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Mechanism of grass-pollen-induced asthma

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Many asthmatics are sensitive to rye-grass pollen, but pollen grains are too large to penetrate the lower airways. Our aim was to investigate the mechanism by which rye-grass pollen causes asthma.

A major allergen of rye-grass pollen, *Lo1 pIX*, is located in intracellular starch granules within pollen grains. In-vitro tests showed that pollen grains are ruptured in rainwater by osmotic shock, each grain releasing about 700 starch granules into the environment. These granules are small enough to enter the airways (< 3 µm in diameter). The starch granules were present in atmospheric samples taken during the pollen season, and showed a 50-fold increase in atmospheric concentration on days following rainfall. Isolated granules elicited IgE-mediated responses in asthmatic patients, and 4 patients with rainfall-associated asthma who

underwent an inhalation challenge test had striking bronchial constriction after exposure to starch granules.

Starch granules released from rye-grass pollen seem to be capable of causing asthma.

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Introduction

Epidemics of asthma have been associated with thunderstorms and related atmospheric events.¹ On two

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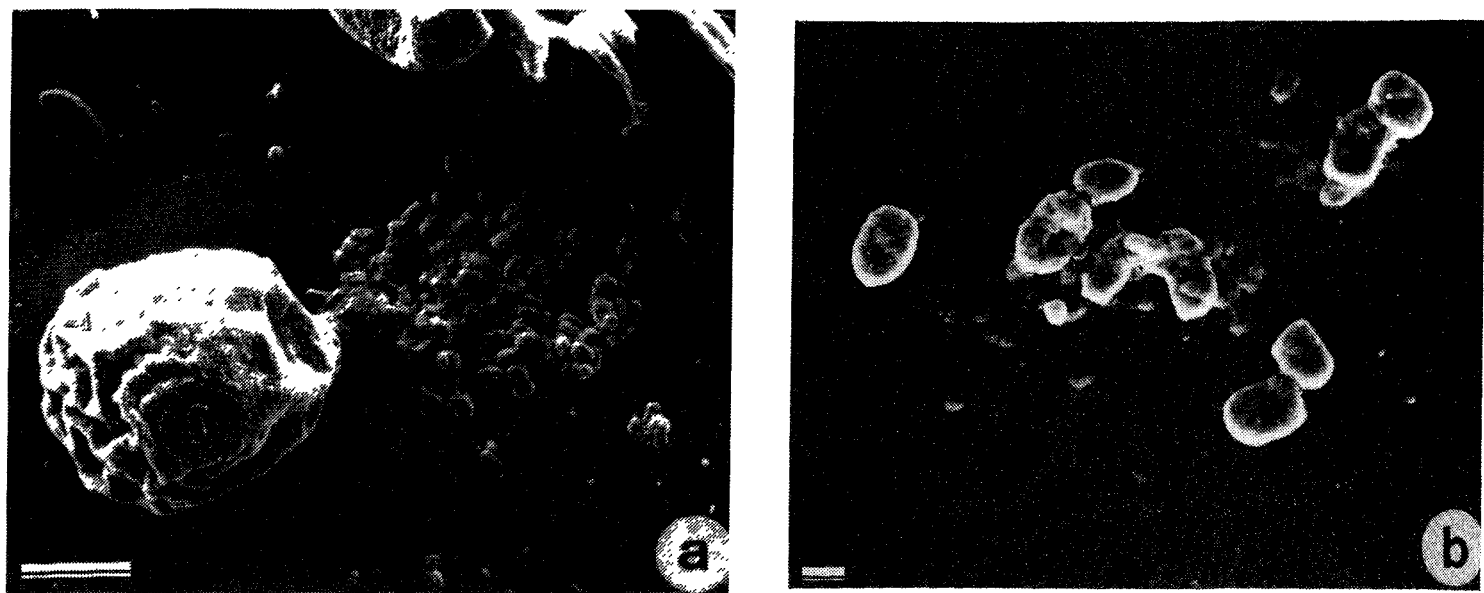


Fig 1—Scanning electronmicrographs of rye-grass pollen grains.

When exposed to water, pollen grains rupture at the single germinal aperture releasing their cytoplasmic contents (a). Prominent among the contents released from each grain are about 700 starch granules (b); these particles are less than 3 μm in diameter. Scale bars represent 10 μm .

consecutive occasions after thunderstorms in Melbourne, Australia, there were 10-fold increases in cases of asthma.² Factors thought to trigger such epidemics include pollution and atmospheric particles³ such as mould spores,¹ but we have shown that storm-affected Melbourne patients were sensitive to rye-grass pollen.² Grass pollen is the most abundant atmospheric allergen in spring,⁴ but the grains are too large (diameter > 35 μm) to penetrate the lower airways.⁵ The mechanism by which pollen triggers asthma involves micronic allergen-containing particles.^{6,7}

A new allergen of rye-grass pollen, *Lol pIX*, has been discovered with molecular cloning.⁸ This allergen is unique in two respects: clones contain a signal peptide with motifs similar to those of chloroplast transit peptides; and immunogold localisation with specific monoclonal antibodies shows that intracellular starch granules within amyloplasts contain the allergen.⁸ By contrast, cDNA clones of the other well-known allergen, *Lol pI*, possess signal peptides specifying secretion, and immunogold labelling shows that the allergen is located in the cytosol from where it could be secreted to the pollen surface. Thus, *Lol pI* is secreted to the surface of pollen, whereas *Lol pIX* is located in small cytoplasmic starch granules. In this report we show that micronic particles have their cellular origin as starch granules within rye-grass pollen grains.

Methods

To osmotically rupture pollen grains, fresh rye-grass pollen, collected from the field, was suspended in distilled water in a syringe, shaken briefly, and passed through 5 μm and 0.45 μm nitrocellulose-mesh filter units (Millipore, Bedford, USA) connected in series. Ruptured pollen grains, retained on the 5 μm filter, and starch granules, retained on the 0.45 μm filters, were resuspended in distilled water and subjected to western blot analysis.⁹ Scanning electronmicrographs of ruptured pollen granules were prepared.

Air sampling was done during the peak of the grass-pollen season in November, 1990. For 2 h in the morning of days with high sunshine hours and northerly winds, which favour high pollen counts,⁸ and in the morning of days with a record of rainfall in the previous day, sampling was done with an air sampler ('MD 8', Sartorius, Göttingen, Germany) operating at 4.5 m³ per h, and particles were collected on 80 mm nitrocellulose filters with a pore size of 0.8 μm . After sampling, nitrocellulose filters were placed

face-up for 1 h on filter paper moistened with phosphate-buffered saline and enclosed in a petri-dish. This procedure facilitates the binding of readily leachable allergenic proteins from pollen and starch granules to the nitrocellulose filter. Nitrocellulose filters were then cut into small portions. Some portions were mounted in iodine/K iodide (IKI) reagent, which stains starch granules blue-black, and were observed by transmitted light microscopy. For quantitative assessment, starch granules on five replicate portions of filter were counted and the total for the entire surface area of the filter was calculated. Division by the volume of air drawn across the filter gives the concentration of starch granules in air. Immunoblot analysis of portions of nitrocellulose filter was done by

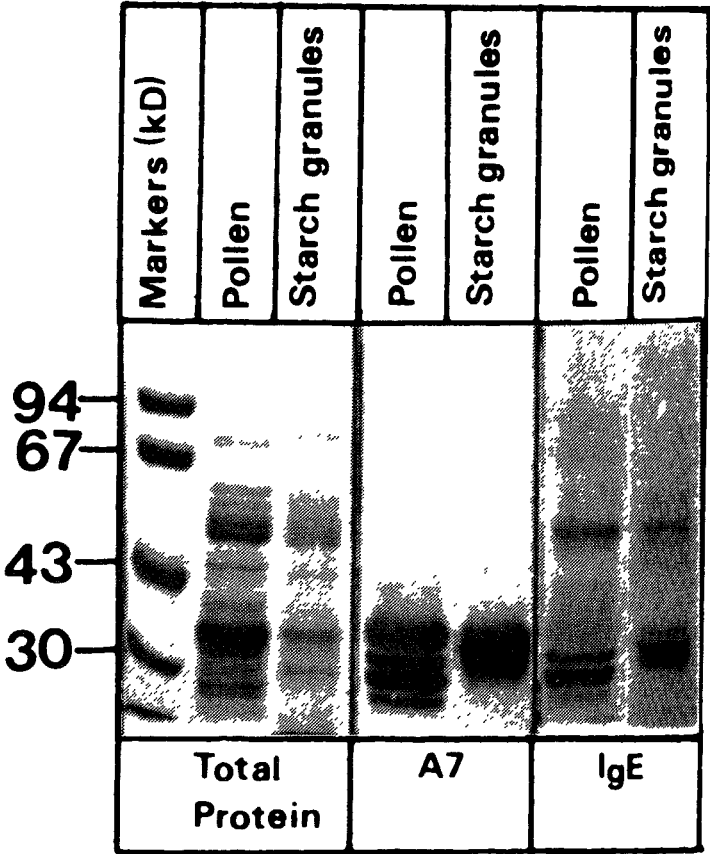


Fig 2—Western blot analysis of pollen and isolated starch granules.

Probes used were monoclonal antibody A7 (12.3) specific to the allergen *Lol pIX* and specific IgE antibodies from the pooled sera of 10 grass-pollen-allergic patients.

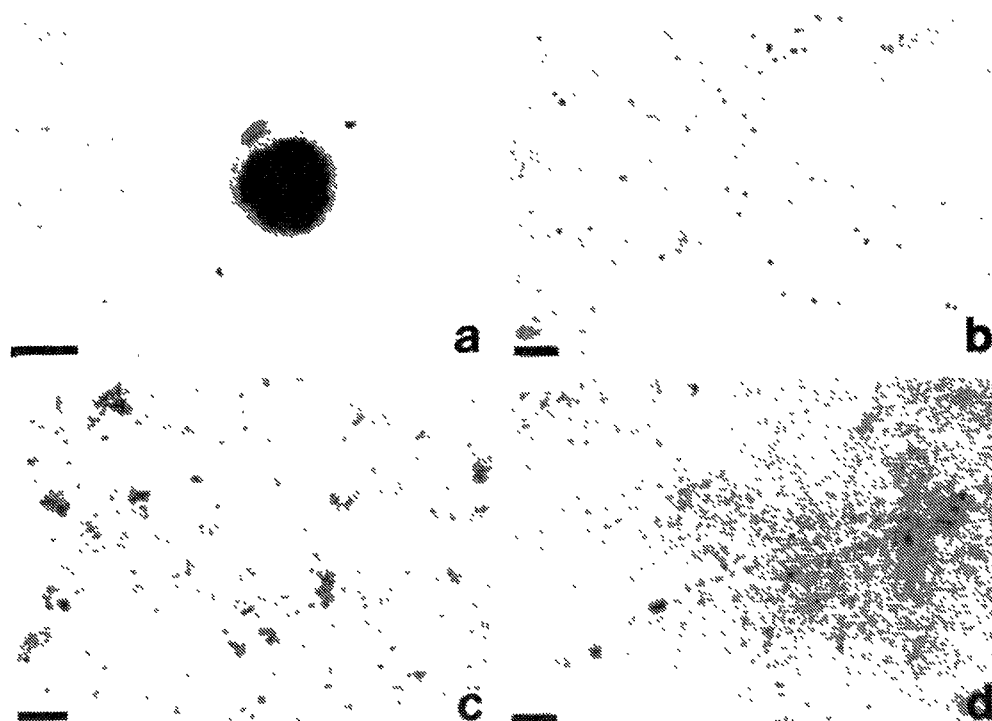


Fig 3—Pollen grains and starch granules obtained by sampling the atmosphere of Melbourne.

Intact rye-grass-pollen grains contain many starch granules (a; IKI stain). Air-filter sample showing starch granules present in the atmosphere on a day following rainfall (b; IKI stain). Preparation of isolated starch granules from rye-grass pollen (c; IKI stain). Portion of same air filter as (c) probed with monoclonal antibody A7 (12.3) showing allergens present in particles of a similar size to the starch granules (d). Scale bars represent 20 μm .

first blocking the free protein-binding sites, then incubating the filters with monoclonal antibody A7 (12.3), which is specific for the allergen *Lol pIX*, followed by incubation with horseradish-peroxidase-conjugated sheep anti-mouse immunoglobulin and visualisation with peroxidase substrate.⁹

Isolated starch granules were prepared under sterile conditions from fresh rye-grass pollen and resuspended in sterile saline to a concentration of 1.6×10^7 starch granules/ml, which is equivalent to 0.34 μg protein/ μl . The suspension was diluted in series from 10^{-1} to 10^{-6} in sterile saline and used for skin-prick tests of IgE-mediated responses in grass-pollen-allergic patients, asthmatics, and controls. Rye-grass pollen extract (Hollister-Stier, Spokane, Washington, USA) was also used in these tests. Bronchial challenge tests were done by standard methods in 4 patients with thunderstorm-associated asthma with a 'Sensor Medics 2400' waterseal spirometer (Sensor Medics, Anaheim, USA). Slow vital capacity, forced vital capacity, forced expiratory volume in 1 s, and peak expiratory flow were measured three times and mean values were calculated. Bronchial challenge tests were done by making a 1 in 1000 dilution of the standard starch-granule suspension in normal saline, nebulising 1 ml of the solution, and delivering the mist by an aerosol mask. Early bronchospasm was treated with 5 mg of nebulised salbutamol.

Results

Rye-grass pollen grains are spheroidal and monoporate, and are ruptured rapidly by osmotic shock, releasing their cytoplasmic contents through the germinal pore (fig 1). Prominent among the contents released from each grain were about 700 starch granules. Each granule was 0.6 to 2.5 μm in diameter (fig 1). Western blot analysis of isolated starch granules with specific monoclonal antibody and specific IgE from pooled sera of grass-pollen-allergic patients showed at least 8 bands including the major allergens (fig 2). We conclude that a single ruptured pollen grain produces a 700-fold amplification of atmospheric allergen which is dispersed in the form of micronic particles.

Air sampled during the grass-pollen season showed the presence of grass pollen grains (fig 3a) and starch granules (fig 3b). Starch granules were found within the grains (fig 3a), and after osmotic rupture in vitro, isolated starch

granules could be readily detected (fig 3c). On a dry day with no record of rainfall in the previous 24 h, the Melbourne atmosphere contained a mean (SD) of 910 (149) starch granules/ m^3 . By contrast, on a dry day with a record of rainfall on the previous day, the filter collected 53 982 (6107) starch granules/ m^3 ($p < 0.001$, t test). Immunolabelling of nitrocellulose membrane blots with monoclonal antibody specific for *Lol pIX* allergen showed that allergenic activity was associated with discrete particles in air samples (fig 3d), which were of a similar size to the starch granules—ie, $< 3 \mu\text{m}$ diameter. It is likely that the allergen-containing micronic particles in the atmosphere are starch granules released osmotically from rye-grass pollen.

Can isolated starch granules trigger an attack of asthma? Available in-vivo tests can demonstrate only whether starch granules initiate specific IgE-mediated responses in grass-pollen-allergic patients. Skin-prick tests with standard rye-grass-pollen extracts and isolated starch granules gave

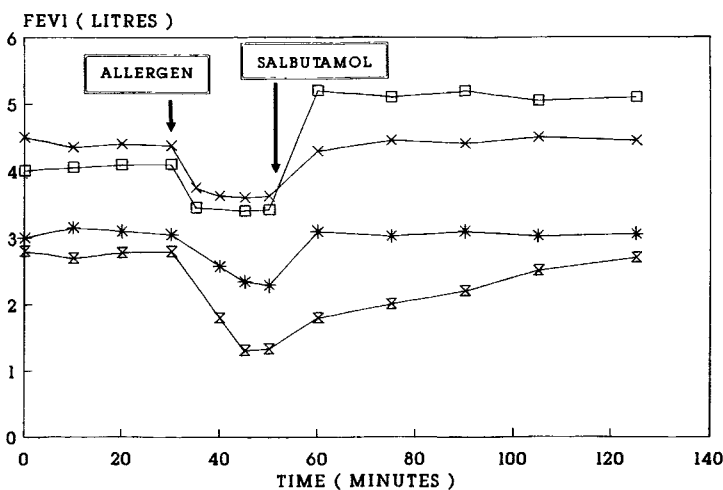


Fig 4—Bronchial response to the inhalation challenge test with isolated starch granule suspensions in 4 subjects with thunderstorm-associated asthma.

Each line represents an individual patient. FEV₁=forced expiratory volume in 1 s.

strong positive results in all of 15 grass-pollen-allergic patients (ie, greater than double the wheal diameter of histamine control in every case) but no reaction in 6 non-allergic subjects (ie, no wheal formation). The most concentrated preparation of granules gave the greatest wheal diameter (data not shown). Positive skin-prick tests were also obtained in all of 12 patients affected by a springtime thunderstorm-associated epidemic of asthma, and in 7 of 13 randomly selected patients with asthma from a hospital outpatient clinic ($p < 0.05$, t test). All of a separate group of 22 asthmatics tested with a standard rye-grass-pollen extract and with the starch-granule suspension were positive to both. In addition, all 4 patients from the cohort of thunderstorm-associated asthmatics who volunteered to undergo an inhalation challenge test showed a typical early response with striking bronchial constriction after exposure to starch granules (fig 4). This response occurred with suspensions containing starch granules but not with supernatant alone. The reaction was completely reversed by salbutamol. It is clear that starch granules can elicit specific IgE-mediated responses in grass-pollen-allergic subjects, including asthmatics.

Discussion

A positive correlation between the grass-pollen count and incidence of childhood asthma has been reported in Melbourne.⁴ In Perth, Western Australia, volumetric sampling of the atmosphere detected micronic particles only after substantial rainfall.⁶ Our study complements this finding by showing that large numbers of starch granules could be detected in atmospheric samples after rainfall but not after dry days. Micronic particles of unknown origin have been detected in the atmosphere of European cities during the grass-pollen season.^{7,10}

The evidence we present strongly suggests a mechanism by which rye-grass pollen grains elicit allergic asthma. The

starch granules contain *Lol pIX* allergen in the starch matrix and are coated with *Lol pI*.⁸ At 0.6 to 2.5 μm diameter the granules are small enough to enter the lower airways.⁵ Tests in asthmatics show that suspensions of granules provoke IgE-mediated responses. We conclude that starch granules released from rye-grass pollen are micronic particles, and suggest that these particles cause asthma.

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Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure

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Nitric oxide (NO), synthesised from L-arginine, contributes to the regulation of blood pressure and to host defence.

We describe in-vitro and in-vivo evidence that NO synthesis can be inhibited by an endogenous compound, N^G,N^G-dimethylarginine (asymmetrical dimethylarginine, ADMA). In man, this inhibitor is found in plasma and more than 10 mg is excreted in urine over 24 h. However, in patients with end-stage chronic renal failure, who have little or no urine output, elimination is blocked and circulating concentrations of the inhibitor rise sufficiently to inhibit NO synthesis.

Accumulation of endogenous ADMA, leading to impaired NO synthesis, might contribute to the hypertension and immune dysfunction associated with chronic renal failure.

Introduction

Nitric oxide (NO) is now recognised as a mediator involved in the regulation of vascular tone, neurotransmission, and host defence.¹⁻⁵ NO is synthesised from L-arginine, and defects in its synthetic pathway have been implicated in several diseases, although the pathogenesis of such defects remains unclear. The synthesis of NO can be inhibited experimentally by some analogues of arginine including N^G-monomethyl-L-arginine (L-NMMA);^{3,6} in animals, inhibition of NO synthesis with L-NMMA leads to hypertension^{6,7} and impaired immune function.^{8,9}

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