

Effects of birch pollen and traffic particulate matter on Th2 cytokines, immunoglobulin E levels and bronchial hyper-responsiveness in mice

E. Fernvik, G. Peltre*, H. Sénéchal* and B. B. Vargaftig

Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur-INSERM U485, *Unité d'Immuno-Allergie, Institut Pasteur, 25 rue du Dr Roux, 75015, Paris, France

Summary

Background Health effects due to air pollution arising from motor vehicles are a major public and political concern world-wide. Epidemiological studies have shown that the manifestations of asthma are increased by air pollution in already affected individuals.

Objective To investigate the potential role of air-polluted tunnel dust (traffic particulate matter, TPM) or pure carbon core particles in the initiation and persistence of experimental allergic inflammation.

Methods BP2 mice were immunized with birch pollen alone (group B) or pollen together with TPM (group A), or with birch pollen and $Al(OH)_3$ (group C), or with birch pollen and carbon core particles (group D). Before methacholine challenge they were challenged intranasally and thereafter bronchial hyper-reactivity (BHR) was evaluated in a whole-body plethysmograph. Levels of Th2 cytokines, fibronectin and lactate dehydrogenase (LDH) were determined, and differential counts were performed in the bronchoalveolar lavage (BAL) fluid. Sera were collected for determination of antibody titres and cytokine levels.

Results Specific IgE titres, BHR, the number of recruited eosinophils and levels of fibronectin and LDH in BAL were increased in mice immunized and challenged with a mixture of birch pollen and TPM. However, mice immunized with birch pollen alone and challenged intranasally with pollen or a mixture of pollen and TPM demonstrated the highest levels of IL-4 and IL-5.

Conclusion This study highlights the importance of the exposure to a combination of particulate matters and pollen allergens, in the induction of allergic disease in the airways, and we have demonstrated that polluted tunnel dust has an effect on both the inflammatory and immunological components of experimental allergy. Immunization and challenge with carbon core particles together with birch pollen increased neither the BHR nor the specific IgE production significantly. Our results therefore strongly suggest that it is most likely to be the organic phase bound to the carbon core of the diesel exhaust particles that might have an important adjuvant effect in the induction of experimental allergy.

Keywords air polluted dust, BHR, birch pollen, BP2 mice, diesel, IgE

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Introduction

Health effects due to air pollution arising from motor vehicles are a major public and political concern world-wide. The role of air pollution in the initiation of asthma is controversial. One hypothesis is, however, that air pollution particles can amplify the pulmonary inflammation associated with different lung diseases. Air pollution has been linked epidemiologically to increased manifestation of signs and symptoms in individuals with established asthma [1].

Asthma often begins in early childhood. Neonatal subjects, however, have an immature immune system and are

predisposed to immune tolerance after repeated exposure to allergen [2,3]. One way of interpreting this observation is that developing individuals need exposure to both allergen and some adjuvant-like cofactors to develop sensitization to allergens.

In Switzerland, the prevalence of hayfever rose from 4.8% in 1958 to 10.0% in 1986 [4], and has recently been demonstrated to have further increased to 11 to 12% [5]. In Sweden, a study in military conscripts has demonstrated an increase in allergic rhinitis from 4.9% in 1971 to 8.4% in 1981 [6]. A change in genetic predisposition cannot explain this rapid increase in atopic disease because genetic changes in any population take thousands of years [7]. It is therefore obvious that environmental factors (e.g. air pollution) are of great importance for the development of atopic disease. Another important factor, which is implicated in the increase of respiratory allergic diseases, is the relative absence of childhood infections and endotoxins [8,9].

Correspondence: B. Boris Vargaftig, Unité de Pharmacologie Cellulaire, Institut Pasteur, 25, rue du Dr Roux, 75015 Paris, France. E-mail: vargafti@pasteur.fr

The epithelial cells in the airways and macrophages are the first cells to come into contact with inhaled particles. In response to those particles, the alveolar macrophages can release a large number of pro-inflammatory mediators, which help to initiate and maintain an acute inflammatory response in the airways. In addition to this initiation, several studies have also demonstrated a role for diesel exhaust particles in enhancing the allergic inflammatory response. Nasal instillation of diesel particles in atopic subjects has been shown to produce a 25-fold increase in IgE mRNA levels, and a 5-fold increase in IgE levels in nasal lavage [10]. Co-instillation of diesel particles and allergen in patients with atopic rhinitis led to a 50-fold increase in allergen-specific IgE levels, as compared with instillation of either allergen or particles alone.

A possible adjuvant effect of the diesel particles can result from their interaction with aeroallergens. Indeed, purified grass pollen allergen has been shown to bind specifically to diesel particles when incubated in a fluid phase *in vitro*. This probably allows the allergens to concentrate onto the surface of the particles and facilitate their presentation to the immune system. These particles may thereafter serve as a carrier for allergens into the respiratory tract [11,12].

The purpose of this study was to investigate whether air polluted dust (traffic particulate matter, TPM) from a heavy car traffic tunnel increases bronchial hyper-responsiveness and titres of IgE antibodies in mice, when used for immunization together with birch pollen. We also wanted to determine whether carbon core particles in themselves could give rise to an enhanced hyper-reactivity in mice immunized and provoked with birch pollen, as diesel particles are complex particles that consist of a non-extractable carbon core with adsorbed chemical substances. The study was performed using a model for allergenic symptoms in a murine selection called BP2 [13], which expresses intensively the different sequelae of asthma after allergenic immunization and provocation [14].

Materials and methods

Birch pollen and traffic particulate matter

Whole birch pollen (Allergon, Pharmacia, Sweden) was used for immunization and challenge. TPM was collected in a ventilation shaft, outside the pollen season (January 1998), in a heavy car traffic tunnel in Prague. The dust was sterilized by gamma-radiation, sieved and then twice milled in a jet mill for 48 h. Pollen and TPM preparations were diluted in saline solution and mixed together for 5 min before use for immunization or challenge.

Preparation of carbon core particles

To determine the influence of the organic compounds adsorbed onto the TPM, a clean model soot aerosol was used. This carbon aerosol was produced by high voltage discharge between two very pure carbon electrodes in an argon atmosphere (soot generator GfG 1000, Palas, Karlsruhe, Germany). The initial particles have a diameter of about 5 nm but agglomerate fast to form larger particles. This soot has a specific surface of 395 m²/g, which is about 10 times more than that of diesel soot [15]. These

particles are pure carbon particles prepared by the Institut für Wasserchemie (Munich, Germany) to mimic the carbon core of diesel particles, and we will refer to them as 'carbon core particles' in the article.

Mice and immunization

Male mice, aged 6 to 7 weeks, of the BP2 selection were used (Centre d'Elevage R. Janvier, BP5, 53940 Le Genest Saint-Isle, France). This mouse selection was used because bronchial hyper-reactivity (BHR) is easily obtained after a single allergenic provocation.

Group A: mice were injected with a mixture of birch pollen (100 µg/mouse) and TPM (4 mg/mouse).

Group B: mice were injected with birch pollen alone (100 µg/mouse).

Group C: mice were injected with a mixture of birch pollen (100 µg/mouse) and Al(OH)₃ (1.6 mg/mouse).

Group D: mice were injected with a mixture of birch pollen (100 µg/mouse) and carbon core particles (1.3 mg/mouse).

As controls, mice were immunized with TPM alone or saline solution.

All injections were made subcutaneously, in the tissue at the base of the neck, with 400 µL of the respective suspension, on days 0, 21 and 35.

Challenge

Group A–C: 1 week after the last immunization (day 42) the mice were lightly anaesthetized (i.v.) with 150–200 µL Immalgene (0.5 mg/mL) and challenged intranasally with 50 µL of either birch pollen alone (100 µg/mouse), birch pollen together with TPM (0.5 mg/mouse) or with TPM alone.

Group D: The mice were challenged intranasally with 50 µL of either birch pollen alone (100 µg/mouse), birch pollen together with carbon core particles (0.2 mg/mouse), or with carbon core particles alone.

The mice were then left for 24 h before the methacholine provocation.

The immunization and challenge protocol is summarized in Table 1.

Table 1. Treatment protocol for the different groups of mice. TPM = traffic particulate matter

Group	Immunization (subcutaneously)	Provocation (intranasal)
A	Birch pollen + TPM	Birch pollen TPM Birch pollen + TPM
B	Birch pollen	Birch pollen TPM Birch pollen + TPM
C	Birch pollen + Al(OH) ₃	Birch pollen TPM Birch pollen + TPM
D	Birch pollen + carbon core particles	Birch pollen Carbon core particles Birch pollen + carbon core particles

Determination of the respiratory function

The BHR was measured 24 h after the intranasal challenge. Pulmonary function was evaluated in a whole-body plethysmograph using enhanced pause (Penh) in response to aerosolized methacholine, as an indicator of airflow obstruction.

Mice were placed in the plethysmographic chamber (Buxco Electronics, Sharon, CT, USA) and after a few minutes of air pressure stabilization an aerosol of methacholine (50 mM) was delivered during 20 s. The airway resistance was recorded for 12 min and is expressed as $\text{Penh} = [\text{Te (expiratory time)} / 40\% \text{ of Tr (relaxation time)} - 1] \times \text{Pef (peak expiratory flow)} / \text{Pif (peak inspiratory flow)} \times 0.67$, according to recommendations from the manufacturer. The Penh-values were used to construct a curve for each group of mice, thereafter the area under each curve was calculated (AUC) with a standard computer program.

Blood collection

Immediately after measuring bronchial hyper-reactivity, blood was drawn from the vena cava of the anaesthetized mice (urethane, 2 g/kg, i.p.). The blood was left in plastic tubes (Sarstedt, Orsay, France) to coagulate and was thereafter centrifuged at 1000 g for 10 min. The sera were stored at -20°C .

Bronchoalveolar lavage

The trachea was cannulated after blood collection. Lungs were lavaged in two successive steps using first 0.5 mL saline solution and thereafter 1 mL. The lavage fluid was harvested by gentle aspiration. Cells were counted using a Coulter Counter ZM (Coultronics, Margency, France). Differential cell counts were performed after cyto-centrifugation (Hettich Universal, Tuttlingen, Germany) and staining with Diff-Quik stain (Baxter Dade AG, Duedingen, Switzerland). A total of 200 cells were counted under oil immersion microscopy. The remains of the lavage fluid were centrifuged at 200 g for 10 min, at 4°C , and the supernatants were collected. They were either used directly for determination of the lactate hydrogenase (LDH) content (a marker of damaged epithelium) or stored at -20°C for later determination of IL-4 and IL-5.

After exsanguination (the lungs were first washed with 5 mL Isoton injected into the heart) the lungs were removed and stored at -20°C , for not more than 24 h, for the determination of eosinophil peroxidase (EPO).

Determination of IL-4 and IL-5

Levels of IL-4 were evaluated in BAL fluid with an enzyme-linked immunosorbent assay technique. Briefly, a 96-well plate was coated with rat anti-mouse IL-4 antibodies (Endogen, Woburn, MA, USA), to which were added dilutions of a mrIL-4 standard (7–1000 pg/mL) or the sample, followed by a biotinylated rat anti-IL-4 antibody (Endogen). The reaction was revealed using a substrate containing *o*-phenylenediamine dihydrochloride and read at 490 nm.

Levels of IL-5 were measured in both serum and BAL fluid using an immunometric method. Briefly, a 96-well plate was coated with a rat anti-mouse IL-5 (TRFK-4), to which were added dilutions of an IL-5 standard (7.8–1000 pg/mL) or the sample, followed by an esterasecholine-labelled tracer, rat anti-mouse IL-5 antibody (TRFK-5). The reaction was revealed

with Ellman's solution and read at 405 nm. The lower limit of detection of both assays was approximately 5 pg/mL of the sample.

Determination of LDH

The levels of LDH were measured in BAL fluid according to the manufacturer (Roche, Germany). The basis of the test is that L-lactate and NAD^{+} are formed from pyruvate, NADH and hydrogen ions when LDH is present after leakage from damaged cells. The reaction was measured as OD at 340 nm and is evaluated in arbitrary units.

Determination of fibronectin

The content of fibronectin in BAL fluid was measured by an ELISA procedure [16,17]. Briefly, a 96-well plate was coated overnight at 4°C with a solution of 300 ng fibronectin per well (ANAWA, Wangen, Switzerland). Undiluted BAL samples and dilutions of a fibronectin standard (0.078–10 µg/mL) were incubated overnight at 4°C with a rabbit-antifibronectin antibody (ANAWA), and thereafter transferred to the fibronectin coated wells. After washing the wells, the antibodies that had not reacted with fibronectin in the BAL samples were revealed by adding sequentially a biotinylated secondary anti-rabbit antibody (ANAWA) and streptavidin (Sigma, St Louis, MO, USA). The TMB (tetramethylbenzidine) microwell peroxidase substrate (KPL, USA) was added for 15 min. The reaction was thereafter stopped with 20% H_2SO_4 , and measured at 450 nm.

As controls a group of non-immunized mice ($n=8$) was provoked with saline solution and challenged with methacholine.

Measurement of serum IgE titres

The specific IgE antibody titres were determined by passive cutaneous anaphylaxis (PCA) in rats [18]. Briefly, diluted mice sera (1:10 to 1:640) were inoculated intradermally on the shaved back of WISTAR rats. Twenty hours later each rat was challenged by an intravenous injection in the tail of a soluble extract of birch pollen (final concentration 2 mg/mL) mixed with Evans blue (0.5%). Thirty minutes later the rats were killed and the reactions were evaluated. The results are expressed as IgE titres, according to the highest dilution of the sera that gave a positive reaction.

Determination of EPO activity

The activity of EPO in the lung tissue was determined as described earlier by Van Oosterhout et al. [19]. Briefly, the washed and frozen lungs were ground in a buffer consisting of Tris-HCl 0.05 M, pH 8.0. After centrifugation the EPO activity of the supernatants was measured in a 96-well plate. Aminotriazole (Sigma) was added to exclude an eventual interference of haemoglobin. The reaction was revealed using a substrate containing *o*-phenylenediamine dihydrochloride and the OD was read at 490 nm.

Statistical analysis

The results are expressed as mean $\pm$ SEM. For statistical analysis the SSPS program was used and data were analysed with one-way ANOVA, $P < 0.05$.

Table 2. Levels of IL-4 and IL-5 in bronchoalveolar lavage (BAL) fluid and serum. The substances used for immunization are indicated within brackets. Values are expressed as mean $\pm$ SEM, $n = 6$. TPM = traffic particulate matter

	IL-4 BAL (pg/mL)	IL-5 BAL (pg/mL)	IL-5 Serum (pg/mL)
Group A (pollen + TPM)			
Provoked with:			
Pollen	1637 $\pm$ 122	207 $\pm$ 40	72 $\pm$ 19
TPM	126 $\pm$ 52	169 $\pm$ 39	106 $\pm$ 21
Pollen and TPM	1295 $\pm$ 231	214 $\pm$ 58	232 $\pm$ 117
Group B (pollen)			
Provoked with:			
Pollen	1604 $\pm$ 104	623 $\pm$ 227*	363 $\pm$ 86*
TPM	75 $\pm$ 49	31 $\pm$ 7	29 $\pm$ 4
Pollen and TPM	1803 $\pm$ 24	1568 $\pm$ 103*	722 $\pm$ 61*
Group C (pollen + Al(OH) ₃)			
Provoked with:			
Pollen	1370 $\pm$ 141	509 $\pm$ 107	224 $\pm$ 44
TPM	27 $\pm$ 14	26 $\pm$ 6	25 $\pm$ 4
Pollen and TPM	1565 $\pm$ 106	390 $\pm$ 93	200 $\pm$ 27
Group D (Pollen + Carbon core particles)			
Provoked with:			
Pollen	1323 $\pm$ 88	230 $\pm$ 65	145 $\pm$ 30
Carbon core particles	71 $\pm$ 28	19 $\pm$ 5	60 $\pm$ 39
Pollen and carbon core particles	1161 $\pm$ 149	161 $\pm$ 34	137 $\pm$ 32

* $P < 0.05$ compared with group A and with mice in group B provoked with TPM alone.

Results

Th2 cytokine levels in BAL fluid and serum

The levels of IL-5 were measured in both serum and BAL fluid, and those of IL-4 in BAL fluid only (as there is normally no IL-4 in serum) (Table 2). Mice immunized with pollen alone (group B) showed the highest levels of IL-5, both in serum and in BAL fluid. The values differed, however, within the group depending on the substance used for challenge. Mice provoked with a mixture of pollen and TPM showed the highest levels (1568 $\pm$ 103 pg/mL in BAL fluid and 722 $\pm$ 61 pg/mL in serum), followed by mice challenged with pollen alone (623 $\pm$ 227 pg/mL in BAL fluid and 363 $\pm$ 86 pg/mL in serum). The levels in mice provoked with TPM alone were significantly lower ($P < 0.05$) both in BAL fluid (31 $\pm$ 7 pg/mL) and in sera (29 $\pm$ 4 pg/mL). The IL-5 levels in group B, in both BAL fluid and serum, were significantly higher ($P < 0.05$) than those of group A (when mice were challenged with pollen or pollen together with TPM). The levels of IL-5 in mice immunized with pollen and TPM (group A) varied between 69 $\pm$ 39 and 214 $\pm$ 58 pg/mL in BAL-fluid, and between 72 $\pm$ 19 and 232 $\pm$ 117 pg/mL in serum. In group C, mice immunized with pollen and Al(OH)₃, the levels of IL-5 varied between 26 $\pm$ 6 and 509 $\pm$ 107 pg/mL in BAL fluid, and between 25 $\pm$ 4 and 224 $\pm$ 44 pg/mL in serum. In mice immunized with pollen and carbon core particles (group D) the values varied between 19 $\pm$ 5 and 230 $\pm$ 65 pg/mL in BAL fluid, and between 60 $\pm$ 39 and 145 $\pm$ 30 pg/mL in serum.

The levels of IL-4 did not differ significantly within the different groups when mice were challenged with either pollen alone

or a mixture of pollen and TPM, neither was there any difference between the groups (1637 $\pm$ 122 pg/mL vs. 1295 $\pm$ 231 in group A, 1604 $\pm$ 104 vs. 1803 $\pm$ 24 in group B, 1336 $\pm$ 167 vs. 1565 $\pm$ 106 in group C, and 1323 $\pm$ 88 vs. 1161 $\pm$ 149 in group D). However, when mice were challenged with TPM alone the levels were significantly ($P < 0.05$) lower in all groups (27 $\pm$ 14 to 126 $\pm$ 52 pg/mL).

Specific IgE levels in serum

The levels of specific IgE antibodies against birch pollen were measured with a PCA assay in rats. The results are expressed in titres reflecting the highest dilution that provided a positive reaction. The highest titres of antibodies were found in mice immunized with pollen and TPM (group A), where the values varied between 120.0 $\pm$ 17.9 and 151.7 $\pm$ 41.2. Next, the titres in mice immunized with pollen and TPM and challenged with pollen were significantly higher ($P < 0.05$) than those in mice immunized with pollen only (group B) or pollen and Al(OH)₃ (group C) and challenged with pollen (Figs 1a–c). In mice immunized with pollen only the titres ranged between 12.5 $\pm$ 4.8 and 87.5 $\pm$ 50.9 (Fig. 1b), and in mice immunized with pollen and Al(OH)₃ they were between 80.0 $\pm$ 0 and 111.7 $\pm$ 30.6 (Fig. 1c). In mice immunized with pollen and carbon core particles we only found low levels of antibodies (8.3 $\pm$ 2.8 to 43.3 $\pm$ 12.8) (Fig. 1d).

Effect of birch pollen, TPM and carbon core particles on bronchial hyper-responsiveness

BHR was evaluated in a whole-body plethysmograph 24 h after the intranasal challenge with the different substances. The Penh values obtained were used to construct a curve for each group of mice, thereafter the AUC was calculated.

The highest BHR was found in group A, in mice immunized with pollen and TPM and challenged with pollen and TPM (78.5 $\pm$ 14.6), as compared with those immunized with the same substances but challenged with pollen only (35.6 $\pm$ 6.7) or TPM only (27.3 $\pm$ 4.7) (Fig. 2a). These mice were also more reactive than those immunized with pollen alone (group B), regardless of what they were challenged with intranasally: 35.0 $\pm$ 10.1 (pollen), 16.5 $\pm$ 2.5 (TPM), 32.6 $\pm$ 12.3 (pollen and TPM) (Fig. 2b). A significant difference in hyper-reactivity between group A and group B was noted when mice were challenged with pollen and TPM ($P < 0.05$). As our hypothesis was that the TPM might act as an adjuvant, we compared its effect with that of a known adjuvant, Al(OH)₃. In fact, the BHR was less pronounced in mice immunized with pollen mixed with Al(OH)₃, regardless of the substance used for the intranasal challenge: 38.2 $\pm$ 2.2 (pollen), 38.2 $\pm$ 5.3 (TPM), 19.1 $\pm$ 8.4 (pollen and TPM) (Fig. 2c), as compared with mice in groups A and B. There was a significant difference between group A and group C when mice had been challenged with pollen and TPM ($P < 0.05$). In mice immunized with carbon core particles (group D) the highest BHR was found in those challenged with pollen alone (43.7 $\pm$ 9.0), compared with those challenged with carbon core particles (22.5 $\pm$ 2.4) or a mixture of the two (29.7 $\pm$ 4.1) (Fig. 2d).

Cell counts in BAL fluid

The numbers of eosinophils and neutrophils were determined in BAL fluid (Table 3).

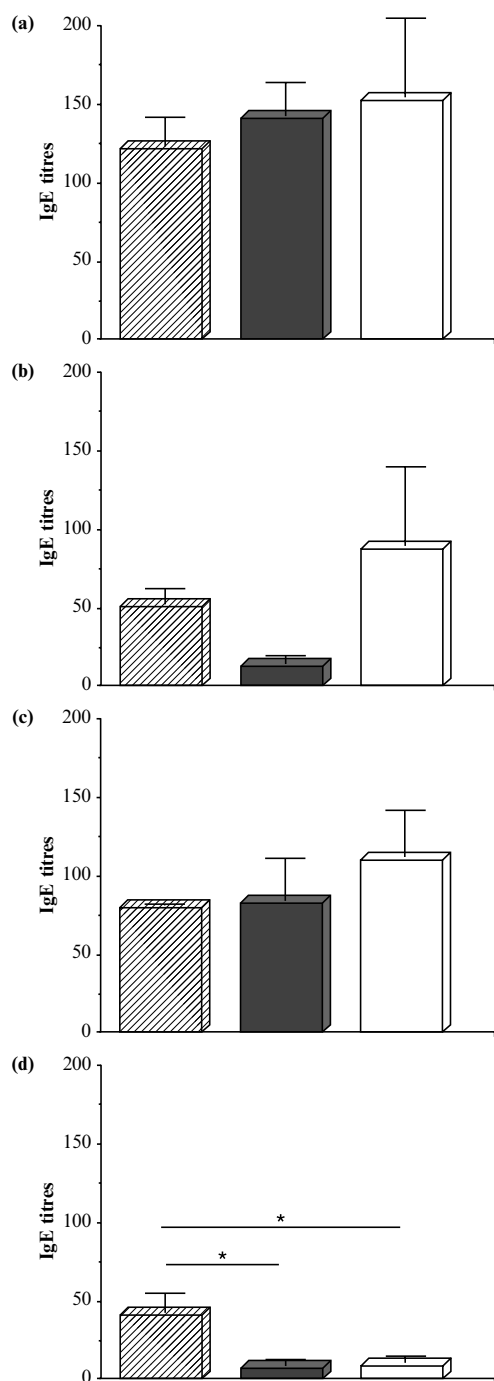


Fig. 1. (a) Production of specific IgE antibodies to birch pollen in response to immunization with birch pollen and traffic particulate matter (TPM) and provocation with pollen (striped bar), TPM (filled bar) or a mixture of pollen and TPM (non-filled bar). The results are expressed as IgE antibody titres and the levels were measured by a PCA assay in rats. Data are shown as mean $\pm$ SEM, $n = 6$, $*P < 0.05$. (b) Production of specific IgE antibodies to birch pollen in response to immunization with birch pollen alone and provocation with pollen (striped bar), TPM (filled bar) or a mixture of pollen and TPM (non-filled bar). (c) Production of specific IgE antibodies to birch pollen in response to immunization with birch pollen and $Al(OH)_3$ and provocation with pollen (striped bar), TPM (filled bar) or a mixture of pollen and TPM (non-filled bar). (d) Production of specific IgE antibodies to birch pollen in response to immunization with birch pollen and carbon core particles and provocation with either pollen (striped bar), carbon core particles (filled bar), or a mixture of pollen and carbon core particles (non-filled bar).

The highest numbers of eosinophils were found in BAL fluid from mice immunized and challenged with a mixture of pollen and TPM (group A) ($0.7 \pm 0.2 \times 10^5$ eosinophils/mL). When mice had been immunized with pollen alone (group B) (0.5 ± 0.1 eosinophils/mL) or pollen and $Al(OH)_3$ (group C) (0.4 ± 0.1 eosinophils/mL), and challenged with pollen and TPM, the levels were somewhat lower. In BAL fluid from mice challenged with TPM only, significantly lower numbers of eosinophils were noted, regardless of the substance used for immunization ($0-0.02 \pm 0.02 \times 10^5$ eosinophils/mL) ($P < 0.05$).

Neutrophils were found in high amounts in the BAL fluids of almost all mice. The highest number was again found in BAL fluid from mice in group A, immunized and challenged with a mixture of pollen and TPM ($13.9 \pm 2.0 \times 10^5$ neutrophils/mL). Mice immunized with a mixture of pollen and $Al(OH)_3$ (group C) and challenged with pollen and TPM also displayed elevated neutrophil numbers ($9.6 \pm 1.1 \times 10^5$ neutrophils/mL). There was a significant increase ($P < 0.05$) in the number of neutrophils recruited to the lungs when the mice were challenged with pollen and TPM compared with pollen alone, regardless of substance used for immunization. In mice immunized with carbon core particles and pollen (group D) there was a significant difference ($P < 0.05$) between mice challenged with pollen or with carbon core particles (9.1 ± 1.3 vs. $1.0 \pm 0.2 \times 10^5$ neutrophils/mL), and mice challenged with carbon core particles and a mixture of pollen and carbon core particles ($1.0 \pm 0.2 \times 10^5$ vs. $7.2 \pm 0.6 \times 10^5$ neutrophils/mL).

Fibronectin levels in BAL fluid

Fibronectin was measured in BAL fluid as a marker of plasma exudation. The highest levels were found in mice challenged intranasally with a mixture of pollen and TPM irrespective of immunization (Figs 3a–c) ($P < 0.05$). In mice immunized with pollen and TPM (group A) the levels amounted to $60.4 \pm 3.2 \mu\text{g/mL}$, followed in rank order by those immunized with pollen and $Al(OH)_3$ (group C), with $54.7 \pm 2.1 \mu\text{g/mL}$, and with pollen alone (group B), with $53.3 \pm 3.6 \mu\text{g/mL}$. The lowest levels were found in mice challenged with TPM alone ($37.5 \pm 2.4 \mu\text{g/mL}$ for group A, 26.7 ± 3.3 for group B and 36.6 ± 2.9 for group C). In mice immunized with carbon core particles (group D) the levels of fibronectin varied between $15.0 \pm 1.6 \mu\text{g/mL}$ (challenged with carbon core particles) and $40.6 \pm 3.4 \mu\text{g/mL}$ (challenged with pollen) ($P < 0.05$) (Fig. 3d).

The control mice, challenged with saline and methacholine, had only $3.9 \pm 0.3 \mu\text{g/mL}$ fibronectin in their BAL fluid.

LDH activity in BAL fluid

The highest LDH activity was found in mice immunized and challenged with a mixture of pollen and TPM (28 ± 14), compared with mice immunized in the same way but challenged with pollen (7 ± 1) or TPM alone (7 ± 1) (group A). In mice immunized with a mixture of pollen and $Al(OH)_3$ (group C) the values varied between 5 ± 0.4 (mice challenged with TPM) and 6 ± 0.3 (mice challenged with pollen alone or a mixture of pollen and TPM). In mice immunized with pollen only (group B) we found no LDH activity at all.

EPO activity in lung tissue

The EPO activity was measured in lung tissue as an indication of trapped eosinophils. The EPO activity in this study was

overall low (Table 3). In mice immunized with pollen and TPM (group A), where we found the highest BHR and the highest number of eosinophils, the activity varied between 0.04 ± 0.01 (in mice challenged with TPM) and 0.5 ± 0.1 (challenged with pollen). There was significantly higher ($P < 0.05$) EPO activity in mice immunized with pollen alone (group B) (0.7 ± 0.1) and mice immunized with pollen and $\text{Al}(\text{OH})_3$ (0.6 ± 0.1) (group C) compared with those in group A (0.2 ± 0.1) when mice were challenged with pollen and TPM. In mice immunized with pollen and carbon core particles (group D) the activity varied between 0 and 0.2 ± 0.05 .

Discussion

In this study we have used polluted dust (TPM), collected in a heavy car traffic tunnel in Prague, or pure carbon core particles, to investigate the effects of particulate matter in the induction of experimental allergic symptoms in BP2 mice [13,14]. These mice express BHR upon a single intranasal challenge with allergen, accompanied by the different indexes we selected, i.e. eosinophils in BAL and in lungs (via EPO), Th2 cytokines, fibronectin as a marker of exudation, and specific IgE antibodies. BHR is an important feature of asthma [20, 21], duplicated in this model in response to a variety of airborne and endogenous stimuli. This mouse model was used to study the effects of air pollution on BHR and experimental allergy development, as it can provide important insights into immune and inflammatory mechanisms of the asthmatics [22–25].

The mice which reacted with the highest BHR, the highest titres of IgE antibodies against birch pollen, the highest number of eosinophils recruited to the lung tissue, and the highest levels of fibronectin and LDH in BAL fluid, were the ones

immunized and challenged with a mixture of birch pollen and TPM (group A). This indicates that the TPM seems to have an impact on the induction of allergy and also that it supports the allergic reaction. Immunization with pollen alone, in the absence of an adjuvant, did not to the same extent increase the IgE titres and BHR upon subsequent challenge with pollen or pollen mixed with TPM. IgE titres and BHR were substantially augmented when TPM was present during immunization and was used for challenge together with pollen, whereas immunization with pollen and TPM, followed by challenge with pollen alone, was not more effective than immunization with pollen alone. Mice immunized with TPM alone had significantly lower BHR, low levels of Th2 cytokines and of inflammatory cells in the BAL fluid, and no detectable IgE antibodies (data not shown). IgG antibodies were also evaluated and did not differ between any of the different groups (data not shown).

The LDH activity was measured in BAL fluids as we wanted to evaluate whether the air pollutants damaged the respiratory tract epithelium. The highest activity was detected in mice immunized and challenged with a mixture of pollen and TPM, suggesting a mechanism that might account for the increased BHR and influx of inflammatory cells into the lungs.

In mice immunized with pollen alone (group B), compared with those immunized with saline or TPM alone (data not shown), we also obtained increased values of all indexes studied, but these values were significantly lower than those obtained in mice immunized with a mixture of pollen and TPM, except for the levels of Th2 cytokines. Indeed, we found that the highest amounts of IL-5 were in the BAL fluids from mice immunized with pollen alone, and challenged with a mixture of pollen and TPM or with pollen alone, as opposed to those immunized with pollen and TPM. This seems a paradox, even more so because the standard adjuvant $\text{Al}(\text{OH})_3$ added to pollen did not provide

Table 3. The number of eosinophils and neutrophils in bronchoalveolar lavage fluid, and eosinophil peroxidase (EPO) activity, expressed as absorbance, in lung tissue for the different groups. The substances used for immunization are indicated within brackets. Values are expressed as mean $\pm$ SEM, $n = 6$. TPM = traffic particulate matter

	No. of eosinophils ($\times 10^5/\text{mL}$)	No. of neutrophils ($\times 10^5/\text{mL}$)	EPO (absorbance)
Group A (pollen + TPM)			
Provoked with:			
Pollen	0.3 ± 0.1	6.9 ± 0.9	0.5 ± 0.1
TPM	0 ± 0	7.1 ± 0.7	0.04 ± 0.01
Pollen and TPM	0.7 ± 0.2	13.9 ± 2.0	0.2 ± 0.1
Group B (pollen)			
Provoked with:			
Pollen	0.3 ± 0.02	4.8 ± 0.3	0.5 ± 0.1
TPM	0 ± 0	7.9 ± 1.3	0.1 ± 0.03
Pollen and TPM	0.5 ± 0.1	8.6 ± 0.5	0.7 ± 0.1
Group C (pollen + $\text{Al}(\text{OH})_3$)			
Provoked with:			
Pollen	0.5 ± 0.1	5.4 ± 0.7	0.7 ± 0.1
TPM	0.01 ± 0.01	6.0 ± 0.4	0.1 ± 0.02
Pollen and TPM	0.4 ± 0.1	9.6 ± 1.1	0.6 ± 0.1
Group D (pollen + carbon core particles)			
Provoked with:			
Pollen	0.3 ± 0.1	9.1 ± 1.3	0.2 ± 0.04
Carbon core particles	0.02 ± 0.02	1.0 ± 0.2	0 ± 0
Pollen and carbon core particles	0.3 ± 0.2	7.2 ± 0.6	0.2 ± 0.05

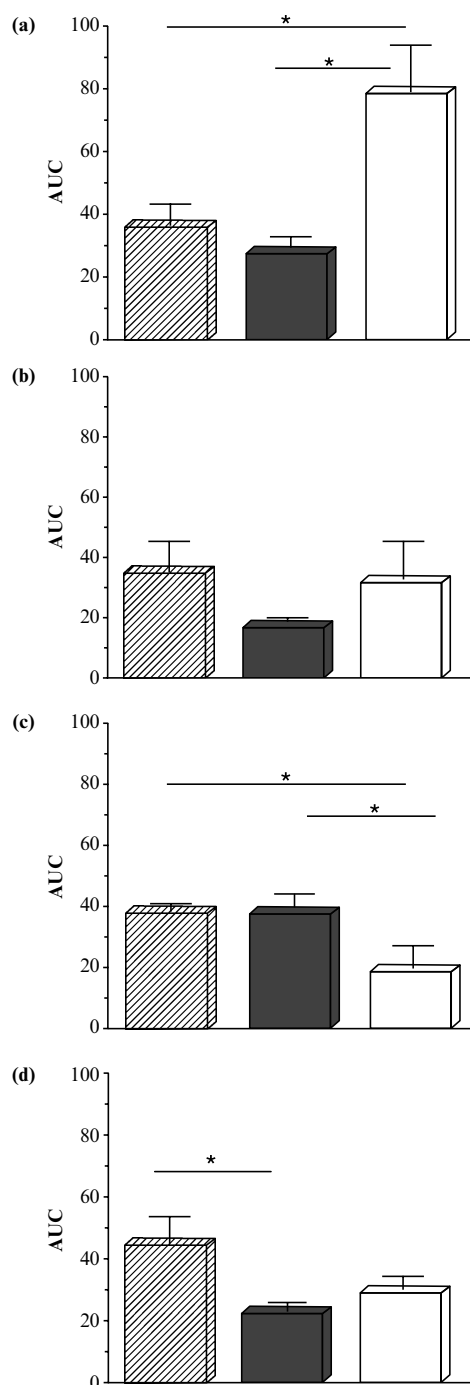


Fig. 2. (a) Analysis of bronchial hyper-reactivity (BHR) in mice immunized with birch pollen and traffic particulate matter (TPM) and thereafter provoked with pollen (striped bar), TPM (filled bar) or a mixture of pollen and TPM (non-filled bar). The BHR was analysed using a whole-body plethysmograph, after provocation with 50 mm methacholine, and is expressed as area under the curve (AUC) (see Material and methods for details). Data are shown as mean $\pm$ SEM, $n = 6$, * $P < 0.05$. (b) Analysis of BHR in mice immunized with birch pollen alone and provoked with pollen (striped bar), TPM (filled bar) or a mixture of pollen and TPM (non-filled bar). (c) Analysis of BHR in mice immunized with pollen and Al(OH)₃ and provoked with pollen (striped bar), TPM (filled bar) or a mixture of pollen and TPM (non-filled bar). (d) Analysis of BHR in mice immunized with pollen and carbon core particles and provoked with either pollen (striped bar), carbon core particles (filled bar) or a mixture of pollen and carbon core particles (non-filled bar).

those elevated IL-5 levels, under conditions where the levels of IL-4 were roughly similar. It is noteworthy that whatever the immunization regimen, the IL-4 and IL-5 levels were low when the challenging agent was TPM alone. This also underlines the essential role of allergen challenge in a given immunization background and that TPM itself does not behave as an allergen. In non-immunized mice, challenged with pollen, TPM or a mixture of both, we did not obtain any IL-4 or IL-5 at all (data not shown).

As one of our hypotheses was that the TPM might exert an adjuvant effect, we also tested the recognized adjuvant Al(OH)₃, which is known to enhance the IgE response in mice [26–28]. Aluminium-adjuvants act mainly by depot formation, allowing a slow release of antigen and thus prolonging the interaction between antigen and antigen-presenting cells and lymphocytes [29,30]. The same mechanism might be valid for air pollution particles, which can in part function as a depot for allergens. Indeed, allergens have been suggested to be absorbed to the surface, e.g. diesel particles, and thereafter released gradually inside the body for long periods of time [11,12]. However, as can be interpreted from our data, lower values were obtained of almost all the parameters studied, when mice were immunized with pollen and Al(OH)₃ (group C) instead of pollen and TPM, with an exception of the Th2 cytokines. This might result from the elevated dose of allergen used (100 μ g/mouse), as compared with natural exposition.

Overall our results compare favourably with those obtained in BP2 mice immunized with the standard allergen ovalbumin dispersed in Al(OH)₃, where an optimal immunization and challenge protocol led to equivalent values for IL-4 in BAL fluid, lung EPO activity and eosinophil recruitment to BAL fluid (Proust, unpublished data). By contrast, in the case of pollen and TPM, BHR was augmented by at least twofold in terms of AUC for a similar duration of evaluation, at least tenfold more neutrophils were present in the BAL fluid, and the levels of IL-5 were also higher. This might mean that when pollen and TPM are used together the more intense recruitment of neutrophils accounts for the additional BHR.

Air pollutants modulate airway responsiveness, and inhalation of NO₂ or ozone causes BHR in normal subjects and triggers asthma in asymptomatic patients [31–34]. Diesel exhaust is one of the major contributors to particulate matter pollution in urban areas, accounting for up to 90% of the ultrafine particulate mass in the ambient air of many cities of the world [35,36]. Diesel particles may be responsible for the increased incidence of allergic diseases of the airways [37,38], and may augment the IgE titres [39–41], mediated, at least in part, by the enhanced production of IL-4 [42,43]. As diesel particles are coated with many chemical substances it is difficult to identify the ones responsible for the observed adjuvant effect. The group of polycyclic aromatic hydrocarbons (PAHs), a product of incomplete combustion of fossil fuels, is suspected to be involved. This hypothesis was strengthened by the results obtained by Bömmel et al. [44], which show that pyrene, a major compound of the PAHs in diesel exhaust particles, was able to induce the production of IL-4, and in that way might promote allergies.

Different studies have already shown the role of diesel exhaust particles in allergy development [39–41] and therefore we chose to include only pure carbon core particles, prepared to mimic the carbon core of diesel particles, in our study. This part

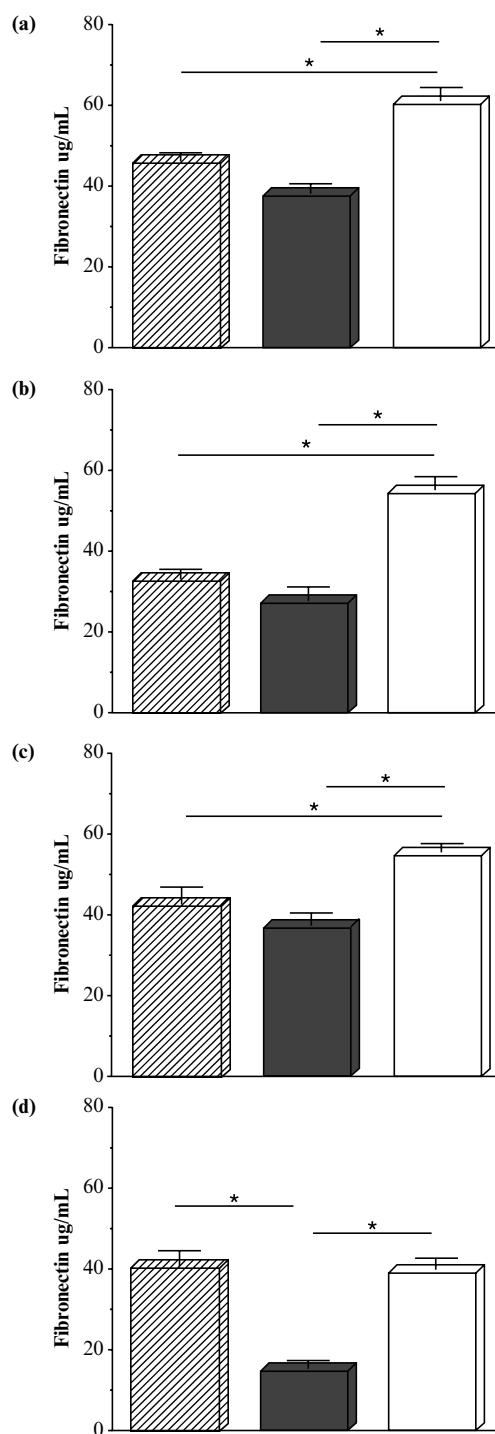


Fig. 3. (a) Levels of fibronectin in bronchoalveolar lavage (BAL) fluid after immunization with birch pollen and traffic particulate matter (TPM) and provocation with pollen (striped bar), TPM (filled bar) or a mixture of pollen and TPM (non-filled bar). Data are shown as mean $\pm$ SEM, $n = 6$, $^*P < 0.05$. (b) Levels of fibronectin in BAL fluid after immunization with birch pollen alone and provocation with pollen (striped bar), TPM (filled bar) or a mixture of pollen and TPM (non-filled bar). (c) Levels of fibronectin in BAL fluid after immunization with birch pollen and $Al(OH)_3$ and provocation with pollen (striped bar), TPM (filled bar) or a mixture of pollen and TPM (non-filled bar). (d) Levels of fibronectin in BAL fluid after immunization with birch pollen and carbon core particles and provocation with either pollen (striped bar), carbon core particles (filled bar) or a mixture of pollen and carbon core particles (non-filled bar).

of the diesel particle has not been extensively studied before [26, 45,46] and our choice enabled us to further investigate which part of the diesel particles is most relevant for the development and maintenance of allergic diseases.

When our mice were immunized with a mixture of pollen and carbon core particles (group D), airway responsiveness did not differ significantly from that obtained in mice immunized with pollen only. The IgE titres, Th2-cytokine and fibronectin levels, as well as the number of eosinophils recruited to the lungs, and EPO activity were also lower, compared with mice immunized with pollen only. Here also, the dissociation between elevated IL-4 levels and relatively low IgE titres is noteworthy. This was somewhat surprising, given the recognized effects of diesel particles in the development of allergic symptoms and diseases [41, 43,47–49]. There are, however, many possible explanations for this. First, the difference in results may be explained by the choice of antigen. Indeed, in this study we used birch pollen, one of the most relevant allergens for humans, but not the most common allergen used in mice models, where sensitization to ovalbumin is more common. Another explanation might be the immunization protocol or our choice of BP2 mice, in view of the recognized differences between mouse strains in allergic airway inflammation, and in the production of allergen-specific immunoglobulins after challenge with diesel particles and allergens [50,51]. A more likely explanation relates, however, to the fact that we have used a special carbon black (carbon core particles), which may be an intermediate between the 'ordinary' carbon black and the complex diesel soot. So, the frequently described adjuvant effect of diesel exhaust particles is probably more likely to be due to the organic moiety, which is bound to the carbon core, than to the carbon core itself.

Concerning the distribution of the TPM in the lungs it has previously been described that depending on the size of the particles they have different fates in the lungs of the animals [52]. Ultra fine particles are, for example, not as readily phagocytosed by alveolar macrophages as are larger particles, and they can consequently penetrate much more rapidly into interstitial sites, possibly including the endothelium [52]. They may even enter the blood circulation, which may result in their translocation to extra pulmonary tissues. Our TPM contained particles ranging from 0 to 50 μm . We therefore assume that we have some particles that are phagocytosed and other particles that are penetrating into the pulmonary interstitium.

In this study we have shown that mice immunized and challenged with birch pollen mixed with TPM demonstrated higher BHR, higher titres of specific IgE antibodies, higher levels of fibronectin and LDH, and more eosinophils in their lungs than those immunized with pollen alone or pollen mixed with $Al(OH)_3$. The highest levels of Th2-cytokines were, however, found in mice immunized with birch pollen alone. This demonstrates an adjuvant effect of TPM, which can have environmental pertinence. As the immunization and challenge with carbon core particles, together with birch pollen, failed to increase the BHR and the specific IgE production, it is more likely to be the organic phase bound to the carbon core of the diesel exhaust particles that displays a marked adjuvant effect for the induction of allergy, and not the carbon core used in this study. In conclusion, we have demonstrated that TPM has an effect on both the inflammatory and immunological components of experimental allergy, highlighting the importance of the exposure to a combination of particulate matters and pollen

allergens in the induction and persistence of allergic diseases in the airways.

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