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Immunologic significance of respirable atmospheric starch granules containing major birch allergen Bet v 1

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Background: Birch-pollen allergens are an important cause of early spring hay fever and allergic asthma. Recently, we reported a mechanism for the release of respirable allergenic particles from birch pollen containing the major allergen Bet v 1. In this study, we aimed to assess the immunologic significance of the released Bet v 1-containing starch granules in the environment.

Methods: A two-site monoclonal antibody-based assay (ELISA) was employed to quantitate Bet v 1 in high-volume air sampler filter extracts, and immunogold-labelling was used on sections of these extracts to localize Bet v 1. Immunoblot analyses were performed with pooled sera from patients sensitive to birch pollen.

Results: Atmospheric starch granules contained Bet v 1, and the concentration increased upon light rainfall. Sera from patients allergic to birch allergens recognized extracts from isolated starch granules.

Conclusions: The clinical implications of these findings are that starch granules released from birch pollen are potentially able to trigger allergic asthmatic reactions to Bet v 1, since the allergen occurs in respirable particles. Thus, clinicians can advise asthma patients to remain indoors on days of light rainfall during the birch-pollen season to avoid high levels of allergen exposure.

In temperate zones, pollen from trees belonging to the family Betulaceae (e.g., birch, alder, hazel, and hornbeam) are among the most important causes of springtime type I allergic reactions such as those of hay fever and allergic asthma (1). In Austria, up to 80% of all pollen-allergic patients are hypersensitive to allergens from the family Betulaceae (2). The major birch (white birch: *Betula verrucosa* Ehrh.= *B. pendula* Roth)-pollen allergen, Bet v 1, is a polypeptide of 17 kDa (3) that can be released from

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hydrated pollen (4). In northern and central Europe, more than 96% of all tree-pollen-allergic patients possess IgE antibodies specific to Bet v 1, and 60% show an exclusive reaction to this major allergen (2). 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 (5). Recently, ribonuclease activity has been attributed to this allergen (6). Current results on the site of synthesis of Bet v 1 suggest localization in the pollen cytoplasm (7, 8). By immunogold-labelling with a monoclonal antibody, Bet v 1 has been located predominantly in the starch granules of mature pollen (9).

Although birch forests do not occur in or around Melbourne, Australia, birch trees are widely grown as exotic ornamental trees in gardens and parks and contribute significantly to the pollen count during spring (10). Because of its size, 22 µm in diameter, birch pollen has a low probability of entering the lower airways (11, 12) and thus triggering allergic asthma in susceptible subjects. Yet, birch-pollen allergens have been shown to occur not only in birch pollen but also as free allergen molecules or associated with fine particles much smaller than birch-pollen grains, even when no birch-pollen grains can be detected in the atmosphere (13). After rainfall, the concentration of these fine particles has been shown to increase (14). Concerning the mechanism of release of allergen-loaded fine particles from pollen grains, several hypotheses have been proposed. It has been suggested that sedimented pollen releases its allergens when moistened (i.e., rainfall), and the allergenic material may become airborne after drying (14); orbicules (0.5–0.8 µm in diameter) may act as carriers for birch-pollen allergens (15). Recently, we have shown that after light rainfall, birch-pollen tubes rupture, releasing ~400 starch granules per grain (16).

For estimation of the clinical significance of Bet v 1-containing starch granules in triggering allergic reactions in patients sensitive to birch-pollen allergens, it is necessary to investigate this mechanism, both qualitatively and quantitatively, and to determine whether the response of susceptible patients to starch granules isolated from birch pollen is IgE-mediated.

Material and methods

Environmental investigations

Presence of birch pollen in the Melbourne atmosphere

Birch-pollen counts were performed during the birch-pollen season of 1997 with a Burkard volumetric trap (Burkard

Manufacturing Ltd, Hertfordshire, UK), according to the manufacturer's instructions. The airflow was set to 10 l/min. Airborne pollen grains were trapped on an adhesive (10% Dow Corning A280 in xylene) on a microscope slide driven by a clockwork motor. The slides were mounted in Calberla's stain, and birch pollen was counted by light microscopy.

Continuous 24-h sampling of particles of different aerodynamic diameters was performed during a part of the birch-pollen season of 1997 (23–31 October) with a high-volume cascade impactor (GMWS-2310, General Motor Works, Inc., Cleves, IA, USA) at a height of about 15 m above ground in Melbourne, Australia. An air stream (1130 l/min) was drawn through fibre filters (Pallflex Co., Putnam, CT, USA). Two fractions of different sized particles were deposited: the first stage collected particles with an aerodynamic diameter above 7.2 µm; the following stage collected particles below 7.2 µm (particle size cutoff at 50% collection efficiency for spherical particles with unity mass density, according to the manufacturer's specifications). Every 24 h, the filters were replaced and samples were frozen at –20°C until required.

Soluble proteins were extracted from particulate filters into PBS by shaking for 3 h on ice. A monoclonal antibody-based, two-site binding assay (enzyme-linked immunosorbent assay, ELISA) was used to quantitate the Bet v 1 concentrations, as described previously (10).

Microscopic analyses of pollen and particles on leaf surfaces

Immunogold-labelling of Bet v 1 in atmospheric samples

Particles with an aerodynamic diameter below 7.2 µm, collected on 25 October 1996, a day with light rainfall and high Bet v 1 concentrations in the particles of <7.2 µm, were extracted from high-volume filters by agitation in acidified 2,2-dimethoxypropane (Sigma Chemical Co., St Louis, MO, USA), infiltrated for 1 week in LR Gold resin (London Resin Co., UK) with continuous rotation, polymerized, and sectioned as described by Taylor et al. (17). Sections, 100 nm thick, were probed with mAb 3B4F11D6, specific to Bet v 1, at 1:500 dilution in PBS/BSA, as described previously (17). Negative controls included fixation control with acetone-extracted particles, tissue specificity control using sections of grass pollen, and antibody control by omission of primary antibody.

Electron microscopy

For direct observation of pollen grains and starch granules settled on leaf surfaces, leaves were collected from local

birch trees after light rainfall during the birch-pollen season of 1997. Leaves were vapour fixed with formaldehyde in a sealed container at 4°C for several days. Pieces of leaf were mounted onto stubs with carbon dag, followed by freezing in liquid nitrogen slush under vacuum. They were then transferred into a Jeol-JSM 840 scanning electron microscope (SEM) on a cold stage at -191°C and sputter coated with gold for 5.5 min before viewing. On days with high birch-pollen count, leaves were harvested from trees, placed on wet filter paper, and enclosed in a Petri dish. Some leaves were briefly sprayed with a mist of distilled water. After 24 h, leaves were fixed and prepared for SEM as above.

Light microscopy

Leaves collected from birch trees on dry and rainy days during the birch-pollen season of 1997 were rinsed with water containing 10% sucrose. After centrifugation at 800 g for 5 min, the pellet was mounted onto a glass microscope slide. Leaves collected on days with high birch-pollen count were incubated on moist filter paper enclosed in a Petri dish for 24 h before rinsing and centrifuging. Freshly dehiscent birch pollen was collected and incubated either in a Petri dish or on a microscope slide in distilled water containing 10% sucrose for 24 h. The pollen grains were regularly observed during the following 48 h with Nomarski optics on an Olympus BH2 light microscope.

Investigation of *in vitro* isolated starch granules

Isolation of starch granules from birch pollen

Pollen grains were manually collected from local birch during the birch-pollen season in 1997. Weighed samples of pollen grains were counted after staining with Calberla's stain by means of a haemocytometer. Aliquots of 2 mg of pollen grains were evenly spread on a Petri dish and sprayed with a mist of distilled water (pH 6) containing 10% sucrose (filter sterilized). The Petri dish was enclosed in a larger Petri dish containing a moist filter paper and incubated for 16 h to allow *in vitro* germination, and then air-dried for 8 h to encourage bursting of pollen tubes. After 24 h, the percentage of germinated birch pollen was assessed. Petri dishes were quickly rinsed with 10 ml distilled water, and samples were immediately passed first through an 8- μ m (Millipore Type SC), followed by a 1.2- μ m (Millipore Type RA) size-exclusive mesh filter (Millipore Corp., Bedford, MA, USA). Particles from both filters were resuspended in 1 ml distilled water (pH 6).

Water-soluble proteins in freshly collected birch-pollen grains, in particles collected on filters (>8 μ m and

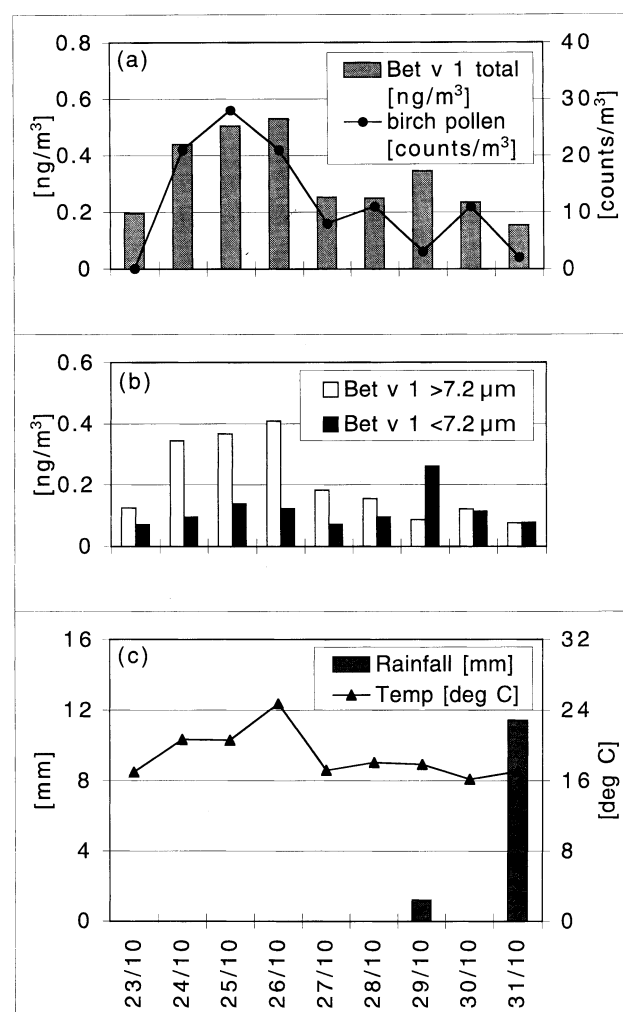


Figure 1. Comparison of birch-pollen count, Bet v 1 concentration in atmospheric samples, and meteorologic parameters in Melbourne (23–31 October 1997). a) Total Bet v 1 concentrations (ng/m³) and corresponding birch-pollen count (counts/m³). b) Bet v 1 concentrations (ng/m³) in two particle size fractions (above and below 7.2 μ m). c) Rainfall (mm) and average temperature (degrees C).

1.2–8 μ m), and in the filtrate (containing particles of <1.2 μ m) were extracted in distilled water (pH 6) by shaking for 3 h on ice. Particles were pelleted by centrifugation (9000 g) and the supernatants submitted to protein assay with bovine plasma gamma globulin as standard, allowing sensitive and accurate detection of proteins of a wide molecular weight range (18), and ELISA as described above.

Results and Discussion

In this study, we were able to evaluate the immunologic significance of starch granules containing Bet v 1 in the Melbourne atmosphere.

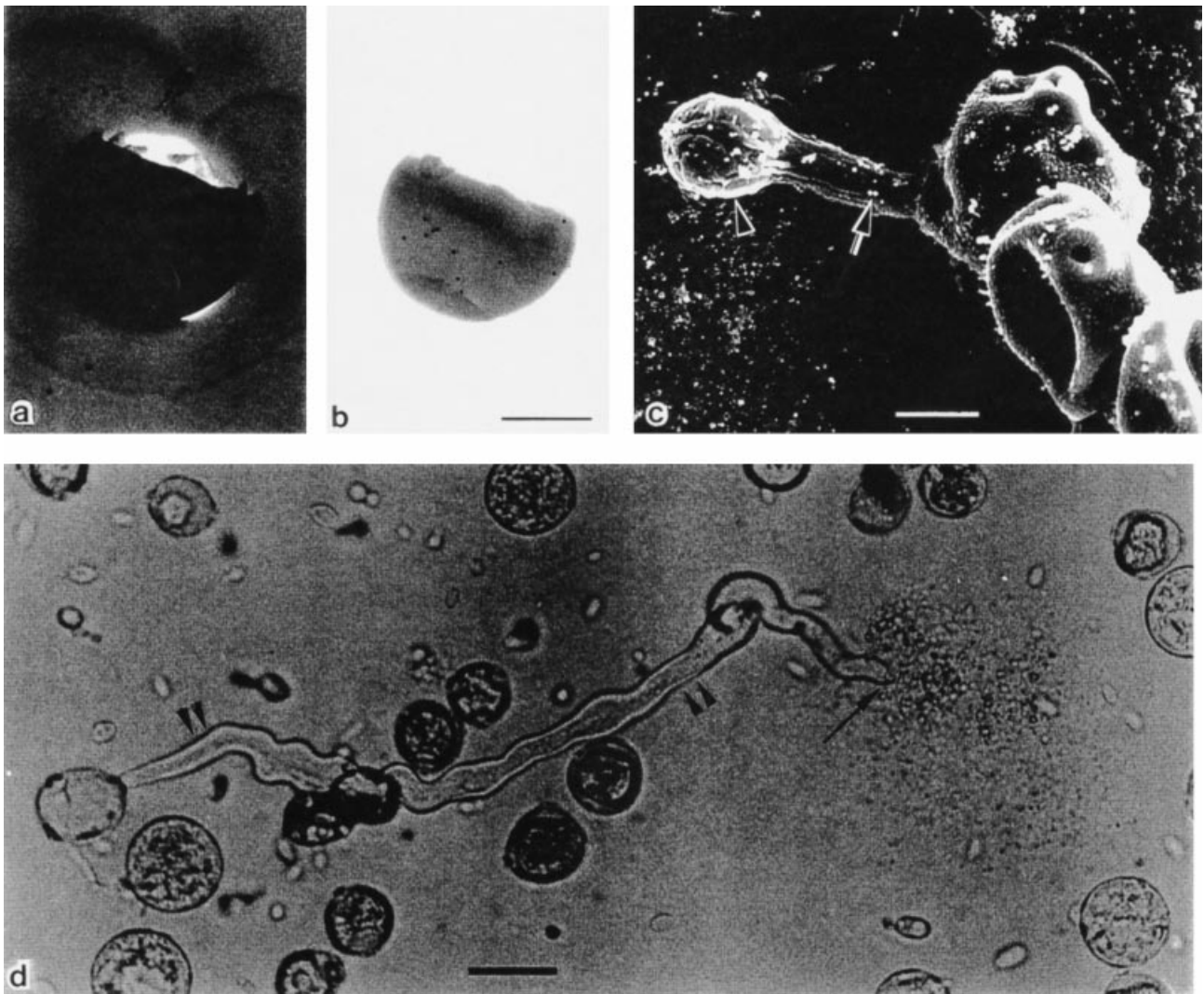


Figure 2. a) Ultrathin section of starch granule isolated from $<7.2\text{-}\mu\text{m}$ atmospheric filter. a) Meniscus envelopes starch granule. b) Underexposed print of panel a showing localization of Bet v 1 in starch granule after sections were immunolabelled (bar: $0.5\text{ }\mu\text{m}$). c) Scanning electron micrograph of part of leaf surface collected from birch tree after light rainfall, showing several collapsed birch-pollen grains. Germinated pollen grain with tube $30\text{ }\mu\text{m}$ long and swollen tip (arrowhead). Numerous starch granules coat surface of both leaf and pollen tube (arrow) (bar: $10\text{ }\mu\text{m}$). d) Light micrograph of birch pollen rinsed from leaf after incubation for 24 h in moist chamber, revealing pollen tube approximately $220\text{ }\mu\text{m}$ long (double arrowheads). During examination with Nomarski optics, tip of pollen tube (arrow) ruptured, releasing within seconds contents of pollen tube (~ 400 starch granules) into surrounding medium. Fungal spores, about $10\text{ }\mu\text{m}$ long, can also be seen (bar: $20\text{ }\mu\text{m}$).

Monitoring birch pollen over four seasons (1994–7) confirmed that birch trees significantly contribute to the pollen count during spring in Melbourne (10) and that birch pollen represents an important potential source of allergens in this region (Fig. 1a). This is also confirmed by the fact that there are patients in Melbourne sensitized to birch-pollen allergens. The birch-pollen major allergen, Bet v 1, has been detected in atmospheric fine particles ($<7.2\text{ }\mu\text{m}$) by a quantitative ELISA of extracts from filters of a high-volume sampler during the season of 1997 (Fig. 1b). Moreover, the concentration of these respirable allergenic particles has been shown to increase significantly after light rainfall, confirming earlier data (Fig. 1c) (14, 19). On a day with high

concentrations of Bet v 1 in particles of $<7.2\text{ }\mu\text{m}$, Bet v 1 could be detected in corresponding filter extracts by immunoblot analysis, qualitatively confirming the ELISA results (data not shown). For the first time, atmospheric starch granules have been shown to contain Bet v 1 by immunogold-labeling (Fig. 2), confirming reports showing predominant localization of Bet v 1 in starch granules *in vitro* (8). Records of germinated birch pollen collected on leaf surfaces after light rainfall, combined with atmospheric monitoring results, supported our proposed model system for the release of Bet v 1-containing particles from birch pollen (16). It has also been shown by others that extracts from leaves and stems of *Parietaria judaica* and *Dactylis*

Table 1. Amounts of protein and Bet v 1 ($\mu\text{g/ml}$) in extracts from particles of $>8\ \mu\text{m}$ (ungerminated and germinated/burst birch-pollen grains), $1.2\text{--}8\ \mu\text{m}$ (including mainly large starch granules), and $<1.2\ \mu\text{m}$ (filtrate; including small starch granules, cytosolic debris, and dissolved proteins). Data represent mean values $\pm$ SD of nine replicates

Particle size range (μm)	Particles $>8\ \mu\text{m}$	Particles $1.2\text{--}8\ \mu\text{m}$	Particles $<1.2\ \mu\text{m}$
Protein ($\mu\text{g/ml}$)	7.8 ± 2.0	1.3 ± 0.7	5.8 ± 1.2
Bet v 1 ($\mu\text{g/ml}$)	1.2 ± 0.3	0.4 ± 0.1	1.0 ± 0.5
Bet v 1 per total protein (%)	15.6 ± 2.5	31.2 ± 7.4	16.6 ± 3.6

glomerata were allergenic to grass- and *Parietaria*-sensitive patients, highlighting the presence of pollen allergens, as microaerosol suspensions, in the atmosphere (20). Moreover, we have recently shown that the major allergen of ryegrass pollen, Lol p 1, interacts with diesel exhaust carbon particles, to gain access to the lower airways to trigger allergic asthma (21). Therefore, the presence of such free allergen molecules, as microaerosol suspensions, allows their interaction with other atmospheric particles, and they can also settle on leaves and parts of other plants and re-enter the atmosphere later. Due to the existence of such free allergen molecules in the atmosphere, pollen counts may not reflect the total airborne allergen exposure; thus, the need for the development of an estimate of the total atmospheric allergen load.

The process of Bet v 1 release was further investigated *in vitro*, to achieve an approximate quantification of the total protein and Bet v 1 contents of released starch granules. An average of 8.6% of the total soluble protein and of 15.6% of the total Bet v 1 was found in the extract of the isolated particles containing predominantly starch granules (Table 1). Counting birch-pollen grains by means of a haemocytometer and estimation of the percentage of germination in the course of the experiment allowed estimations of protein (0.04 pg) and Bet v 1 (0.01 pg, 31.2% of soluble proteins) contents per starch granule. These values represent conservative estimations of the actual concentrations. This is because some soluble proteins, including Bet v 1, had been leached out during the germination/filtration process and had been found in the filtrate. Yet, they indicate the association of Bet v 1 with isolated starch granules, despite isolation in an aqueous medium. Bet v 1 was predominantly concentrated in starch granules (31.2% of total protein) compared with whole pollen grains (15.6%) (Table 1). Consequently, the amount of Bet v 1 detected within respirable particles on a day with light rainfall is equivalent to 26 000 starch granules per m^3 of air. We have previously shown that ryegrass-pollen grains rupture in rainwater by osmotic shock, each grain releasing about 700 allergen-

loaded starch granules (22). On days with rainfall during the grass-pollen season of 1996/7, the number of starch granules from grass pollen was estimated as up to 100 000 per m^3 (data not shown).

Birch leaves are apparently not the only source of secondary emissions of these micronic particles. Leaves of other plants also appear to act as pollen traps and, along with window ledges (23) and even road surfaces underneath trees, may be important sources of the allergenic starch granules contributing to the atmospheric particle load. The starch granules extracted from the atmosphere which showed Bet v 1 labelling originate from pollen of the Betulaceae family, probably from nearby birch trees. Starch granules from pollen sources other than birch are also expected to occur in the atmosphere. The techniques developed in this study, along with available antibodies that recognize specific allergens, can be used to determine the origin of atmospheric starch granules.

Western blot analysis has shown that sera from patients allergic to birch allergens recognized extracts of starch granules isolated from germinated birch pollen (data not shown), indicating the potential of starch granules as triggers of allergic responses in susceptible subjects.

The clinical implications of these findings for tree-pollen allergy are that starch granules released from birch pollen may cause allergic asthmatic reactions to Bet v 1, since the allergen occurs in respirable particles. They appear in high concentrations on days of light rainfall during the birch-pollen season. In summary, patients with specific IgE to Bet v 1 are at high risk of rain-related asthma, particularly those not protected by medication. Thus, clinicians can use the information presented here to advise birch-allergen-sensitive asthma patients to remain indoors on days of light rainfall during the birch-pollen season in order to minimize exposure to Bet v 1-containing respirable particles. Indeed, a recent study comparing outdoor pollen levels with allergenic activity measured both outdoors and indoors provided scientific support for the recommendation to pollen-sensitive patients to remain indoors during seasons with high outdoor levels of pollens (24).

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