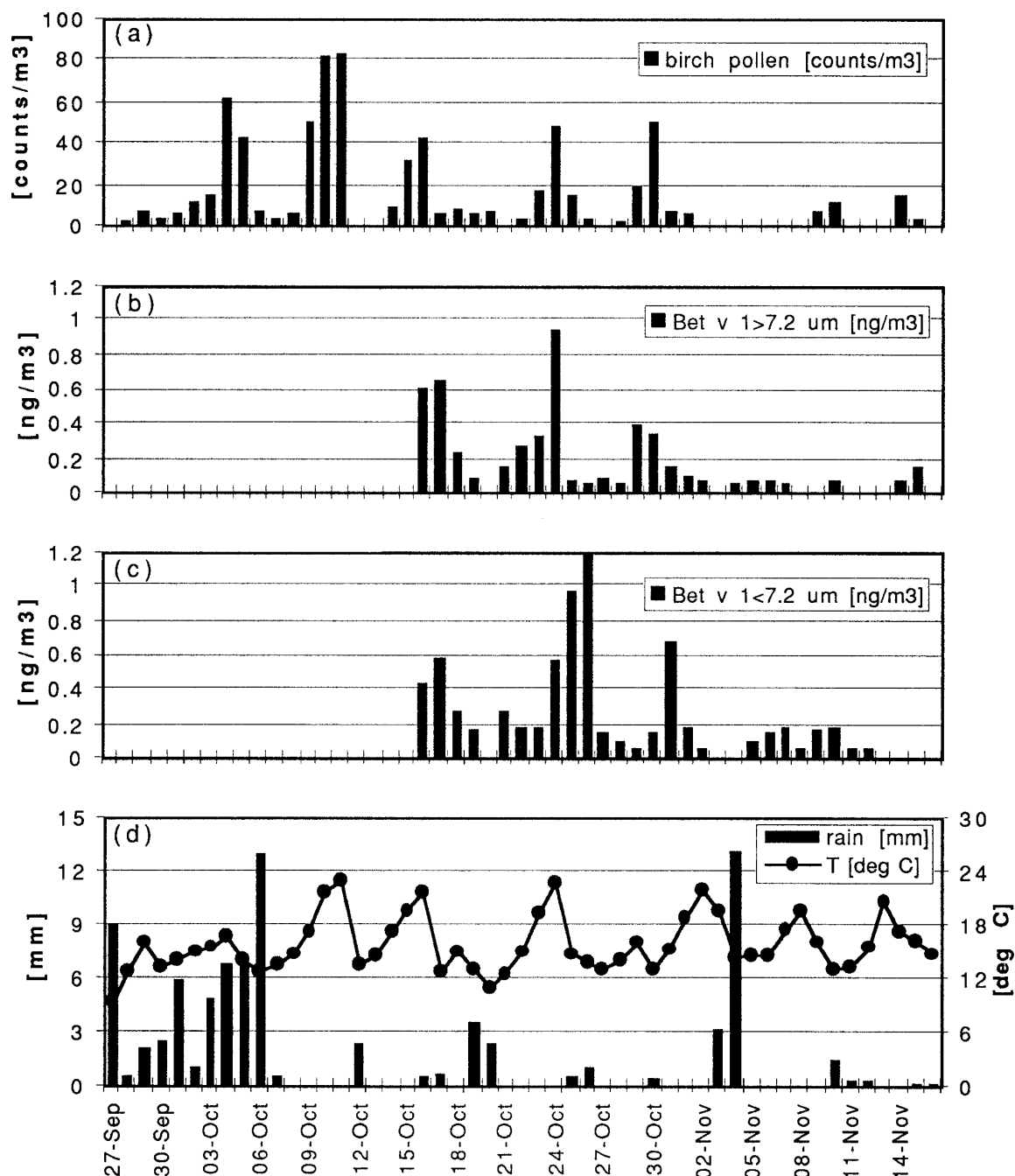


Fig. 2 **a** Birch pollen counts (number/m³); **b** Bet v 1 concentrations (ng/m³) in particles greater than 7.2 µm in diameter; **c** Bet v 1 concentrations (ng/m³) in inhalable particles less than 7.2 µm in diameter; **d** rainfall (mm) and temperature (deg C) in the atmosphere of Melbourne during the 1996 birch pollen season. Bet v 1 concentrations were determined from 16 October–15 November 1996. Bet v 1 data for 20 October and 3 November are not available

pollen grains were generally characterized by elevated Bet v 1 concentrations in the large-size particle fraction. Again, the peaks in Bet v 1 in the large-size particle fraction are associated with the temperature peaks (Fig. 2d). On 3 days, the highest estimates of Bet v 1 in large particles were within the range of 0.6–0.95 ng/m³.

In the inhalable particle fraction, Bet v 1 concentrations ranged up to 1.2 ng/m³ on 26 October 1996, which followed 3 days after the pollen count peak and average temperature peak (24 October). In contrast, the peak on 31 October (0.7 ng/m³) followed only 1 day after the pollen count and temperature peaks. Conspicuously both these of peaks in fine particles followed days with light rainfall (<1 mm). The mean Bet v 1 concentrations in fine particles on days following light rainfall is 0.57 ± 0.16 ng/m³ ($n=8$); this is significantly different ($P<0.05$) from the Bet v 1 concentrations on the second and following dry days after rain (0.15 ± 0.02 ng/m³, $n=10$) and also from days with heavy rainfall and the following first dry days (0.12 ± 0.01 ng/m³, $n=8$).

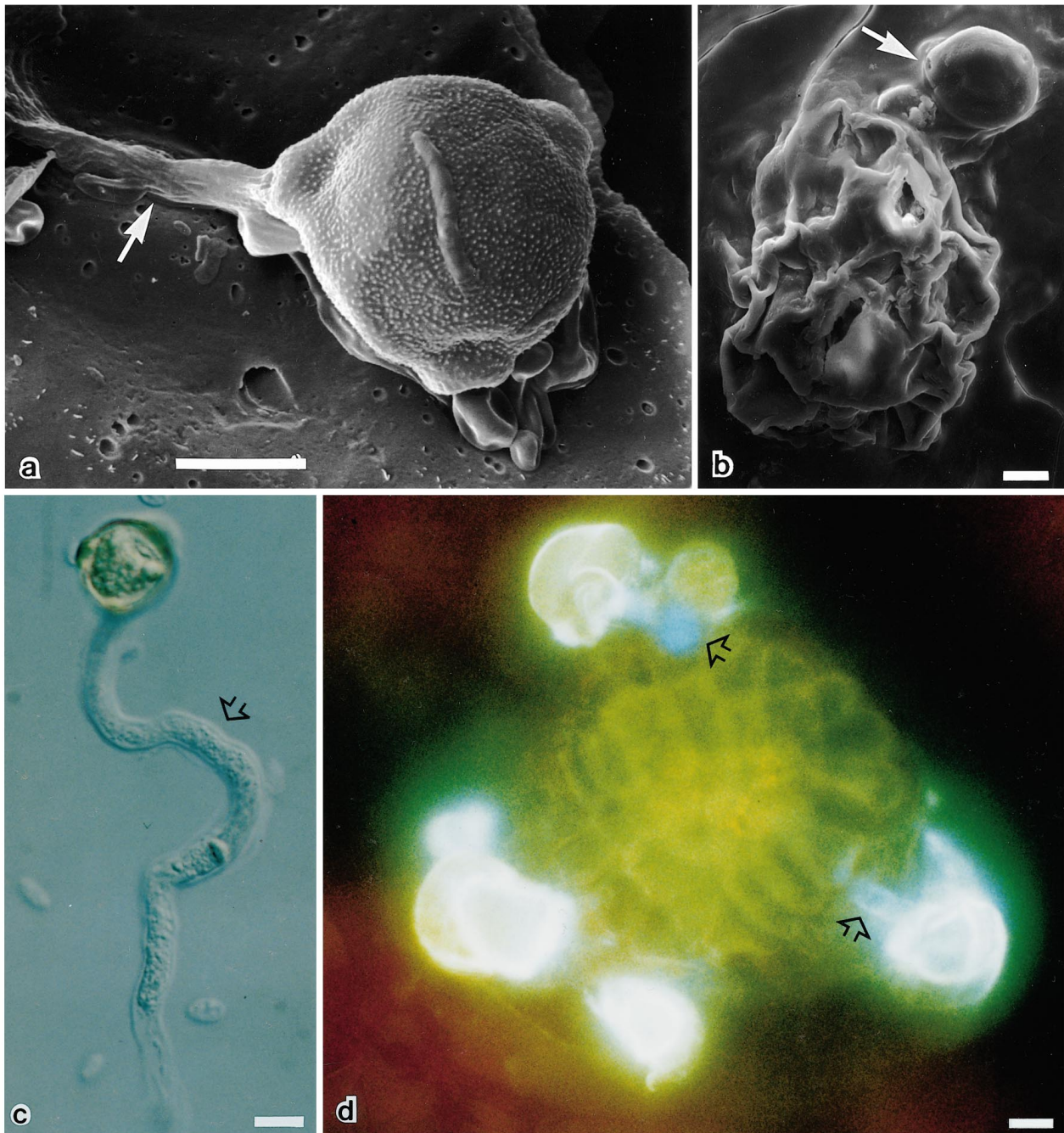


Fig. 3 Micrographs of germinating birch pollen and bursting pollen tubes. **a** Scanning electron micrograph of a triporate birch (*Betula verrucosa*) pollen grain on a birch leaf. The pollen grain has germinated, producing a pollen tube (arrow). **b** SEM of birch leaf gland with one birch pollen grain nearby (arrow). **c** A germinated birch pollen grain with a pollen tube (arrow) about 90 μm long showing dense cytoplasmic contents. This was viewed with Nomarski optics after being washed off a birch leaf. **d** Germinating pollen accumulates around the birch leaf gland. Each grain produces a pollen tube (arrows). Within hours of germination, the pollen tube tips rupture, releasing the cytoplasmic contents. Prominent amongst this discharged cytoplasm are numerous starch granules. Bars 10 μm .

We found that, unlike grass pollen grains, birch pollen grains do not rupture in water, even after an incubation period of 5 h. The question then arises as to how fine particles loaded with birch pollen allergens can be released into the atmosphere and thus become respirable, and able to trigger asthma attacks.

Airborne birch pollen grains settle on nearby leaf surfaces

We observed that, after being shed into the atmosphere, large numbers of birch pollen grains settle on nearby surfaces such as leaves of birch trees (Fig. 3a). Birch leaves possess multicellular surface glands (Esau 1965) that rend-

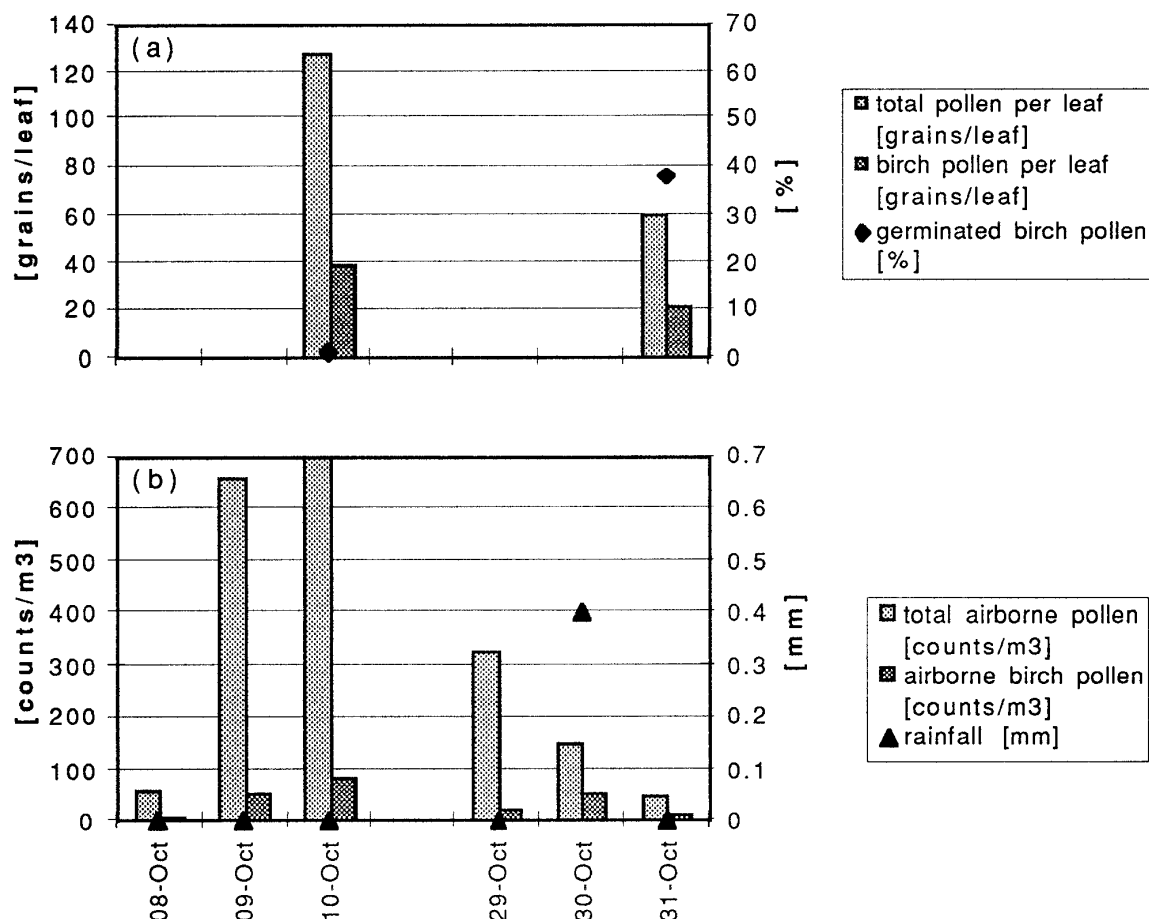


Fig. 4 **a** Total number of pollen grains per leaf, number of birch pollen grains per leaf, and percent of germinating birch pollen grains observed on leaves collected from birch trees growing on campus of the University of Melbourne on 10 and 31 October 1996. **b** Total pollen count/m³, birch pollen count/m³ and rainfall (mm) in the atmosphere of Melbourne (8–10 October and 29–31 October 1996)

er the leaf surface sticky (Fig. 3b, d). Examination of materials washed from birch leaves showed a large amount of pollen (Fig. 4). On 10 October 1996, a day with a high pollen count (a total of 700 pollen grains including 81 birch pollen grains per m³), we found a mean of 127 ± 44 pollen grains per leaf, of which 38 ± 15 were birch pollen ($n=10$). On the 31 October 1996, a day with a low pollen count (a total of 45 pollen grains including eight birch pollen grains per m³), we found a mean of 60 ± 24 pollen grains per leaf, of which 21 ± 11 were birch pollen ($n=10$).

Following light rainfall pollen grains germinate

Within at least 5 h following light rainfall (0.5 mm) on 25 October 1996, we observed that up to 80% of the birch pollen had germinated on the leaves (Fig. 3a, c, d). In Melbourne, at the time birch pollen was shedding (October and November 1996), there were 8 days when light rain followed dehiscence. On these occasions, conditions were ap-

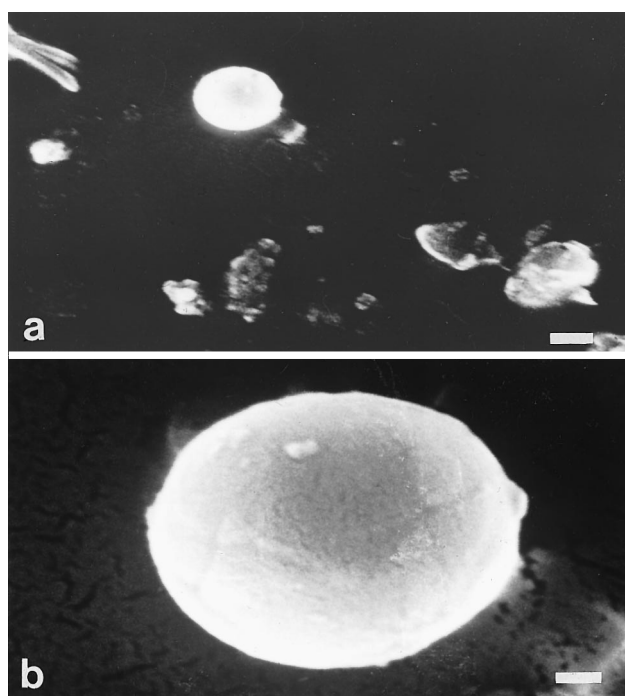


Fig. 5 **a** Microscope slide exposed in an Andersen sampler and viewed by SEM showing particles in the size range of approximately 1–2 μm . Bar 1 μm . **b** Starch granule from the same sample. Bar 250 nm

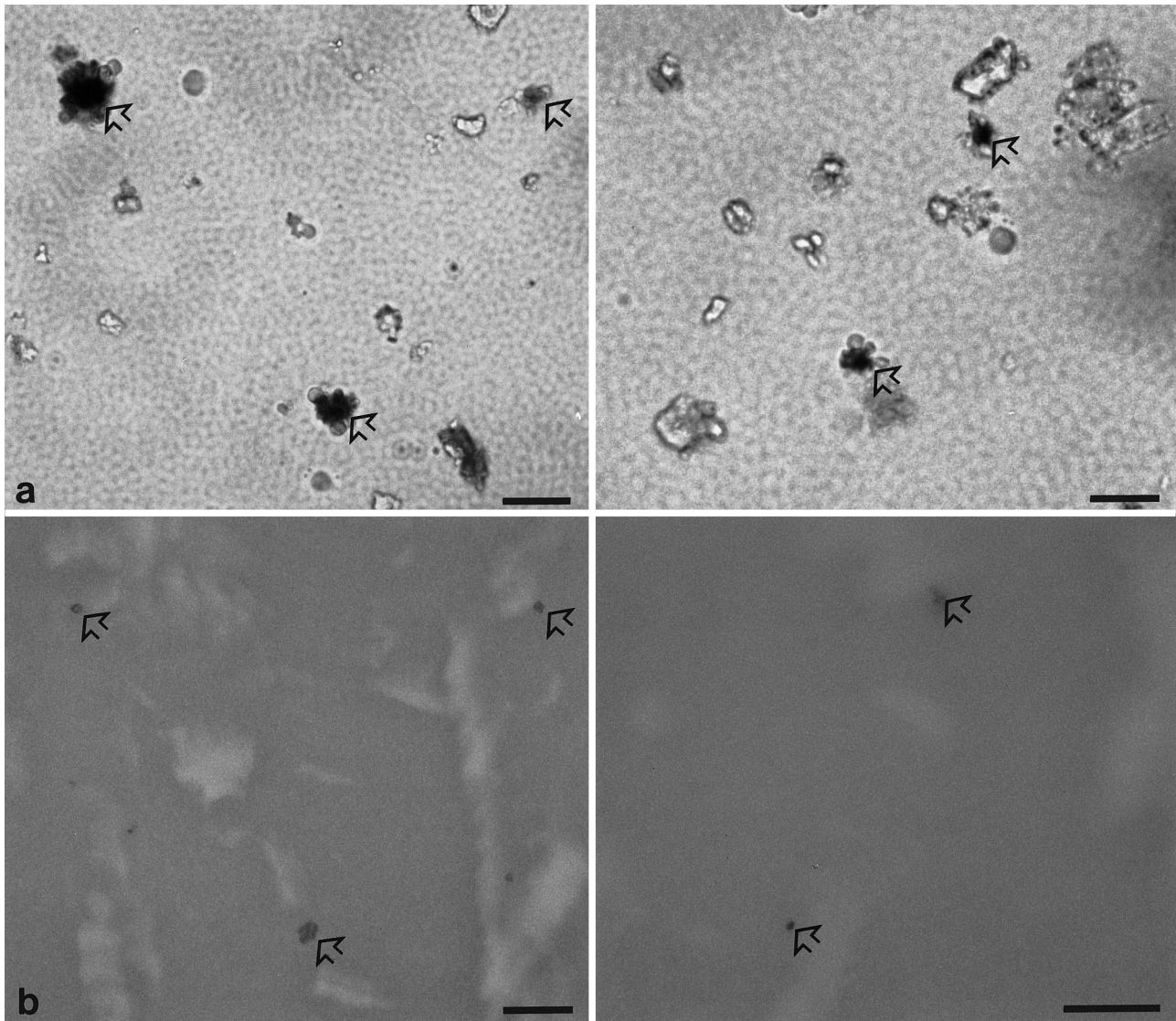


Fig. 6 **a** Microscope slides exposed in an Andersen sampler and mounted in IKI reagent staining starch particles blue-black in colour, viewed by bright-field light microscopy. **b** Corresponding immunoblots performed with monoclonal antibodies specific to Bet v 1. IKI-stained with Bet v 1-positive particles indicated by arrows. Bars 5 µm

parently suitable for pollen germination on leaf surfaces. Examination of materials washed from birch leaves confirmed these observations; on 10 October 1996 less than 1% of the detected birch pollen had germinated whereas on 31 October 1996, after a light rainfall of 0.4 mm, we found that 38% of the observed birch pollen had germinated (Fig. 4).

Pollen tubes burst, releasing inhalable particles carrying Bet v 1 into the atmospheric aerosol

Observations made on birch leaves *in vivo* on 25–27 October 1996 showed that the resultant pollen tubes grew up to 300 µm long, then burst, releasing their cytoplas-

mic contents. These contents consisted mainly of up to 400 starch granules per tube that were coated in cytosolic debris.

In vitro experiments confirmed these observations. Fresh birch pollen grains were on the one hand placed in distilled water and observed over a 72-h period, and on the other hand placed onto collected birch leaves and incubated at 100% relative humidity in an enclosed petri dish and observed over a 72-h period. In both cases, birch pollen grains were observed to germinate after a minimum of 5 h incubation. After at least 5 and up to 48 h, the pollen tube tip of germinated pollen grains burst, releasing the entire contents of the tube within seconds.

Bet v 1 has recently been shown by immunocytochemical studies to occur in starch granules (El-Ghazaly et al. 1996). These respirable particles are now shown to occur in the atmospheric aerosol. SEM examinations of glass slides used as collectors of inhalable particles from the Andersen sampler showed particles in the size class 0.65–2.1 µm. Particles morphologically similar to starch

granules had an incidence of $50.1 \pm 9.6\%$ while orbicule-like particles had an incidence of $12 \pm 6.4\%$ ($n=12$; Fig. 5). We showed using immunoprinting, that starch granules are able to form allergen-loaded inhalable particles in the atmosphere. The microscope slides mounted in IKI reagent stained starch particles blue-black in colour. Comparison of Bet v 1 immunoblots and IKI-stained slides showed the co-localisation of starch granules and Bet v 1 in air samples (Fig. 6).

Discussion

This study reveals a mechanism for the formation of natural fine particles loaded with birch pollen allergen and their occurrence in the aerosol. Birch pollen grains are shed, settle on nearby leaves and other surfaces and, after light rainfall, start to germinate. The pollen tubes burst and release allergen-loaded fine particles, which, after drying, can be suspended in the aerosol, enhanced, e.g., by air movements.

Fine particles loaded with birch pollen allergen have been recorded in the aerosol, even when no birch pollen grains were present (Schäppi et al. 1996). The concentration in arbitrary units of these fine allergenic particles has been found to increase after rainfall (Rantio-Lehtimäki et al. 1994). No explanation of such results has been provided previously. The first observed case of natural fine particles was that of grass pollen grains, which rupture in rainwater (Suphioglu et al. 1992) and release their cytoplasmic contents through the single germinal pore (Fig. 1a).

Although bursting of pollen tubes appears to be the main origin of birch allergen-loaded inhalable particles, these water-soluble allergens may also be eluted from intact sedimented pollen grains and resuspended into the atmosphere (Rantio-Lehtimäki et al. 1994). They may also interact with settled fine particles from other sources prior to their resuspension. Also, allergen-loaded fine particles may adsorb to other fine particles (such as diesel particles) in the aerosol (Knox et al. 1997). Orbicules – irregular bodies that surround the pollen sacs of intact anthers – have been hypothesised as carriers of birch pollen allergens (El-Ghazaly et al. 1995). Allergens have been localised in orbicule walls of the gymnosperm *Cryptomeria japonica*. (Takahashi et al. 1991; Miki-Hirosige et al. 1994). However, current results on the site of synthesis of birch allergens show that they are specifically localised in the pollen cytoplasm (Swoboda et al. 1995; Emilson et al. 1996). It is therefore highly unlikely that freshly shed orbicules would be loaded with large amounts of allergen. Also, our results show that the incidence of orbicule-like particles in the aerosol is only 25% that of particles morphologically similar to starch granules.

Immunoprinting with monoclonal antibodies specific to Bet v 1 has successfully confirmed that the atmospheric starch granules can carry this major allergen; this supports recent studies showing predominant localization of Bet v 1 in starch granules by immunogold labelling

(El-Ghazaly et al. 1996). Previous immunoprinting studies detected atmospheric allergenic activity associated with pollen grains and smaller particles, including orbicules (Schumacher et al. 1988; El-Ghazaly et al. 1995; Ekebom et al. 1996).

There are implications for the interpretation of results of allergenic activity detected in vegetative tissue of birch and other species (Fountain et al. 1992). When screening for allergens with ELISA or RAST (radio allergosorbent test) on non-reproductive tissue such as leaves, bark or twigs, the presence of pollen grains or fine particles from pollen tubes must be considered. Otherwise, conclusions about the expression of allergens in various tissues or species may be misleading.

These observations may explain both the cause of allergic reactions deep within the lungs, which cannot be reached by birch pollen because of its aerodynamic diameter ($22 \mu\text{m}$), and continuing symptoms during periods when little or no birch pollen is present in the atmosphere. As important complementary information to the pollen count, these findings will enable clinicians to estimate the quite different risks for asthma and hay fever patients sensitive to Bet v 1. They can advise asthma patients to avoid outdoor exposure in times of light intermittent rain during or shortly after the birch pollen shedding season.

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