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Reactions of Airway Epithelial Cells to Birch Pollen Grains Previously Exposed to In Situ Atmospheric Pb Concentrations: A Preliminary Assay of Allergenicity

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Abstract A growing body of evidence suggests that interactions between pollen grains and environmental pollutants, especially air pollutants, could be of critical importance with regard to the increase in allergic responses observed in the past decades. Using birch pollen grains (BPG), a major allergy source in European countries, and lead (Pb), a highly toxic metal trace element (MTE) present in urban areas, the immune response of human epithelial cells exposed to BPG or to Pb-associated BPG was compared. The cellular response after exposure either to BPG, BPG exposed to 30 mg/L of Pb (BPG-30), or BPG exposed to 60 mg/L of Pb (BPG-60) was evaluated after two time lapses (2 and 6 h) by measuring mRNA levels of four mediators, including two inflammatory (interleukin-8 and interleukin-6) and two allergic (interleukin-5 [IL-5] and interleukin-13) cytokines. After 2 h of exposure, significant upregulation of the IL-5 gene was observed after exposure to BPG-60 in comparison with exposure to BPG and BPG-30 ($N_{IL\text{-}5}$=1.9, Mann–Whitney test, p=0.003). After 6 h of exposure, significant upregulation of the IL-5 gene was observed after exposure to BPG-30 with $N_{IL\text{-}5}$=1.8 and to BPG-60 with $N_{IL\text{-}5}$=2.3 (Mann–Whitney test, p=0.0029) in comparison with exposure to BPG. This first attempt to investigate the influence of pollution by MTE on pollen grain showed a dose–time-dependent increase in IL-5 gene expression after exposure to BPG combined to Pb.

Keywords Airway cells · RT-qPCR · Birch pollen · Pb · Atmospheric Pb exposure · Allergenicity test

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Introduction

Exposure to allergenic pollen is an increasing public health problem in occidental societies where pollinosis has become widespread [1, 2]. Among all the hypotheses raised to explain the increased prevalence of allergic diseases observed in the industrialized world during the past decades, the role of air pollutants and their interaction with pollen grains has become a subject of investigation [3–8].

Metal trace elements (MTE) are predominantly present in urban areas and at industrial sites through wet deposition in damp temperate climates [9]. Lead (Pb) is a predominant MTE from industrial and traffic sources [10, 11]. Traffic-derived Pb is emitted mainly by vehicle exhaust systems, tires, brakes, greases, and oils [12]. MTE can come into contact with and combine with pollen grains, which are biological components able to cover great distances [13] and are present in large quantities in the atmosphere during the pollen season. Consequently, pollen grains collected in polluted areas are generally associated with MTE [14].

The respiratory epithelium is the first tissue to encounter inhaled components and is of critical importance in the development of allergic or inflammatory reactions. Moreover, in

vitro cellular models are known to be particularly helpful in providing a better understanding of pathogenesis. However, there has been no report on the response of human lung epithelial cells after stimulation with MTE-associated pollen grains. Inhaled MTE–pollen grain combinations may have more powerful allergen potential than pollen grains alone because MTE may have intrinsic adjuvant activity when associated with pollen grain [15–17].

The present experiment was made up of two steps. In the first step, Pb-associated birch pollen grains (BPG) were created by exposing collected BPG, which are a major allergy source in European countries [18], to low atmospheric Pb concentrations to replicate in situ conditions. The second step investigated airway epithelial cell responses after stimulation with BPG and Pb-associated BPG by quantifying the mRNA of inflammatory (interleukin-8 [IL-8] and interleukin-6 [IL-6]) and allergic (interleukin-5 [IL-5] and interleukin-13 [IL-13]) mediators using reverse transcription quantitative polymerase chain reaction (PCR).

Methods

Pollen Grains Collection

Pollen grains used in the experiments were freshly collected from pollinating *Betula pubescens* (white birch). Collection took place in a French village (Frasne, 46°51′23″ N, 6°09′37″ E; mean altitude of 846 m) of 1,700 inhabitants in a temperate climate (under the double influence of oceanic and continental climates with a mean annual temperature of 10.2 °C) with abundant precipitations (1,110 mm/year, with a national mean of 770 mm/year), at 500 m from a road with car traffic below 1,600 vehicles per 24 h. After collection, pollens were separated from the flowers by sieving. Following separation, samples were stored unwashed in glass bottles in the dark.

Pb-Associated Pollen Grains

To investigate the effects of Pb-associated pollen on airway cells, BPG were placed in an experimental device simulating wet atmospheric Pb pollution. The pollen mass used in each exposure weighs 1 g, divided into three subsamples of 0.3 g to make three replicates for measuring Pb concentration. The experimental device used is somewhat similar to the one presented by Desalme et al. [19] but was adapted to create polluted mist. It consisted of one cylindrical nebulization chamber of 15 dm^3 (Altuglass®; 20 cm in diameter, 50 cm high). The air entered the exposure chamber at the top and passed through a nebulizer where the air and liquid combined to make a fog. Nebulization and dispersion of a solution inside the hermetic chamber were done using a nebulizer adapted to contain a liquid (10 mL/h), a pump (KNF Neuberger, Village Neuf, France; N811KN.18, 11.5 L/min), and an electric fan. The solution used was $PbCl_2$ dissolved by ultrasound in pure water. BPG were exposed for 1 h to this solution. The Pb concentrations used were 30 and 60 mg/L of Pb. The choice of these concentrations was made according to concentrations measured in BPG collected in situ <50 m from roads with moderate to heavy traffic (2,000 and 44,600 vehicles per day, respectively) in a medium-sized city with a population density of 1,800 people per square kilometer. This city (Besançon, located in eastern France) was chosen because its air quality is below legislative norms, polluting industries are few, and the main air pollutants are from traffic vehicles [8]. The residual chloride concentrations were checked and were negligible.

Detection of Pb on Pollen Grains

After exposure to controlled conditions, three subsamples of 0.3 g of pollen were removed from each sample to make three replicates. Each subsample was treated with 65 % HNO_3 (RPE Carlo Erba), placed in a mineralizer (80 °C for 45 min and 120 °C for 120 min), and diluted to obtain a final volume of 10 mL. Pb-associated pollen grains were determined with an atomic absorption spectrometer (AAS Varian) that was equipped with a graphite oven atomization (method of additional calibration). Each measurement was repeated three times. Pollen-free blanks were systematically mineralized and measured every 15 samples. The resulting concentrations were negligible for Pb with regards to the total amount (0.06 ppm) and undetected for other MTE from urban traffic (cadmium, nickel, and zinc).

Airway Cell Pollen Exposure

The quantity of BPG given to airway cells was calculated, taking into account the following criteria: the number of confluent cells per well (2×10^6 cells), cell size (5 μm), the mean surface area of respiratory epithelium in an adult (≈150 m^2), size of BPG (27 μm), the quantity of inhaled air for an adult at rest (10 L/min), and the quantity of BPG triggering an allergic reaction (100 BPG/m^3/day). A dose of 1,000 BPG/mL was used for exposure, which reflects the quantity of BPG that could be inhaled per day by an adult participating in a moderately intense activity during a high peak of birch pollen. Solutions containing 1,000 BPG/mL in culture media were prepared for BPG, BPG exposed to 30 mg/mL of Pb (BPG-30), and BPG exposed to 60 mg/mL of Pb (BPG-60).

Cell Lines

The alveolar epithelial cell line A549 (DSMZ, Braunschweig, Germany) was cultured as described previously [20]. Confluent cells (about 2×10^6 cells per well) were inoculated with 1,000 BPG/mL either of BPG, BPG-30, or BPG-60. Each

Table 1 Primer sequences used in this study

	Sense primers	Antisense primers
P0	GGC GAC CTG GAA GTC CAA CT	CCA TCA GCA CCA CAG CCT TC
TBP	TGC ACA GGA GCC AAG AGT GAA	CAC ATC ACA GCT CCC CAC CA
IL-8	CAC CGG AAG GAA CCA TCT CAC TGT	TCC TTG GCA AAA CTG CAC CTT CA
IL-6	CAA TCT GGA TTC AAT GAG GAG AC	CTC TGG CTT GTT CCT CAC TAC TC
IL-5	GAA CTC TGC TGA TAG CCA ATG AGA C	CTC CAG TGT GCC TAT TCC CTG A
IL-13	ATC ACC CAG AAC CAG AAG GCT C	GAT TCC AGG GCT GCA CAG TAC A

experiment was performed three times. To investigate the early immune reaction, cell exposure was terminated after 2 and 6 h.

RNA Quantification

RNA was extracted using the RNA MagNa Pure Compact Isolation Kit (Roche Diagnostics®, Meylan, France). Reverse transcription was carried out as previously described [20]. Sterile water was used to dilute the cDNA (1/20), which was then stored at −20 °C until amplification.

The primers used are listed in Table 1. Real-time PCR was carried out as previously described [21]. Quantitative values were obtained from the cycle threshold (C_t) number. Samples from three separate experiments were analyzed in duplicate. Each sample was normalized on the basis of its content compared with the reference gene, P0, also known as 36B4 (GenBank accession no. NM001002) and encodes acid ribosomal phosphoproteins. The results, expressed as the *N*-fold difference in target gene expression relative to P0 (termed N_{target}), were determined according to the following formula: $N_{target} = 2^{\Delta C_t \text{ sample}}$.

IL-5 Dosage in Cell Supernatants

To determine if synthesized IL-5 was released into the culture supernatant of cells exposed to BPG-30 and BPG-60, IL-5 levels were evaluated using a specific enzyme-linked immunosorbent assay (ELISA) kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Each sample was measured in duplicate. The detection limit of the assay was 3 pg/mL.

Statistical Analysis

Data are presented as the means±SEM from three separate experiments. Statistical analyses were performed using XLSTAT 2010. Differences were considered statistically significant for $p<0.05$. Nonparametric tests (a Kruskal–Wallis test followed by a Mann–Whitney test) were used to detect significant variations in the quantification of mRNA levels.

Results

Pb Quantification in Collected Pollen Grains

BPG collected in rural areas, before exposure, contained a maximum of 0.2 mg/kg of Pb (mean, 0.1 mg/kg). Successful combination of BPG and Pb was also checked and Pb concentrations in Pb-associated pollen grains were related to Pb concentrations used for exposure: mean of 2.4 mg/kg of Pb associated with pollen grains exposed to 30 mg/L and mean of 4.4 mg/kg of Pb associated with pollen grains exposed to 60 mg/L (Table 2).

Relative mRNA Quantification

After 2 h of exposure, significant upregulation of the IL-5 gene was observed after exposure to BPG-60 in comparison with exposure to BPG and BPG-30 ($N_{IL\text{-}5}=1.9$, Mann–Whitney test, $p=0.003$; Fig. 1).

After 6 h of exposure, significant upregulation of the IL-5 gene was observed after exposure to BPG-30 with $N_{IL\text{-}5}=1.8$ and to BPG-60 with $N_{IL\text{-}5}=2.3$ (Mann–Whitney test, $p=0.0029$) in comparison with exposure to BPG (Fig. 2).

In contrast, no significant difference in mRNA levels was observed for the other three mediators examined (IL-8, IL-6,

Table 2 Pb concentrations in Pb-associated pollen grains

[Pb] of exposition (mg/L)	[Pb] in Pb-associated pollen grains (mg/kg)	Mean of [Pb] (mg/kg)	Standard deviation (mg/kg)
0	0.2	0.1	0.1
0	0.0		
0	0.0		
30	2.9	2.4	0.5
30	2.3		
30	2.0		
60	3.9	4.4	0.7
60	4.0		
60	5.2		

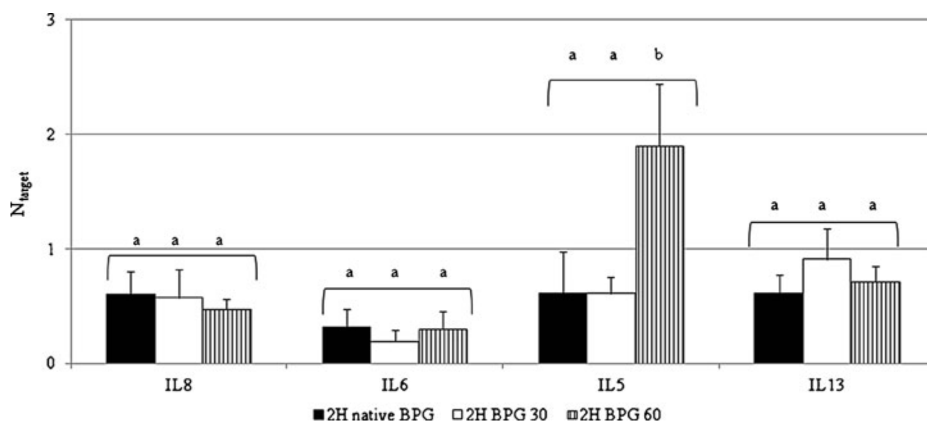

Fig. 1 Standardized mRNA expression from A549 cells of the genes encoding IL-8, IL-6, IL-5, and IL-13 after 2 h of exposure to BPG, BPG-30, or BPG-60. The results were calculated using the ΔC_t method with two reference genes (P0). Data are presented as the means ±SEM. *a* and *a* no significant difference ($p>0.05$), *a* and *b* significant difference ($p<0.05$) [Mann–Whitney test for each interleukin (n per test=9)]

and IL-13) after exposure to BPG, BPG-30, or BPG-60 (Figs. 1 and 2).

Concentrations of Synthesized IL-5 in Cell Supernatants

Synthesized IL-5 was not detected in the cell supernatants after exposure to BPG, BPG-30, or BPG-60.

Discussion

This preliminary assay was intended to evaluate the influence of Pb on the allergenicity of pollen grains. We observed an increase in IL-5 gene expression after exposure to BPG-30 or BPG-60 that was dose and time dependent.

The cytokine IL-5 promotes eosinophil migration and proliferation and is characterized as a Th2-type cytokine involved in allergic reactions [22, 23]. The dose–time-dependent upregulation of the IL-5 gene observed in these experiments suggests that the presence of Pb particles may increase the allergic response, but only moderately. The fact that in situ conditions were respected may explain the weak variations observed: (1) given the size difference between whole BPGs (27 µm) and epithelial cells (5 µm), it is possible that contact was not optimal and (2) BPGs were exposed to relatively low Pb concentrations. That the IL-5 protein could not be detected in cell supernatants using a specific ELISA assay, whereas a significant increase in the amount of IL-5 mRNA was detected, may indicate a lack of sensitivity in these assays, either on the part of the ELISA kit or improper folding incomplete spatial conformation of the IL-5 protein produced by A549 cells, resulting in the absence of recognition by specific IL-5 antibodies.

As pollen grains release substances structurally similar to inflammatory lipid mediators [15, 24] and the upregulation of IL-8 and IL-6 gene expression is often observed in innate immune reactions of A549 cells [20, 21], the absence of an inflammatory response in the present experiment was surprising. Indeed, the fold change of IL-8 and IL-6 mRNA levels compared to our reference genes are clearly under the control value of 1 during the first 2 h postexposure. Thus, we can hypothesize that Pb may have an inhibitory effect on the inflammatory ability of BPG, as was previously described for sulfur dioxide [15]. This decreased inflammatory response could be due either to the effect of Pb on cellular cytokine production or to modified BPG allergenicity because of the presence of Pb.

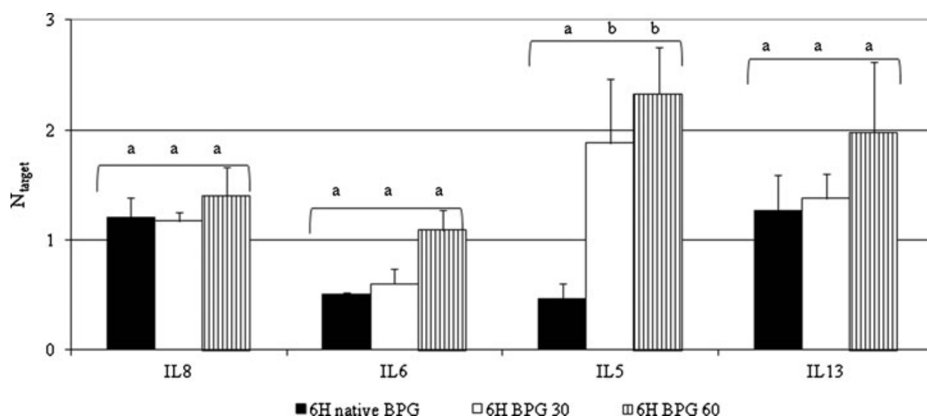

Fig. 2 Standardized mRNA expression from A549 cells of genes encoding IL-8, IL-6, IL-5, and IL-13 after 6 h of exposure to BPG, BPG-30, and BPG-60. The results were calculated using the ΔC_t method with two reference genes (P0). Data are presented as the means ±SEM. *a* and *a* no significant difference ($p>0.05$), *a* and *b* significant difference ($p<0.05$) [Mann–Whitney test for each interleukin (n per test=9)]

Conclusions

This first attempt to investigate the influence of pollution by MTE on pollen grain showed a dose–time-dependent increase in IL-5 gene expression after exposure to BPG combined to Pb. The fact that probably only fragments of pollens–MTE combinations actually reach the epithelial respiratory cells as well as the uncertainty about quantity and time of exposure illustrate the limitations of this study and the complexity to mimic in situ conditions.

Conflict of Interest The authors declare no conflict of interest.

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