

Ability of Pollen Cytoplasmic Granules to Induce Biased Allergic Responses in a Rat Model

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Key Words

Cellular responses • Humoral responses •
Pollen cytoplasmic granules • Timothy grass pollen

Abstract

Background: Grass pollen is one of the most important aeroallergens in Europe. It highly contributes to respiratory allergic diseases, mainly allergic rhinitis. In contact to water or airborne pollutants, pollen grains can release pollen cytoplasmic granules (PCGs) containing allergens. Because of their size ($<5 \mu\text{m}$), PCGs may penetrate deeper into the lungs to induce higher allergic responses, such as asthma. They have been associated with thunderstorm-related asthma. The aim of this study was to evaluate, with Brown Norway rats, the allergenic potential of isolated PCGs and to compare it with the allergenicity of whole timothy grass pollen. **Methods:** Rats were sensitized (day 0) and challenged (day 21), in controlled comparative conditions, with pollen grains (0.5 mg) or PCGs (4.5×10^7 and 0.5 mg). At day 25, blood samples, bronchoalveolar lavage fluid (BALF) and bronchial lymph node were collected. IgE and IgG1 levels in sera were assessed by ELISA. Alveolar cells, protein and cytokine concentrations were quantified in BALF. T cell proliferation, in response to pollen or granules, was performed by lymph node assay. **Results:** The results showed that proliferative

responses of lymph node cells were similar in PCG- and pollen-sensitized rats. IgE and IgG1 levels were higher in pollen- than in PCG-sensitized rats. However, eosinophils, lymphocytes and pro-allergy cytokines in BALF were higher in PCG- than in pollen-sensitized rats. **Conclusions:** Thus, PCGs, able to deeply penetrate in the respiratory tract, induced local and strong allergic and inflammatory responses more linked with asthma- than rhinitis-related allergic symptoms.

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Introduction

Respiratory allergic diseases, such as allergic rhinitis and asthma, are currently a major public health problem worldwide, in particular in industrialized countries. Over the last decades, epidemiological studies have shown that the prevalence of allergic diseases has dramatically increased, especially in the younger generations and in people living in urban areas [1].

These atopic diseases are complex immune and inflammatory disorders influenced by both genetic and environmental factors, among which pollen is quantitatively one of the most important aeroallergens. However, although the symptoms associated with these disorders coincide with the pollination season, it is now well estab-

lished that a direct relationship does not exist between these two phenomena and that many other factors must be taken into account.

In parallel, several studies have shown that after light rainfall or thunderstorm, a single pollen grain can release 700–1,000 small particles called pollen cytoplasmic granules (PCGs) [2]. PCGs consist of starch granules and polysaccharide-particles [3]. In fact, starch granules have been isolated from the Melbourne atmosphere with 10- to 50-fold increase following rainfall [4, 5]. Furthermore, our team has previously shown that after exposure to gaseous pollutants such as ozone or nitrogen dioxide, pollen grains release PCGs [6]. The likelihood that whole grass pollen grains (15–60 µm) will reach the deeper human airways is low, but PCGs (<5 µm) might penetrate into the lower respiratory tract and could induce symptoms of bronchial asthma [7, 8]. Several grass pollen allergens have been found in PCGs by immunogold labeling and/or Western blot studies, such as Lol p 5 and Lol p 9 for rye grass [9], and Phl p 4, Phl p 5 and Phl p 6 for timothy grass [10–12]. As well, small particles derived from birch pollen contain the major allergen Bet v 1 [4].

It has been shown in previous in vivo studies that timothy grass PCGs can induce high humoral and cellular responses in pollen- and PCG-sensitized rodents [13–15]. Moreover, isolated PCGs can elicit a positive skin prick test in asthmatic patients and play a role in 'thunderstorm asthma' either directly [16, 17] or indirectly through interaction with respirable particulate matter present in the atmosphere, such as diesel exhaust particles [18].

The aim of this study was to compare, in clearly defined conditions, the allergenic potential of PCGs and timothy grass pollen. Rats' immunizations performed with isolated size-controlled PCGs and pollen grains were processed in parallel, in the same experimental design. Allergic and inflammatory responses have been assessed following intratracheal instillation of PCGs or whole pollen in Brown Norway rats, a relevant animal model of allergy [19, 20]. Systemic pollen-specific IgE and IgG1 responses were quantified, and the local immune and inflammatory responses were evaluated in bronchial lymph node and bronchoalveolar lavage fluid (BALF) (cytokines and alveolar cells).

Materials and Methods

Animals

Six-week-old male Brown Norway rats were obtained from Charles River Laboratories (L'Arbresle, France). Animals were housed in an air-conditioned room (22°C, 60% relative humidity)

with restricted access. They received conventional laboratory diet and tap water was available ad libitum. They had a light-dark circadian rhythm of 12 h. Their acclimation had begun one week before use in the animal facility. Rats were handled in accordance with French and European State Council guidelines for the care and use as well as ethical approval of laboratory animals (Decree 2001-486,187 and directive 86/609/EEC, respectively). The experiments were approved by the Institutional Animal Care and Use Comity at the INERIS. The rats were randomly divided into 6 groups of 6 rats each.

Pollen and PCGs

Timothy grass pollen (*Phleum pratense*) from the 2006 harvest was obtained from Allerbio AB (Varennes-en-Argonne, France) and immediately after the harvest sent to our laboratory without conservative treatment. Pollen grains were stored at 4°C. Pollen grains were used for both rats' immunization and PCG isolation.

PCGs (0.6–5 µm) were isolated from whole grass pollen following exposure of intact pollen grains to osmotic shock, according to Motta et al. [15] with slight modifications. Timothy grass pollen (500 mg) was suspended in 5 ml distilled water to allow the release of PCGs. After 1 h of gentle agitation, the suspension was centrifuged (10,000 g, 10 min) through a 5-µm mesh filter (Ultrafree-MC; Millipore Corp., Bedford, Mass., USA). PCG pellet was collected and washed twice in 5 ml distilled water and resuspended in 1 ml of saline solution (NaCl 0.9%).

The size and the number of PCGs were assessed with a particle counter (Z3 Multisizer™ Coulter Counter®, Beckman Coulter, Villepinte, France). For pollen grains, a particle counter (Z2™ Coulter Counter®, Beckman Coulter) was used for the size evaluation and the counting.

Endotoxin Assay

The presence or the absence of endotoxins/lipopolysaccharide in pollen extract, PCGs extract and saline solution were determined by Limulus Amebocytes Lysate test (E-Toxate™ Kit; Sigma, St. Louis, Mo., USA).

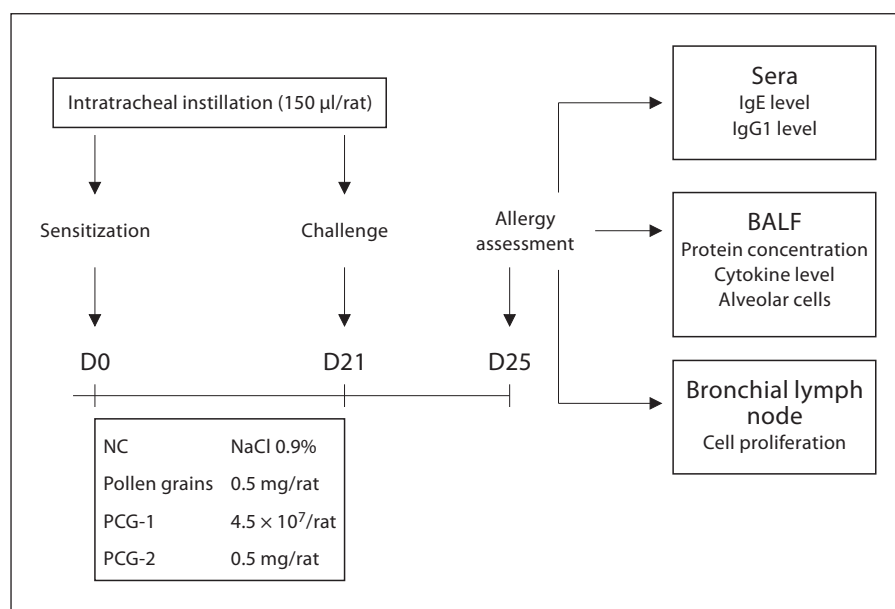
Sensitization and Challenge of the Rats

Animals were anesthetized by an intramuscular injection of a mixture of ketamine hydrochloride (0.5 mg/kg body weight), atropine (0.1 mg/kg body weight) and xylazine (1 mg/kg body weight). Rats were then sensitized on day 0 and challenged on day 21 by intratracheal instillation of 150 µl of aeroallergen (pollen grains or PCGs), which were suspended in saline solution (NaCl 0.9%) and used as allergen sources as described in figure 1. Pollen-sensitized rats correspond to the rats immunized by 0.5 mg of whole pollen grains. Two rats' groups were sensitized with PCGs: PCG-1 and PCG-2. PCG-1 corresponds to the rats immunized with 4.5×10^7 PCGs, corresponding to the number of PCGs released with 0.5 mg of pollen if 100% of pollen burst. The rats of the PCG-2 group were immunized with 0.5 mg of PCGs, containing 9×10^7 PCGs. Negative control (NC) animals received saline solution.

Autopsy and Sampling

At day 25, rats were anaesthetized by intraperitoneal injection of pentobarbital (150 mg/kg body weight). They were exsanguinated by cutting the inferior vena cava after blood taking. Bronchoalveolar lavage was then performed. A cannula was inserted

Fig. 1. Schematic representation of the experimental design. By intratracheal instillation, rats were sensitized at day 0 and challenged at day 21 with pollen grains or PCGs (PCG-1 and PCG-2). Negative control (NC) animals received saline solution. At day 25, humoral and cellular allergic and inflammatory responses were assessed. Total number of rats studied: 24.



in the trachea and the lungs were washed 3 times with 10 ml of phosphate-buffered saline (pH 7.2). Bronchial lymph nodes were removed and immediately plunged in 5 ml of ice-cold RPMI-1640 medium (Invitrogen, Cergy Pontoise, France).

After collection, blood was kept at 4°C for about 2–4 h for clotting. The blood samples were then centrifuged for 10 min at 2,000 g at 4°C. Serum was removed and stored in 500 µl aliquots at –80°C until use.

Fluid collected from the bronchoalveolar lavage of each animal was centrifuged at 150 g for 10 min. Cell-free BALF was concentrated using Amicon Ultra tubes® (Millipore) until the volume reached 1 ml.

Protein Assay

The protein concentrations in water-soluble extracts of pollen grains and PCG suspension and those of BALF were determined by the Lowry method [21]. Bovine serum albumin was used to obtain the calibration curve.

ELISA for Specific Anti-Timothy Pollen IgE and IgG1

Water-soluble pollen extract was prepared according to Mahler et al. [22]. This extract contained together the water-soluble proteins from pollen and PCGs. Pollen extract was labeled with digoxigenin-3-O-succinyl-ε-aminocaproic acid-N-hydroxy-succinimide ester (DIG-NHS) using a DIG protein labeling kit (Roche Diagnostics, Mannheim, Germany). Pollen extract (1 mg/ml) was mixed with DIG-NHS according to the manufacturers' restrictions. Labeled proteins were collected and concentrated using centrifugation (2,000 g, 4°C) in Amicon Ultra tubes® (Millipore) until the volume was equal to 1 ml and stored in 50-µl aliquots at –80°C.

ELISA was performed according to Motta et al. [15]. Briefly, 96-well microtiter plates were coated with 1:500 diluted mouse anti-rat-IgE or anti-rat-IgG1 antibody (Zymed, San Francisco, Calif., USA). After night incubation at room temperature, wells

were washed and saturated with a solution of phosphate-buffered saline containing 1% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20. After 1 h incubation, rat sera were added to the wells (in duplicate) at the proper dilution (1:2 and 1:1,500 for IgE and IgG1 detection, respectively) and incubated for 1 h at 37°C. After washing, wells were incubated with a pollen-DIG solution (dilution 1:800). Thereafter, a horseradish peroxidase-conjugated anti-DIG antibody was added (Roche Diagnostics; dilution 1:625). The amount of IgE or IgG1 was measured by adding peroxidase substrate and the absorbance values were measured at 450 nm with a multichannel photometer (Tecan, Lyon, France).

BALF Alveolar Cells

After centrifugation, cells were suspended in 800 µl RPMI-1640 medium. The collected cells (diameter ranges from 9 to 20 µm) were counted with Z2 Coulter Counter (Beckman Coulter) and applied to a slide by centrifugation using a Shandon Cytospin 2 (Pittsburgh, Pa., USA) at 150 g for 5 min. Cell differential counts were performed after May-Grünwald Giemsa staining and a minimum of 400 cells were counted per slide.

Evaluation of Cytokines in BALF

Cytokines were quantified in BALF using bio-Plex kit for IL-1α, IL-1β, IL-6 and IFN-γ (catalog No. 171K11070; Biorad, Marnes-la-Coquette, France) and multiplex immunoassay kit for IL-5, IL-13 and Rantes (catalog No. RCYTO-80k-04; Linko, Millipore, France) according to the manufacturer's instructions.

Bronchial Lymph Node Cell Assay

The bronchial lymph node assay previously described [15] was slightly modified. Briefly, isolated lymph nodes were forced through a sterile 70-µm mesh nylon cell strainer (Falcon; Becton and Dickinson Company, Franklin Lake, N.J., USA). The collected cells were centrifuged twice at 300 g for 10 min at 4°C. After removing the supernatant, the cell pellet was resuspended in 1 ml

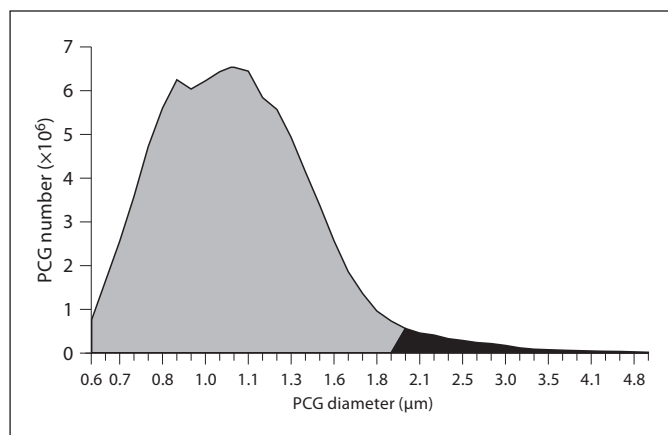


Fig. 2. Particle size distribution of cytoplasmic granules from timothy grass pollen grains was performed by Z3 Multisizer Coulter Counter (Beckman Coulter). The grey area corresponds to PCGs counted between 0.6 and 2 μm . The black area corresponds to the PCGs counted between 2 and 5 μm .

of 10% fetal calf serum medium, and counted with Z2TM Coulter Counter[®]. Cells were incubated in U-bottomed 96-well plates (Cellstar; Greiner bio one, Frickenhausen, Germany), containing 50 μl 10% fetal calf serum medium, 1.5 μM 2 β -mercaptoethanol, with and without allergen sources (pollen: 10 and 100 $\mu\text{g}/\text{ml}$; PCGs: 9×10^5 and 9×10^6 PCGs/ml), for 3 days in humidified atmosphere at 37°C and 5% CO_2 . Then, cells were incubated 24 h with 10 $\mu\text{l}/\text{well}$ [³H]-thymidine (Amersham, Little Chalfont, UK; 37 kBq/ml) and harvested for scintillation counting in a β -plate counter (1205 BetaplateTM; Wallac, Turku, Finland) to measure cell proliferation. T cell proliferation was expressed as counts per minute.

Statistical Analysis

The results of all studied parameters are expressed as means $\pm$ SEM. For statistical analysis nonparametric tests (Kruskal-Wallis and Mann-Whitney) were used. The results were considered significant when a p value <0.05 was found.

Results

PCG Size Distribution

The average diameter of a timothy grass pollen grain, measured by Z2 Coulter, was $31 \pm 2 \mu\text{m}$. By such a counting type, we determined that 1 μg of pollen contained roughly 90 pollen grains and a single pollen grain contains 1,000 PCGs [2], that is, 1 μg of pollen should correspond to 9×10^4 PCGs. However, experimentally, only 2% of PCGs can be released in aqueous solution. The size distribution of PCG, evaluated by Z3 Multisizer (Beck-

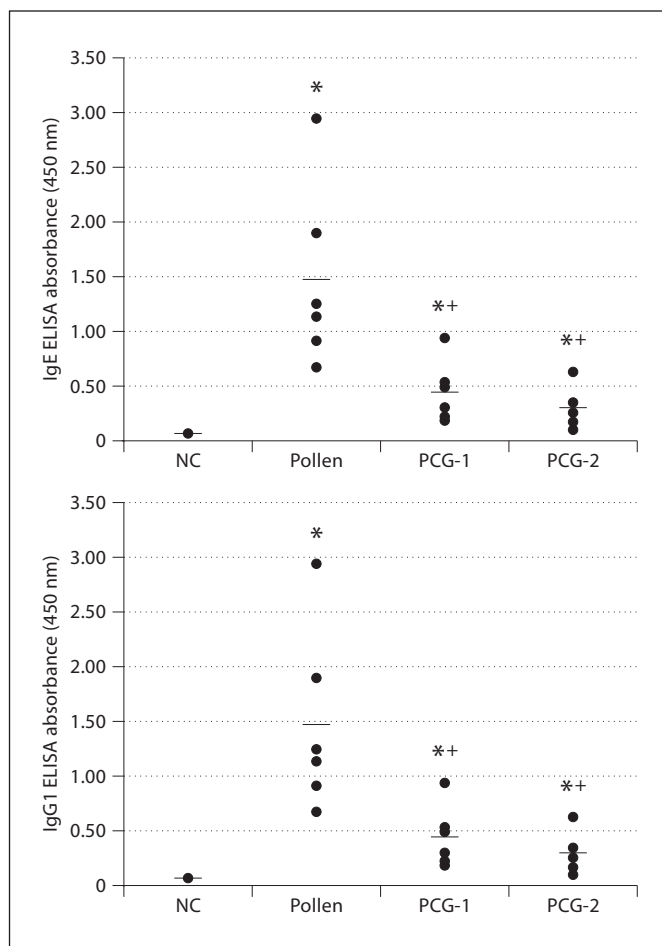


Fig. 3. Timothy grass pollen-specific IgE and IgG1 levels in sera (mean $\pm$ SEM). * Significantly different to NC (p < 0.05); + significantly different to pollen group (p < 0.05).

man Coulter), is reported in figure 2. The average diameter of PCGs was 1.1 μm .

Proteins and Endotoxins in Instilled Solutions

The water-soluble protein concentration in pollen suspension, used for rats' immunization, was 76 $\mu\text{g}/\text{ml}$. For the 2 PCG suspensions used for immunization of rats' groups PCG-1 and PCG-2, the water-soluble protein concentrations were 6.8 ± 0.03 and $3.6 \pm 0.08 \mu\text{g}/\text{ml}$, respectively. After each aqueous wash of PCG, a decrease of the protein concentration in the supernatant was observed. After 5 washes, water-soluble protein concentration was near 0 (data not shown).

Neither PCGs extract nor saline solution contains endotoxins, whereas they are found in pollen extract.

Pollen-Specific IgE and IgG1 Levels in Serum

The IgE and IgG1 levels are presented in figure 3. Sensitization with 0.5 mg of whole pollen generates significant pollen-specific IgE and IgG1 levels. Rats sensitized with 4.5×10^7 and with 0.5 mg of PCGs had higher IgE and IgG1 levels than the NC group. All PCG-sensitized rats produced lower IgE and IgG1 responses than those of the pollen group.

Protein Concentrations in BALF

As an evaluation of in situ inflammation and immune response, protein concentrations were measured in BALF in differentially sensitized animals (fig. 4). Pollen grain- and PCG-sensitized rats showed a significant increase in protein concentrations in BALF. No significant difference was found between protein concentration in BALF from 4.5×10^7 PCG- and pollen-sensitized groups. A two-fold increase in protein concentration was observed in BALF of rats sensitized with 0.5 mg of PCGs compared to the pollen group.

Cytokines in BALF

Several cytokine (IL-1 α , IL-1 β , IL-5, IL-6, IL-13, IFN- γ , Rantes and TNF- α) concentrations were determined in the different rat groups (table 1).

All BALF cytokine concentration values were higher than NC in pollen- and PCG-sensitized rats, but some differences were statistically significant: (1) pollen grains significantly increased IL-6, IFN- γ and TNF- α concentrations; (2) 4.5×10^7 PCGs significantly increased IL-1 β , IL-5, IL-13, Rantes and TNF- α concentrations; (3) all cytokine concentrations increased significantly in 0.5 mg of PCG-sensitized rats.

Compared to pollen-sensitized rats, 4.5×10^7 PCG-sensitized rats showed higher IL-5, IL-13 and Rantes concentrations in BALF, but lower IL-6 and IFN- γ cytokine concentrations. These differences were statistically significant for Rantes only.

For the rats sensitized with 0.5 mg of PCGs, all BALF cytokine concentrations were significantly increased compared to pollen-sensitized rats, except for IL-1 α .

Alveolar Cells in BALF

Pollen-sensitized rats had a significantly higher number of macrophages, eosinophils and neutrophils than NC (table 2). Moreover, the rats' immunization performed with PCGs (4.5×10^7 and 0.5 mg) leads to a significant increase in all alveolar cell types in BALF compared with the NC group.

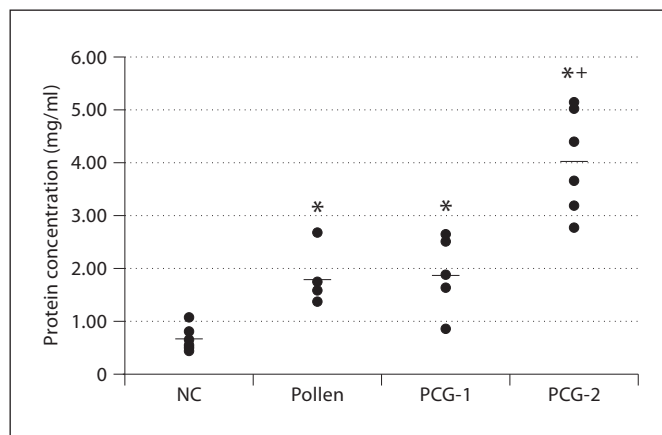


Fig. 4. Protein concentration (mean $\pm$ SEM) in BALF. * Significantly different to NC ($p < 0.05$); + significantly different to pollen group ($p < 0.05$).

Table 1. Cytokine levels in BALF

	NC	Pollen	PCG-1	PCG-2
IL-1 α	4.8 $\pm$ 1.9	8.4 $\pm$ 1.8	9.9 $\pm$ 1.5	12.4 $\pm$ 2.4*
IL-1 β	120 $\pm$ 20	186 $\pm$ 31	257 $\pm$ 40*	554 $\pm$ 62*,**
IL-5	13 $\pm$ 6	142 $\pm$ 51	316 $\pm$ 110*	1,483 $\pm$ 110*,**
IL-6	21 $\pm$ 8	98 $\pm$ 20*	55 $\pm$ 18	670 $\pm$ 95*,**
IL-13	15 $\pm$ 6	43 $\pm$ 10	61 $\pm$ 21*	1,030 $\pm$ 307*,**
IFN- γ	6.9 $\pm$ 5.7	41.8 $\pm$ 11.9*	16.6 $\pm$ 6.0	124 $\pm$ 10*,**
Rantes	6.8 $\pm$ 1.8	9.9 $\pm$ 2.2	22.4 $\pm$ 3.1*,**	273 $\pm$ 58*,**
TNF- α	12 $\pm$ 8	71 $\pm$ 11*	72 $\pm$ 11*	233 $\pm$ 41*,**

The cytokine concentrations are expressed as pg/ml. NC = Negative control group.

* Significantly different to NC ($p < 0.05$); ** significantly different to pollen group ($p < 0.05$).

When compared to pollen-sensitized rats, (1) rats sensitized with 4.5×10^7 PCGs showed a smaller macrophage number and, at the same time, a higher number of eosinophils and lymphocytes, this last one being the only statistically significant number, and (2) the highest dose of PCGs (0.5 mg) induced a significantly higher number of eosinophils and lymphocytes and leads to a smaller number of macrophages (table 2).

Lymph Node Cell Proliferation Induced by Pollen or PCGs

Lymph node cell of pollen- and PCG-sensitized rats showed a greater proliferation than NC after in vitro ad-

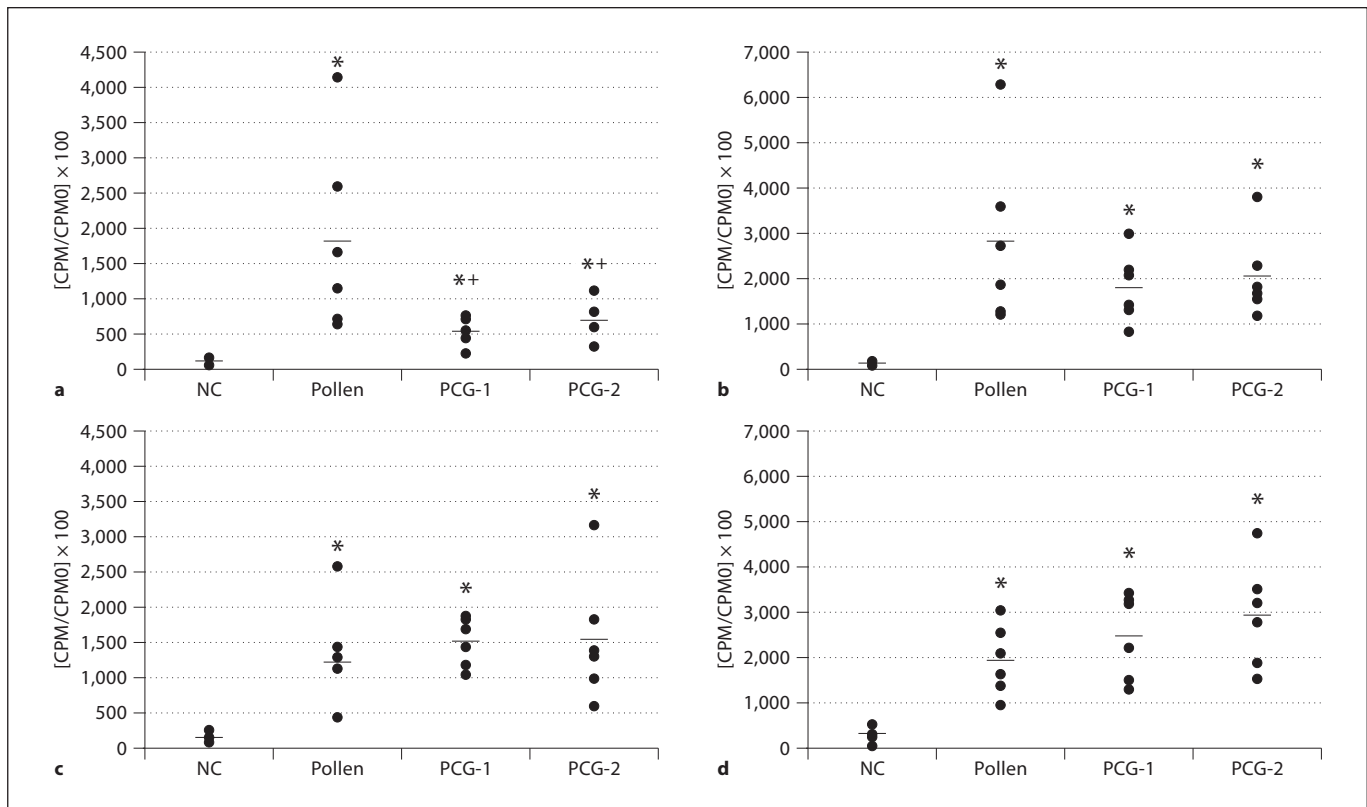


Fig. 5. Pollen- and PCG-induced proliferation of lymph node. Cells from NC, pollen- or PCG-sensitized rats were cultured with [^3H]-thymidine in the presence of pollen (10 and 100 μg , **a** and **b**, respectively) and PCGs (9×10^5 and 9×10^6 PCGs, **c** and **d**, respectively). CPM = Thymidine incorporation by antigen-stimulated cells; CPM0 = thymidine incorporation in control cells. * Significantly different to NC ($p < 0.05$); + significantly different to pollen group ($p < 0.05$).

Table 2. Number and type of alveolar cells in BALF

	NC	Pollen	PCG-1	PCG-2
Macrophages ($\times 10^6$)	3.39 ± 0.44	$7.85 \pm 1.33^*$	$6.95 \pm 0.50^*$	$5.40 \pm 1.58^*$
Eosinophils ($\times 10^6$)	0.40 ± 0.09	$4.00 \pm 0.83^*$	$5.32 \pm 0.49^*$	$7.00 \pm 1.33^{*,**}$
Lymphocytes ($\times 10^6$)	0.21 ± 0.06	0.11 ± 0.09	$0.63 \pm 0.19^{*,**}$	$0.98 \pm 0.21^{*,**}$
Neutrophils ($\times 10^6$)	0.03 ± 0.01	$0.25 \pm 0.11^*$	$0.29 \pm 0.13^*$	$0.36 \pm 0.10^*$

Data are expressed as means $\pm$ SEM. NC = Negative control group.

* Significantly different to NC ($p < 0.05$); ** significantly different to pollen group ($p < 0.05$).

dition of pollen grains (10 and 100 $\mu\text{g}/\text{ml}$, fig. 5a and b, respectively) or PCGs (9×10^5 and 9×10^6 PCGs/ml, fig. 5c and d, respectively).

After in vitro addition of 10 μg of pollen, significantly less proliferation was observed in lymph node cells from animals sensitized to PCGs than in those issued from

pollen-sensitized rats. A slight increase, not significantly different, in the lymph node cell proliferation from pollen-sensitized rats, compared to those from PCG-sensitized animals, was observed after in vitro addition of 100 μg of pollen. Conversely, after in vitro addition of PCGs (9×10^5 and 9×10^6 PCGs/ml), the proliferation of

lymph node cells from PCG-sensitized rats was slightly higher than the proliferation of those from pollen-sensitized rats, but not significantly.

Discussion

In this study, pollen cytoplasmic granules of timothy grass, as well as pollen grains, were shown to induce allergenic and inflammatory responses in Brown Norway rats. PCGs induced lower systemic immune responses than pollen grains, but the local immune and inflammatory responses varied according to the different parameters studied.

For a better comparison with 0.5 mg of whole pollen, used to sensitize rats, two PCG doses were chosen: (1) in terms of quantities, 4.5×10^7 PCGs, which corresponds to the PCGs number released from 0.5 mg of pollen when 100% of pollen grains are broken, and (2) in terms of mass, the same amount as the instilled pollen grains dose (0.5 mg). The immunization route that we used, that is intratracheal instillation, might be considered similar to what happens between humans' tracheal and/or bronchial walls and the aeroallergens (PCGs and pollen grains) because the tracheal diameter is large enough to accommodate whole pollen grains. This method is a simple and rapid technique, allowing the introduction of a controlled dose of the allergenic product. Intratracheal instillation can also minimize risk accidents related to the manipulation of toxic and allergenic products for laboratory workers.

In our study, by a single sensitization and one challenge, PCGs administered by intratracheal instillation can induce an increase in IgE (marker of allergy) and IgG1 (marker of inflammation) levels in sera (systemic responses). In several studies on allergy, it has been shown that allergenic sources such as pollen grains could increase IgE and IgG1 levels in animal sera [19, 23]. Motta et al. [15] also showed an increase in these parameters after a sensitization and two challenges with PCGs from timothy grass pollen administered by intratracheal instillation, but this increase was higher than what has been obtained in the present study. This difference to Motta's work could mainly be explained by the use of an oversensitizing dose due to an underestimation of the PCG number [15].

In our study, PCGs induce lower systemic immune and inflammatory responses than pollen grains. The ratio between IgE levels in PCG-sensitized rats and in pollen-sensitized rats was 33% (fig. 3). This ratio was close to

the humoral responses of Motta's studies [14, 15], but with different experimentation protocols, 2 or 4 challenges for PCG- and pollen-sensitized rats, respectively. These differences in systemic responses, between PCG- and pollen-sensitized rats, could be explained by the quantity and quality of allergens present on isolated PCGs versus the whole pollen ones. In fact, a high amount of water-soluble proteins in pollen grain suspension, higher than those in PCG suspensions, were observed. On the other hand, several grass pollen allergens have been found in PCGs by immunogold labeling and/or Western blot studies, such as Lol p 5 and Lol p 9 for rye grass [9] or Phl p 4, Phl p 5 and Phl p 6 for timothy grass [10–12]. Finally, proteomic analysis, that is, two-dimensional gel electrophoresis followed by mass spectrometry analysis, allowed the detection of Phl p 5a and Phl p 5b in PCG extracts (data not shown).

Beside the systemic responses, different parameters of local immune responses were assessed: (1) pro-allergy cytokine concentrations, (2) eosinophil numbers, (3) lymphocyte numbers in BALF and (4) lymph node cell proliferation. For these four parameters, PCGs have induced similar and/or higher responses than pollen grains.

Concerning the pro-allergy cytokines (IL-5, IL-13 and Rantes), pollen-sensitized animals presented no significant difference from NC, while rats sensitized with 4.5×10^7 PCGs showed increase in the pro-allergy cytokine levels compared to NC and pollen-sensitized rats. The second PCG dose (0.5 mg) drastically increased all pro-allergy cytokine levels compared to whole pollen. In previous human studies, increases in IL-5 and IL-13 concentrations were shown in allergic patients [24, 25]. Asthmatics also presented an increase in the Rantes level in BALF during the pollen season compared to their Rantes level before the pollen season [26]. Moreover, asthmatic patients, but not seasonal allergic rhinitis patients, have increased levels of Rantes in sputum compared to healthy subjects [27].

In our experiments, 4.5×10^7 PCGs and 0.5 mg of PCG doses induced a greater infiltration of eosinophils in lungs than what is obtained with NC and pollen-sensitized rats. However, only 0.5 mg of PCGs showed a significant difference, for eosinophil levels in BALF, with NC and pollen-sensitized rats. The results reported herein are similar to previous studies. Bacsí et al. [13] reported that mice sensitized intraperitoneally with 150 µg of ragweed pollen and challenged intranasally with 25 µg of PCGs showed an accumulation of eosinophils in the peribronchial area. On the other hand, in thunderstorm-related asthma patients, where PCGs are suspected to be a

causative agent, elevated sputum eosinophils were observed [28]. Moreover, a previous study showed that eosinophil number in sputum is significantly increased with asthma severity [29]. Furthermore, the difference in the induction of eosinophils could be partly explained by the presence (in pollen) or absence (in PCGs) of endotoxins in the suspensions used for rats' immunizations. In fact, endotoxins could increase Th1 cytokines, such as IFN- γ , which have an inhibitory effect on the production of Th2 cytokines, such as IL-5 and IL-13, inducers of eosinophil recruitment [30].

In our study, PCG sensitizations induced an increase in lymphocyte number in BALF in contrast to pollen sensitization. In human studies, dense lymphocyte infiltrations were observed in the bronchial walls of patients with severe asthma and in asthma-related deaths [31].

It also has to be noted that, compared to NC, our findings reveal that PCGs and pollen grains induced lymph node cell proliferation *in vitro*. The same types of results were previously obtained by Motta et al. [14, 15]. In our study, however, a slight increase in the lymph node cell proliferation from pollen-sensitized rats, compared with those from PCG-sensitized animals, was observed when we added *in vitro* pollen grains. In the same way, a slight T cell proliferation increase from PCGs-sensitized rats was observed when PCGs were added *in vitro*. These findings suggest, for the first time, the existence of some specific allergenic determinants in each of the allergenic sources, pollen and PCGs. Although PCGs are included in whole pollen grains, when they are isolated, availability and/or relative abundance of specific allergens may differ.

All these local immune responses were stronger in PCG-sensitized rats than in pollen-sensitized rats. Among the 4 parameters, pro-allergy cytokines (mainly Rantes), eosinophils and lymphocytes in BALF showed statistically significant increases. These results suggest that PCGs could induce asthmatic rather than rhinitis symptoms in contrast to pollen. Other experimental data support this hypothesis [32]: by inhalation challenge, pollen grains elicited nasal symptoms and starch granules triggered lower airway inflammation in patients with allergic rhinitis or mild intermittent asthma. In another study, asthmatic volunteers showed a striking bronchial constriction following exposure to starch granules only [17]. Bronchoconstriction was not assessed herein because Brown Norway rats are not a good model for this parameter [33, 34].

Among local inflammatory parameters, protein and pro-inflammatory cytokine (IL-1 α , IL-1 β , IL-6 and IFN-

γ) concentrations, macrophage and neutrophil numbers were studied in BALF. Differences in response between PCG- and pollen-sensitized rats were observed according to the PCG doses used for sensitization. (1) The lowest PCG dose (4.5×10^7 PCGs) induced similar inflammatory responses as pollen grains, except for IL-6 and IFN- γ ; (2) the rats sensitized with the strongest PCG dose (0.5 mg of PCGs) had higher local inflammatory responses than those obtained in pollen-sensitized rats. In mouse model allergy, PCGs and pollen are potent inducers of inflammation in lower airways because of their antigenic content and NAD(P)H oxidase-generated oxidative stress [13, 35]. Moreover, Rylander et al. [36] highlighted the presence of (1 $\rightarrow$ 3)- β -glucan in PCGs and pollen grains, which could explain airways inflammation.

In conclusion, PCGs could induce systemic and local, immune as well as inflammatory, responses in Brown Norway rats. In contrast to pollen-sensitized rats, systemic responses were lower in PCG-sensitized rats, but local cellular responses were higher. The local immune responses suggest that PCGs might play a main role in inducing asthma symptoms in contrast to pollen more involved in rhinitis.

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