

Intratracheal Instillation of Cytoplasmic Granules from *Phleum pratense* Pollen Induces IgE- and Cell-Mediated Responses in the Brown Norway Rat

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Key Words

Allergy · Asthma · Granules · Pollen

Abstract

Background: Release of cytoplasmic granules from grass pollen upon contact with water is thought to be an important source of airborne allergens. **Objectives:** To investigate the humoral and cellular responses to intratracheal instillation of *Phleum pratense* (timothy grass) pollen cytoplasmic granules (PCG) in the Brown Norway rat. **Methods:** PCG were purified from timothy grass pollen by filtration through 5-μm-mesh filters. Rats were sensitized (day 0) and challenged (day 21) intratracheally with purified PCG suspended in saline (6×10^6 PCG/rat). Rats were then challenged 4 weeks later (1.5×10^6 PCG/rat). Blood samples, bronchial lymph nodes and lungs were collected from the rats 4 days after the second challenge. PCG-specific IgE and IgG1 levels and specificity were determined by ELISA and Western blotting. Pollen, pollen extract and PCG-induced proliferation of lymph node cells were monitored by [³H]-thymidine incorporation in a lymph node assay. Histopathological examination was carried out on the lungs. **Results:** Specific IgE and IgG1 were present in the sera. Cultured lymph node

cells proliferated in the presence of pollen, pollen extract and PCG. Western blots showed that all major pollen allergens are recognized by IgE and IgG1 from PCG-treated rats. Histopathological examination revealed features of a mild allergic reaction. **Conclusions:** In our rat model of allergy, purified timothy grass PCG instillation induced specific antibodies and lymph node cell responses, comparable to those obtained with intact pollen.

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Introduction

Grass pollen is a major source of airborne allergens. Although the pollen grain itself cannot penetrate the deep airways because of its size [1], it is a source of respirable size aeroallergens [2–4]. Of particular interest are the pollen cytoplasmic granules (PCG), consisting of starch granules and pollen wall precursor polysaccharide particles (p-particles). These micronic particles (<5 μm) are released from pollen grains of several grass and tree species upon contact with water or following germination tube formation [5–7]. Immunogold labeling revealed the presence of allergens on the surface of pollen starch granules, includ-

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ing the major allergens Lol p 5 for rye grass [5, 8], Phl p 4 and Phl p 5 for timothy grass [9, 10] and Bet v 1 for birch tree [11, 12]. The Phl p 6 allergen (timothy grass pollen) has been found on p-particles [13]. In vivo studies have shown that starch granules isolated from rye grass pollen can trigger an IgE-mediated response in grass-pollen allergic patients [14]. Moreover, we have shown that purified timothy grass PCG can provoke a secondary allergic response in pollen-sensitized rats [15]. Once released from the grain, PCG can form a respirable aerosol. This was found for small particles derived from birch tree pollen ($<7.2 \mu\text{m}$) which have been reported to contain Bet v 1. These particles were present in high quantity in the atmosphere, during episodes of light rainfall, following dry periods associated with high pollen counts [16]. PCG are also suspected to be implicated in episodes of acute asthma epidemics occurring during periods of thunderstorms associated with high atmospheric pollen counts, so-called thunderstorm asthma [17–20]. The aim of this study was to verify the PCG potency to prime for specific humoral and cellular reactions following intratracheal instillation in the rat. Therefore, Brown Norway (BN) rats were sensitized (day 0) and challenged (day 21) with PCG purified from timothy grass pollen. The induced reactions were characterized by assessing PCG-specific IgE and IgG1 levels in the serum, as well as in vitro pollen, pollen extract and PCG-induced lymph node cell proliferation. The specificities of the IgE and IgG1 antibody responses to PCG were tested by Western blotting and compared to those obtained in pollen-sensitized rats. In addition, histopathological examinations of the rat lungs were carried out.

Materials and Methods

Animals

Six- to 8-week-old male BN rats were obtained from Harlan-Olac (Horst, The Netherlands). The animals were housed in Macrolon type II and III cages. Food pellets and tap water were available ad libitum, and all animals were allowed to adapt for at least 1 week before the experiments started. All experiments were approved by the Dutch Animal Ethics Committee, and the principles of laboratory animal care were followed.

Pollen Grains and Pollen Extract

Pollen grains from *Phleum pratense* (timothy grass) were purchased from Allergon, Angelholm, Sweden. Lyophilized extracts from this pollen were obtained from ALK Benelux (Houten, The Netherlands). For fresh pollen extracts, 50 mg of pollen were incubated on a rotating drum for 30 min at room temperature, with 450 μl of distilled water and then centrifuged for 10 min at 10,000 g. The supernatant was centrifuged a second time and used immediately for digoxigenin (DIG) labeling and electrophoresis.

PCG Isolation

Phleum pratense pollen (50 mg) was suspended in 500 μl of distilled water to facilitate PCG release and incubated for 1 min with gentle agitation. The suspension was then filtered by centrifugation through a 5- μm -mesh filter at 10,000 g for 5 min (Ultrafree-MC; Millipore, Bedford, Mass., USA). The PCG pellet was collected and washed twice in 500 μl of distilled water, filtered again through a 5- μm filter and resuspended in 100 μl of distilled water.

Sensitization and Challenge of the Rats

The rat allergy model described earlier has been slightly modified [15]. Briefly, rats ($n = 5$) were anesthetized and, using a cannula, a suspension of PCG (0.2 ml, 3×10^7 PCG/ml in saline) was intratracheally instilled on day 0. This was repeated on day 21 for the challenge. Rats were then challenged 4 weeks later (50 μl of PCG suspension, same concentration). Negative control animals received saline. Rats were killed 4 days after the second challenge and lungs, blood samples and bronchial lymph nodes were taken.

Serum Samples

After collection, the blood was kept at 4°C for clotting for 2–4 h. Blood samples were then centrifuged at 800 g at 4°C for 15 min. Serum was removed and stored in 1-ml aliquots at –20°C until use.

ELISA for PCG-Specific IgE and IgG1

Previous studies have already shown that PCG contain pollen allergens. Thus, pollen-specific IgE or IgG1 ELISA were used to determine PCG-specific humoral responses in PCG-sensitized rat sera. ELISA assays were performed according to the pollen-DIG protocol described previously [21]. Briefly, mouse anti-rat-IgE or anti-rat-IgG1 antibody diluted 1:500 was used to coat 96-well microtiter plates (Zymed, San Francisco, Calif., USA). After washing and saturation of the wells with a 1% (w/v) skimmed milk powder solution (incubated for 1 h at room temperature in PBS + Tween 0.1%), rat sera were added to the wells (in triplicate) at the proper dilution (1:2 and 1:1,500 for IgE and IgG1 detection, respectively), incubated for 1 h at 37°C and washed. Wells were then incubated with a pollen-DIG solution at a 1:800 dilution (pollen extract labelled with DIG according to the instructions for the DIG antibody labelling kit; Boehringer, Mannheim, Germany). After washing, a horseradish-peroxidase-conjugated anti-DIG antibody was added (Roche Diagnostics, Mannheim, Germany, dilution 1:625). IgE or IgG1 levels were assayed by adding peroxidase substrate and reading the absorbance values at 450 nm with a multichannel photometer. All dilutions of sera, pollen-DIG and anti-DIG antibody were made in 0.5% (w/v) milk powder solution. Absorbance values are expressed as the mean $\pm$ SEM of the 5 rats.

Bronchial Lymph Node Cell Assay

At autopsy, lymph nodes were removed and immediately placed in 5 ml of ice-cold RPMI-1640 medium (Invitrogen, Paisley, UK), supplemented with 2 mM L-glutamine, 1% antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml, amphotericin B 1 $\mu\text{g}/\text{ml}$) and 5% heat-inactivated fetal calf serum (FCS). The lymph nodes were then forced through a sterile 70- μm -mesh nylon cell strainer (Falcon, Becton Dickinson, Franklin Lake, N.J., USA). The cells were collected in a 50-ml Falcon tube, in a final volume of 25 ml 5% FCS. Single cell suspension was centrifuged at 310 g for 10 min at 4°C. Supernatant was discarded, and the pellet was resuspended in 25 ml of 5% medium. After a second centrifugation (310 g, 10 min, 4°C),

the supernatant was discarded and cells were resuspended in 1 ml of 10% FCS, counted and diluted in 10% medium to a final concentration of 4×10^6 cells/ml. Then, 100 μ l of the cell suspension (4×10^5 cells) were cultured in U-bottomed 96-well plates (Cellstar, Greiner Bio One, Frickenhausen, Germany) containing 50 μ l 10% medium + 1.5 μ M 2- β -mercaptoethanol, 10% medium + 1.5 μ M 2- β -mercaptoethanol + allergen (pollen extract or pollen: 10 or 100 μ g/ml or PCG: 10^5 , 10^6 or 10^7 PCG/ml), in triplicate. Cells were incubated for 3 days in humidified atmosphere at 37°C and 5% CO₂, followed by a 24-hour pulse with 10 μ l/well [³H]-thymidine (Amersham, Amersham, UK, 37 kBq/ml) and harvested for scintillation counting in a β -plate counter (1205 Betaplate™, Wallac, Turku, Finland) to determine cell proliferation. Proliferation is expressed in counts per minute (cpm) as the mean $\pm$ SEM of the 5 rats.

Western Blotting

Isoelectric focusing was performed in 5% acrylamide precast gel (Pharmacia Biotech, Uppsala, Sweden). The dehydrated gel was reswelled prior to utilization with an ampholyte solution (Pharmacia Biotech, 2% w/v in distilled water, pH gradient: 4.45–9.6). Fresh soluble extract from timothy grass pollen was loaded 1 cm from the anode. After isoelectric focusing, a pressure print was taken of the gel on a nitrocellulose membrane, which was cut into strips to be used for immunodetection. Strips of nitrocellulose were saturated in milk powder solution (5% w/v, in PBS + Tween 0.05%) for 1 h at room temperature. Strips were then incubated 1 h at room temperature with pooled serum from PCG-sensitized rats or pollen-sensitized rats as a positive control. The sera from pollen-sensitized rats were obtained in previous experiments, using the same immunization protocol. Sera were diluted 1:5 in 5% milk powder solution. For IgG1 detection, strips were washed several times in PBS-Tween and then incubated with horseradish-peroxidase-conjugated mouse anti-rat IgG1 monoclonal antibodies diluted 1:400 (Zymed) for 1 h at room temperature. Strips were then washed several times in PBS-Tween 0.05% and revealed with a peroxidase substrate (DAB; Sigma-Aldrich, St. Louis, Mo., USA). For IgE detection, strips were washed in PBS-Tween and incubated with a mouse anti-rat IgE antibody diluted 1:500 (isotype IgG1, Zymed), 1 h at room temperature. After washing, strips were incubated with an horseradish-peroxidase-conjugated goat anti-mouse IgG antibody, diluted 1:7,500 (ZyMax grade, Zymed). Strips were then washed several times in PBS-Tween 0.05% and revealed with peroxidase substrate.

Histopathology

Lungs were fixed in situ in 4% phosphate-buffered formaldehyde and embedded in Paraplast. Five-micrometer sections were prepared and routinely stained with hematoxylin and eosin. The histopathological lesions were semiquantitatively scored from absent (0), minimal (1), slight (2), moderate (3), marked (4) to strong (5). This scoring system included the incidence and the extent of the lesions.

Results

Pollen-Specific IgE and IgG1 Levels

Pollen-DIG ELISA was used to determine PCG-specific IgE and IgG1 in PCG-sensitized rat sera (fig. 1). The ELISA for IgE showed a significantly increased absor-

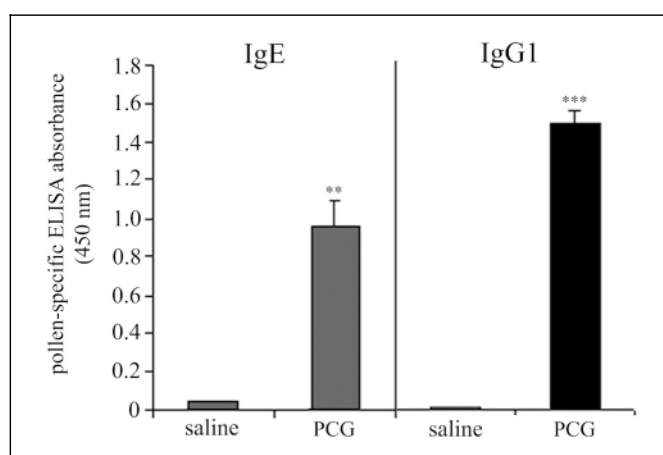


Fig. 1. Timothy-grass-pollen-specific IgG1 and IgE ELISA. Sera from saline- or PCG-sensitized rats ($n = 5$) were tested for pollen-specific IgE and IgG1 at dilutions of 1:2 and 1:1,500, respectively. The mean $\pm$ SEM are shown. ** $p < 0.01$ and *** $p < 0.001$.

bance for the rats in the PCG group compared to the saline-treated controls (0.88 ± 0.12 and 0.04 ± 0.002 , respectively). The PCG-specific IgG1 also showed a significant increase, as the absorbance reached 1.5 ± 0.07 for PCG-treated rats compared to 0.007 ± 0.003 for the saline-treated rats.

Lymph Node Assay

As shown in figure 2, lymph node cell proliferation in the PCG-sensitized rats was significantly increased when cultured in the presence of pollen extract (100 μ g/ml) and pollen (100 μ g/ml) as well as PCG (10^6 and 10^7 PCG/ml). The same cells cultured with medium alone or with a non-related allergen (house dust mite) did not proliferate. Lymph node cells from negative control rats (immunized with saline) did not proliferate when cultured either with medium alone, pollen extract (100 μ g/ml), pollen (100 μ g/ml) or PCG (10^7 PCG/ml): 190 ± 10 , 297 ± 30 , 221 ± 60 and 330 ± 110 cpm, respectively.

IgE and IgG1 Specificity

IgE (fig. 3a) and IgG1 (fig. 3b) from the sera of PCG-sensitized rats were tested by Western blotting for pollen antigen specificity. Blots incubated with pooled serum from saline-treated rats remained negative (lanes 1a and 1b). Lane 2a shows the classical allergen recognition pattern by IgE of pollen-sensitized BN rats, based on at least 5 different pollen-immunized rats (data not shown). The major allergens Phl p 1, acidic and basic Phl p 5 and Phl p 6 can be recognized at their respective pI (6, 5.1–5.8,

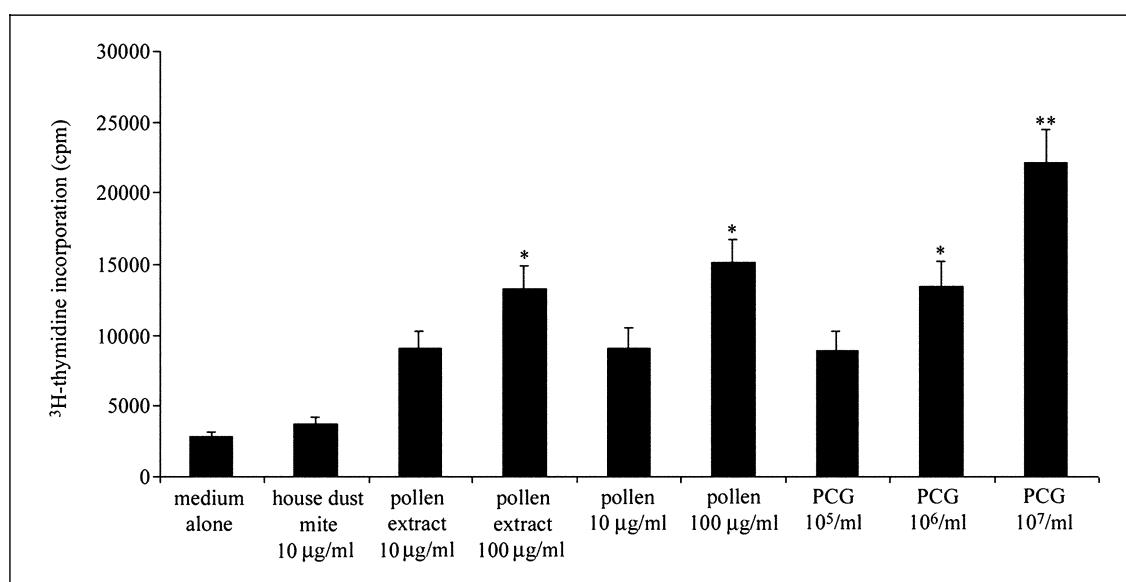


Fig. 2. PCG-induced proliferation of bronchial lymph node cells (means $\pm$ SEM). Cells from PCG-immunized rats ($n = 5$) were cultured in the presence of either medium alone, house dust mite (10 μ m/ml), pollen extract (10 or 100 μ g/ml), pollen (10 or 100 μ g/ml) or PCG (10⁵, 10⁶ or 10⁷ PCG/ml) for 3 days. Proliferation was assessed by measuring [³H]-thymidine incorporation following a 24-hour pulse. * $p < 0.05$ and ** $p < 0.01$, vs. medium alone.

7.2–7.8 and 5.5, respectively). In pollen-sensitized rats the specificity of the IgG1 antibodies showed in at least 5 different animals the same profile than the one obtained by IgE as well as in lane 2b with pooled sera. Pooled serum IgE and IgG1 (lanes 3a and 3b) from PCG-sensitized rats showed complete homology with pollen-sensitized rats.

Histopathology of the Lungs

The histological changes induced in the rat lungs are shown in table 1. Control animals showed minimal peribronchiolar/perivascular infiltrates and hypertrophy of mucous bronchiolar cells and neither alveolitis, eosinophil influx nor granuloma (fig. 4a). After 4 days, instillation of PCG resulted in slight perivascular infiltration and hypertrophy of bronchiolar mucous cells as well as a slight alveolitis and granuloma. In the aforementioned infiltrate, a slight influx of eosinophils was observed. The peribronchiolar infiltrate remained minimal (fig. 4b).

Discussion

PCG from grass pollen are released upon contact of the pollen grain with rainwater. They can then become airborne and form an inhalable aerosol, capable to reach the

deep airways due to the micronic size of PCG and evoke an IgE response. Previous studies proved the presence of major allergens on the surface of PCG and their capacity to trigger IgE-mediated responses in allergic patients. We have previously shown that purified PCG could elicit a secondary allergic response in BN rats sensitized to pollen [15]. In this study, we showed the allergenic potential of granules from timothy grass pollen. For the first time, purified PCG were intratracheally instilled in rats, which triggered both humoral and T-cell-mediated specific responses. Moreover, lymph node cells of PCG-sensitized rats proliferated when cultured in the presence of pollen extract or intact pollen. Thus, PCG are effective pollen allergen carriers, able to prime an immune response in a rat model. The amount of PCG in the air is related to the pollen concentration. However, conditions like light rainfall or thunderstorm, associated with high pollen counts, can lead to the presence of PCG in the atmosphere without presence of pollen, once these have been washed away [11, 17]. Knowing their allergenic capacity, the importance of PCG in pollen-related airway pathologies should be borne in mind, and the relevance of atmospheric PCG should be taken into account for seasonal allergy forecasts. In our model of allergy, intratracheal administration of PCG in BN rats triggered humoral and cellular reactions, similar

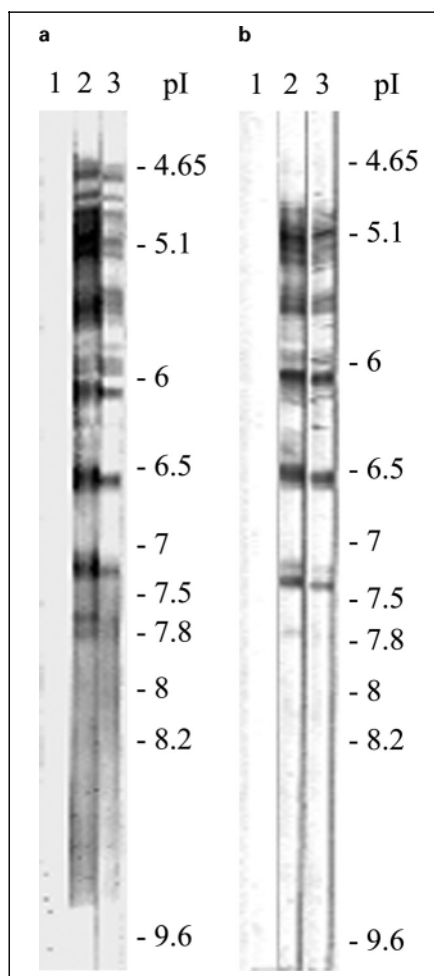


Fig. 3. Specificity of humoral responses of PCG-sensitized rats. A timothy grass pollen extract was separated by isoelectric focusing and blotted on a nitrocellulose membrane. The membrane was cut in strips, and each strip was incubated with pooled sera from saline-, pollen- or PCG-sensitized rats (lanes 1, 2 and 3, respectively). Specific detection of IgE (**a**) and IgG1 (**b**) was then carried out.

to those observed following pollen administration using the same protocol [15]. However, the histological changes in the lungs of rats exposed to purified granules were not as dramatic as those previously observed in rats instilled with pollen [21]. Indeed in our model, pollen grains and PCG instilled intratracheally reach the lungs, where they are processed by the immune system. We hypothesize that the differences observed in histopathology are mainly due to the difference in size between pollen (25–60 μm) and PCG (<5 μm). It has been shown that pollen PCG strongly bind to human alveolar macrophages and monocyte-derived dendritic cells [22]. Airway dendritic cells are known to

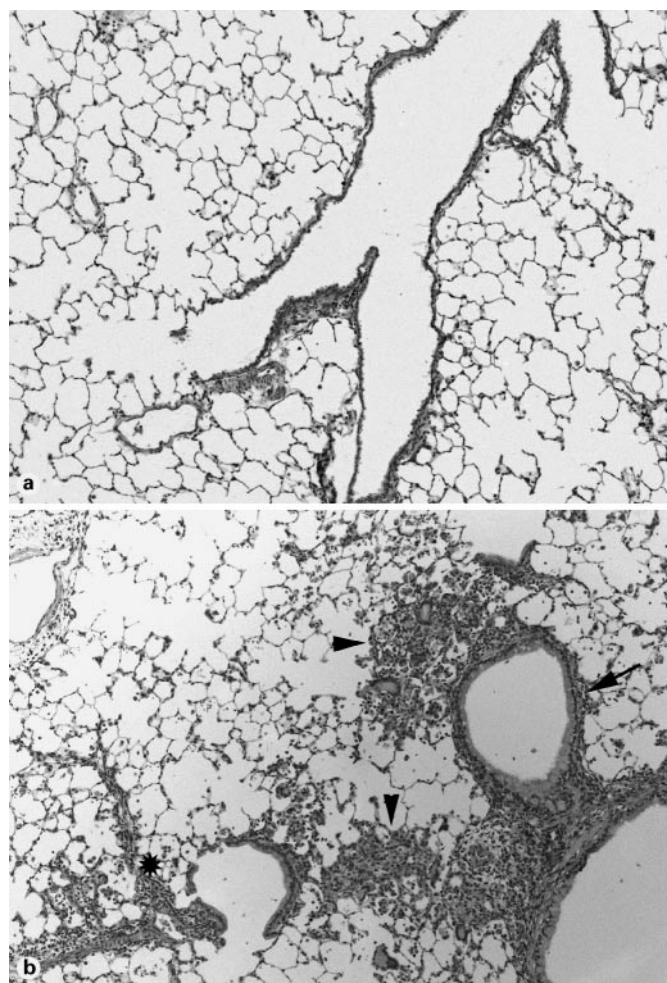


Fig. 4. Histological changes in the lungs. **a** In a lung of a saline-treated BN rat, a bronchiole with an extensive alveolar area is shown. Neither inflammation nor granuloma is present. HE. $\times 150$. **b** Lung of a PCG-treated BN rat. A slight peribronchiolar (arrow) and perivascular (asterisk) infiltrate is present. Some granuloma with giant cells are situated in the alveolar area (arrowhead). The bronchiole has been covered with slightly hypertrophied mucous epithelium. HE. $\times 150$.

Table 1. Histological changes in the lungs of rats exposed to saline or PCG

Changes	Saline	PCG
Peribronchiolar infiltrate	1	1
Perivascular infiltrate	1	2
Hypertrophic bronchiolar mucous cells	1	2
Alveolitis	0	2
Eosinophil influx	0	2
Granuloma	0	2

0 = Absent; 1 = minimal; 2 = slight.

underlay the epithelial bronchial cells and to take up particular antigens in the alveolar space via expansions through the epithelial barrier [23, 24]. Granules are thus quickly taken up by alveolar macrophages and dendritic cells, and do not have time to trigger a local inflammatory response. In contrast, pollen grains intratracheally administered remain in the alveolar space, surrounded by inflammatory cells for several days and trigger much more lesions [21]. So far, most studies showing the presence of pollen allergens at the surface of PCG have used monoclonal antibodies against one or several specific allergens [5, 9–11, 14], which was also demonstrated by the inhibition of a pollen-specific IgE ELISA with timothy grass starch granules [15]. In this work Western blotting was applied to reveal the whole spectrum of pollen allergens

recognized by IgE and IgG1 in the sera of PCG-sensitized rats. We were able to show that all allergens recognized by pollen-sensitized rat IgE are also recognized by IgE from PCG-sensitized rats. The IgG1 spectrum was also similar in pollen-sensitized rats and PCG-sensitized rats.

In conclusion, instillation of PCG in BN rats triggers humoral and cell-mediated responses comparable to those obtained following instillation of intact pollen.

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